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Alessia VIRZI

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Phosphoproteomic characterization of Hepatitis B virus infection reveals a new role of collagen VI and linker histone on fibrosis development and viral life cycle

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THÈSE dirigée par :

Dr. Joachim LUPBERGER   HDR, Université de Strasbourg
Prof. Robert THIMME   Professor of Medicine, University Hospital of Freiburg

RAPPORTEURS :
Dr. Anna SALVETTI   DR, Université de Lyon
Prof. Tarik ASSELAH   PU-PH, Université de Paris Diderot

AUTRES MEMBRES DU JURY :
Dr. Irwin DAVIDSON   DR, Université de Strasbourg
Dr. Christine NEUVEUT   DR, Université de Montpellier

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Key Abbreviation

4E-BP1	Eukaryotic translation initiation factor 4E-binding protein 1
AAV	Adeno-associated virus
ADV	Adefovir dipivoxil
Akt	Akt serine/threonine kinase
ALD	Alcoholic liver disease
ALT	Alanine aminotransferase
anti-HBs	HBsAg antibody
AST	Aspartate aminotransferase
Au-Ag	Australia antigen
cccDNA	Covalently closed circular DNA
CDK	Cyclin-dependent kinase
CDKN2C	Cyclin-dependent kinase 4 inhibitor C
ColVI	Collagen VI
CHB	Chronic hepatitis B
CHC	Chronic hepatitis C
CTGF	Connective tissue growth factor
DAA	Direct-acting antiviral
DAMP	Damage-associated molecular pattern
DNMT	DNA methyltransferase
DR 1/2	Direct repeat 1/2
ECM	Extracellular matrix
EGFR	Epidermal growth factor receptor
EN1/2	Enhancers 1/2
ETV	Entecavir
GPC5	Glypican 5
GSK-3β	Glycogen synthase kinase-3 beta
H3K4me3	Trimethylation of lysine 4 on histone H3
H3K9me3	Trimethylation of lysine 9 on histone H3
HA	Hyaluronic acid
HAT	Histone acetyltrasferase
HBcAg	HBV core antigen
HBcrAg	HBV core-related antigen
HbeAg	HBV e-antigen
HBsAg	HBV surface antigen
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HDAC	Histone deacetylase
HDV	Hepatitis D virus
HIV	Human immunodeficiency virus
HPV	Human papillomavirus
HSC	Hepatic stellate cell
HSPG	Heparan-sulfate proteoglycan
HSV	Herpes simplex virus
IFN	Interferon
KC	Kupffer cells
LAM	Lamivudine
LdT	Telbivudine
LN	Laminin
LSEC	Liver sinusoidal endothelial cells
M2BPGi	Mac-2-binding protein glycosylation isomer
MAPK	Mitogen-activated protein kinase

MOI	Multiplicity of infection
mTOR	Mammalian target of rapamycin
NAFLD	Non-alcoholic fatty liver disease
NG2	Chondroitin sulfate proteoglycan 4
NPC	Non-parenchymal cell
NTCP	Na$^+$ taurocholate co-transporting polypeptide
Nucleos(t)ide analogues	NAs
ORF	Open reading frame
PAHO	Pan American Health Organization
PD-1	Programmed death-1
PDGF	Platelet derived growth factor
Peg-IFN	Pegylated Interferon
pgRNA	Pre-genomic RNA
Pol	Polymerase
pRb	Retinoblastoma-associated protein
PTM	Post-translational modification
rcDNA	Relaxed-circular DNA
RIG-I	Retinoic acid-inducible gene 1
RNP complex	Ribonucleoprotein complex
STAT3	Signal transducer and activator of transcription 3
SVP	Subviral particle
TAF	Tenofovir alafenamide fumarate
TDF	Tenofovir disoproxil fumarate
TGFβ	Transforming growth factor β
TGFβR1	Transforming growth factor β type 1
VEGF	Vascular endothelial growth factor
WHO	World Health Organization
WHV	Woodchuck hepatitis virus

"Science is magic that works" Kurt Vonnegut

CHAPTER 1

"Now, why is the stomach surrounded by the liver? Is it in order that the liver may warm it and it may in turn warm the food? This is indeed the very reason why it is closely clasped by the lobes of the liver, as if by fingers." – Galen, ca. 200 A.D

OVERVIEW OF THE LIVER

In antiquity, the liver was considered the center of the soul. The interest of priests and theologians of the ancient Babylonia in finding the location of the soul in the human body brought to the first liver anatomical studies about 3,000 years BC. This can be assumed to be the begin of the study on liver anatomy. It is extraordinary that most of the interpretation about the future, or public and private events, has been conceived according to the shape or size of the liver or its components. Throughout the years then, science started to develop and to look for non-divine natural explanation (Cavalcanti de & Martins, 2013; Evans, 1908). The Greek physician Galen, born in the 129 AD, had reasons to believe that the liver perpetually produced the blood which was then filtered through the heart. Only at the beginning of the 17th century, with larger access to human cadavers less limited by previous dogma, the central galenic role of the liver was displaced by Harvey's studies on the movement of heart (Aird, 2011; T. S. Chen & Chen, 1988). The importance of the liver as central organ of the human body increased more and more throughout the centuries, and every scientific contribution was crucial for our understanding. The current knowledge on the functional anatomy of the liver, essential for performing safe hepatic surgery, previously underlined by Henri Bismuth (Bismuth, 1982), is largely due to the work of Claude Couinaud, French surgeon and anatomist who described his detailed work in "*Le Foie: Études anatomiques et chirurgicales* (*The Liver: Anatomic and Surgical Studies*)" in 1957 (Abdel-Misih & Bloomston, 2010; Sutherland & Harris, 2002).

STRUCTURE AND FUNCTIONS

The liver is a real body's engine, playing a key role in metabolism and detoxification, storage and hematopoiesis. It exerts important secretory and excretory functions, representing the bigger gland of the human body. Anatomically, this organ is classically divided in four lobes and constitutes 2-3% of average body weight (Bismuth, 1982). It receives simultaneously oxygenated blood from the cardiac output through the hepatic artery, and venous blood via the portal vein. The strategic position of the liver, situated in the right upper quadrant of the

abdominal cavity, and its vascular and biliary connections, underline its central role for the entire body. Moreover, the liver interfaces closely with the lymphatic network and it is regulated by both sympathetic and parasympathetic neural system (Abdel-Misih & Bloomston, 2010; Burchill, Goldberg, & Tamburini, 2019).

The classical functional unit of the liver is the lobule (Fig. 1). It is formed by chords of hepatocytes organized in a hexagonal shape with the center being a central vein. At the periphery, it is defined by branches of the hepatic artery and the portal vein, bile ducts, lymphatics and nerves. Within the lobules, the hepatocytes are elegantly organized radially as cords, and separated by the sinusoids, highly fenestrated vessels which host Kupffer and hepatic stellate cells (HSCs) (Fig. 1) (Vernon, Wehrle, & Kasi, 2022). The different cell types that constitute the liver will be described in the next section.

CELLULAR ORGANIZATION WITHIN THE LIVER

The human liver operates thanks to a complex interplay between different cell types, which together exert a plethora of functions. The hepatocytes are the major parenchymal cells of the liver and account for 70% of the liver cells (B. Gao, Jeong, & Tian, 2008). They play a pivotal role for glucose storage, protein synthesis and other metabolic processes. However, they are also involved in the secretion of innate immune proteins (Z. Zhou, Xu, & Gao, 2016). Beside hepatocytes, cholangiocytes represent another type of epithelial cells of the liver and are distributed on the intrahepatic and extrahepatic bile ducts (Banales et al., 2019). To exert all their functions, hepatocytes depend on a complex interplay with the non-parenchymal cells (NPCs) of the liver. The liver sinusoidal endothelial cells (LSECs) have mainly scavenger and endocytic functions. They are located on the liver sinusoids and characterized by *fenestrae*, which dynamically regulate the trafficking of macromolecules between the space of Disse and the sinusoidal blood and control the endocytic capacity of the cells. Moreover, LSECs regulate the blood flow, interacting with the HSCs (Poisson et al., 2017). HSCs are located within the

Disse space between the hepatocytes and the LSECs. This cell type is characterized for the presence of lipid droplets and thin protrusions which are extended around the blood sinusoids. HSCs activation after liver injury leads to the secretion of extracellular matrix (ECM) proteins with consequent formation of scar tissue (Kordes, Bock, Reichert, May, & Haussinger, 2021). A more precise overview about the mechanisms of activation of HSCs is presented in the section ''Mechanisms of stellate cells activation'' (page). Kupfer cells (KCs) are the liver resident macrophages which localize in the sinusoidal lumen (Fig. 1). This strategical location allowed them to develop clearance functions and host defense mechanisms, being the first liver cell population to enter in contact with the absorbed substances from the gastrointestinal tract (Nguyen-Lefebvre & Horuzsko, 2015). Recently, proteomics and single-cell analysis have been conducted on the major types of human liver cells. This reveals a peculiar protein expression profile for every cell type (Nguyen-Lefebvre & Horuzsko, 2015). At the same time, functional cooperation among the different cells appears to be essential to carry out the vital functions of the liver (Aizarani et al., 2019).
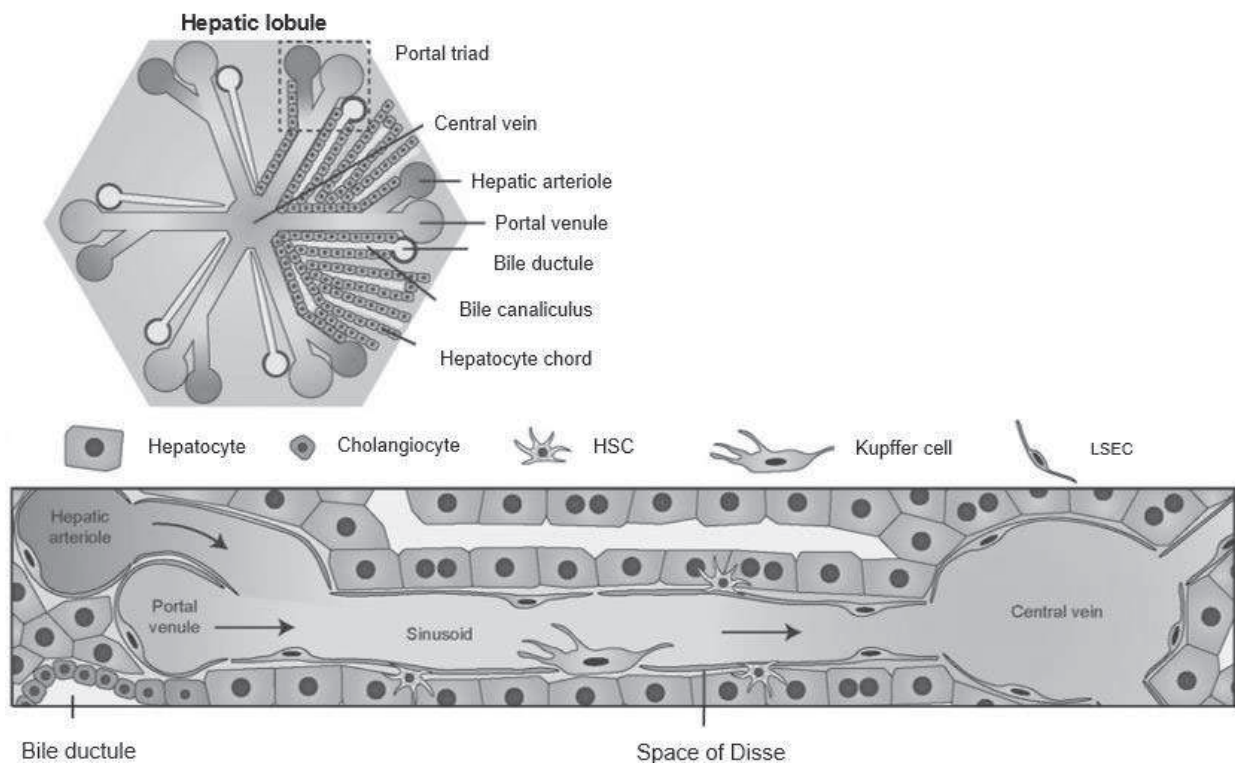
Figure 1. Schematic view of the liver lobule and its cellular composition. Image modified from (Peng, Kraaier, & Kluiver, 2021).

THE GLOBAL HEALTH BURDEN OF CHRONIC LIVER DISEASES

As emerged from the previous section, the liver orchestrates several life-sustaining functions and represents a key organ for the whole human organism. Indeed, liver dysfunctions often cause systemic damages such as vasodilatation and extrahepatic complications regarding organs, e.g., heart, kidney and lungs (Moller, Henriksen, & Bendtsen, 2014). Liver damage is caused by different etiologies, some of them still uncharacterized such as the cryptogenic cirrhosis (Mercado-Irizarry & Torres, 2016). Liver disease comprehends chronic viral hepatitis, alcoholic liver disease (ALD) and non-alcoholic fatty liver disease (NAFLD). In addition, hepatic diseases may be characterized by genetic disease and autoimmune disorders. Comorbidities (e.g. HBV/HCV co-infections) are common and accelerate the risk of fibrosis progression (El-Zayadi, 2009). Recent evaluations indicate chronic liver diseases to account for 2 million deaths per year worldwide. This is prevalently due to complications such as decompensated cirrhosis and hepatocellular carcinoma (HCC) (Asrani, Devarbhavi, Eaton, & Kamath, 2019; Cheemerla & Balakrishnan, 2021).

Viral hepatitis and World Health Organization strategy

Every year, viral hepatitis and the associated complication are responsible for 1 to 4 million deaths globally. When becoming chronic, viral hepatitis can lead to end-stage liver diseases such as cirrhosis and HCC (Boulahtouf et al., 2022). In general, chronic hepatitis induces epigenetic imprinting and persistent deregulation of signaling pathways in the host cells, which can be associated with the development of fibrosis, cirrhosis and HCC (Boulahtouf et al., 2022; Virzi, Gonzalez-Motos, Tripon, Baumert, & Lupberger, 2021; P. Zhao, Malik, & Xing, 2021). Approximately 354 million people worldwide are currently infected by HBV or HCV (Lim et al., 2020; Roudot-Thoraval, 2021). In 2019, around 296 million people were chronically infected with HBV, according to the World Health Organization (WHO) estimation (WHO, 2021). In terms of mortality, HCV and HBV are among the top four global infectious agents, together with human immunodeficiency virus (HIV), malaria and tuberculosis. Patients with

HBV are at risk for HDV co-infection which can lead to chronic HDV, the most severe form of chronic viral hepatitis (Asselah et al., 2020). According to the WHO estimation, approximately 5% of people with chronic hepatitis B (CHB) infection are affected by HDV (Pouri et al., 2020).

The World Health Assembly supported the WHO strategy which aims to reduce new hepatitis infections by 90% and deaths by 65% by 2030 implementing vaccination, diagnostic tests, medicines and prevention campaigns, which nowadays remain beyond reach in many countries (Cox et al., 2020; D. L. Thomas, 2019; WHO, 2017). Especially in African regions, where less than 2% of HBV-infected people are diagnosed, the elimination of chronic hepatitis would be more realistic by 2060 (de Villiers et al., 2021; Sonderup & Spearman, 2022). Moreover, CHB burden affects in large part also the Western pacific regions (WHO, 2017). Progresses to reach HBV elimination are uneven in the world (Sonderup & Spearman, 2022). According to some estimation, HBV eradication by 2030 will only be possible for the Pan American Health Organization (PAHO) regions (de Villiers et al., 2021).

CHAPTER 2

"*Nothing seems quite so dramatic as the unexpected Eureka moment, when, escorted by the gods of good fortune, scientists somehow stumble upon answers to questions they never knew to ask. This is the story that Baruch S. Blumberg tells in Hepatitis B: The Hunt for a Killer Virus*" - Carolyn Abraham, Toronto Globe and Mail

HEPATITIS B VIRUS

The discovery of the Australia antigen in 1965 (Blumberg, Sutnick, & London, 1968), then associated with HBV and identified as HBV surface antigen (HBsAg) two years later (London, Sutnick, & Blumberg, 1969; Sutnick, Millman, London, & Blumberg, 1972), is often considered as the beginning of the HBV research. However, the discovery of HBV involved a long history of observation, studies and challenges. The physical manifestation of jaundice has been first recorded in Babylonian tablets, mentioned in the Old Testament, and described by Hippocrates in Occident. Using the term *ikteros* and *kirros,* the hyppokratic doctors associated for the first time the symptoms of the hepatitis to a pathological condition of the liver. However, the infectious aspect of the disease was still ignored, and the aetiologias associated to a "humoral" imbalance (Papavramidou, Fee, & Christopoulou-Aletra, 2007). Across the centuries, jaundice was spreading widely through the population and the armies. Especially during World War II, the administration of yellow fever vaccine containing human serum caused a massive plague of HBV in the USA army (R. E. Thomas, Lorenzetti, & Spragins, 2013). Considerable progresses in the understanding of the hepatitis have been made by inoculating volunteers with material from soldiers who developed jaundice after yellow fever vaccination. The distinction between "infectious" and "serum" hepatitis, associated then with two different filtrable agents, became clearer by the 1920s, and only in 1947 these terms have been translated in hepatitis A and B, respectively (Block, Alter, London, & Bray, 2016). However, although the hepatitis A had meanwhile been well characterized in the early 1960s and associated to insufficient hygiene and oral-fecal transmission, hepatitis B was still poorly understood since more silent and less epidemic. It will be fortuitously discovered by the geneticist Dr. Baruch Samuel Blumberg and collaborators in 1965. By the screening of over 20,000 sera specimens, they identified a new antigen, initially called "red antigen" for its staining properties or "Australia antigen" (Au-Ag) from the serum of an Australian aborigine. At the beginning, Dr. Blumberg observed that leukemic patients exhibited higher frequency of Au-Ag, speculating that the antigen may predict leukemia risk. Many years of investigation followed. Individual observations of patients with Down syndromes, laboratory members and

wide screening brought to the conclusion that the presence of Au-Ag was from infectious origins associated to hepatitis (Blumberg, Sutnick, London, & Millman, 1970). The possibility to carry out retrospective studies to detect Au-Ag confirmed that HBV was in part associated to the burden of chronic liver diseases in different parts of the world (Okochi & Murakami, 1968; Prince, 1968). One of the links between Au-Ag and HBV was found detecting fluorescein-labeled anti-Au antibody in the nuclei of hepatocytes in liver biopsies (Millman, Zavatone, Gerstley, & Blumberg, 1969). In 1970, Prof. Dane and colleagues observed virus-like particles in serum of patients with Au-Ag associated with hepatitis (Dane, Cameron, & Briggs, 1970). Later on, Dr. Blumberg received the Nobel prize in 1976 in Physiology or Medicine for the discovery of HBV.

Even before that the link between Au-Ag (today called HBsAg) and HBV has been well established, it has been considered necessary to perform a blood screening for all the donors (H. J. Alter, Holland, & Schmidt, 1970). After the discovery of HBV, the persistence of the post-transfusion hepatitis cases allowed identification of "non-A, non-B" hepatitis, later called HCV. The discovery of HCV is mainly attributed to Michael Houghton, Nobel prize for Medicine in 2020. A novel HBV antigen distinct from HBsAg, core and HBV e-antigen (HbeAg), associated with severe liver damage, has been identified by Prof. Mario Rizzetto and collaborators and today it is known as HDV (Rizzetto et al., 1977; E. Thomas, Yoneda, & Schiff, 2015).

EPIDEMIOLOGY AND TRANSMISSION

Since the discovery of HBV, much work has been done for the understanding of its viral life cycle and natural history (Tsukuda & Watashi, 2020). However, despite the efforts in scientific research, HBV remains still a global health burden. Approximately 1.5 million new HBV infections occurred only in 2019, and around 820,000 deaths were due to HBV-related cirrhosis or HCC (WHO, 2021).

To clarify the epidemiology and the transmission modality of HBV, it is useful to consider that the age at which the HBV exposition occurs constitutes one of the main risk

factors for the development of chronic liver diseases. 90% of the HBV-infected newborn develops CHB, compared to 5% of the HBV infected in the adulthood (Indolfi et al., 2019). These, however, are more at risk of fulminant HBV (Ichai & Samuel, 2019). In the hyperendemic regions of the Western pacific and sub-Saharan Africa, the majority of HBV infection occurs in early childhood, due the absence of effective prevention of mother-to-child-transmission and birth dose (Sonderup & Spearman, 2022). In the North America countries, sexual transmission causes 30-40% of HBV infection (WHO, 2015) and a main part of HBV infection is also due to percutaneous transmission by intravenous drug use, tattoos, acupuncture, and piercings (M. J. Alter, 2003; Goldstein et al., 2002; S. Yang et al., 2015). In other words, in the highly endemic countries transmission occurs mainly at birth or during the first years of life, by mother to child at birth (perinatal transmission) or from infected to non-infected children (horizontal transmission) which is probably due to the immaturity of the adaptive immune system (Fattovich, Bortolotti, & Donato, 2008). On the other hand, in the low endemic countries the sexual or occupational exposure are the main causes of transmission, which occurs during adulthood. This difference in transmission modalities heavily influences the development of chronic liver disease. Other factors that complicate the eradication of HBV in patients are the comorbidities and the distribution of HBV different genotypes (Sunbul, 2014). Thus, despite the implementation of a universal vaccination program, eradication of HBV-associated diseases is difficult to achieve especially in endemic regions. Moreover, clear estimations about HBV epidemiology are difficult to carry out because of changes in vaccination policy and migratory flux (Tout, Loureiro, & Asselah, 2021). More details about the global impact of the HBV prophylactic vaccination are provided in the next section.

HBV VACCINATION

The first HBV vaccine was introduced by Dr. Blumberg and his team in the 1965 (Blumberg, 1977). The recent vaccines contain recombinant HBsAg produced in yeast or mammalian cells (Das et al., 2019). Current vaccination has more than 95% efficacy for children or young adults with completed vaccination cycle (Bialek et al., 2008; Floreani et al., 2004). Protection against HBV is achieved by HBsAg antibody (anti-HBs) titers greater than or equal to 10 mIU/ml (Francis et al., 1982; Seeff et al., 1978; Szmuness, Stevens, Zang, Harley, & Kellner, 1981).

WHO aims to eradicate HBV by the 2030 by scaling up the vaccination coverage to 90% to prevent horizontal and vertical transmission respectively by increasing the administration of HBV vaccine series (HepB3) in children, and by the administration of the timely birth dose (HepB-BD) within 24 hours of birth in newborns (de Villiers et al., 2021; Keane, Funk, & Shimakawa, 2016; World Health, 2019). This objective is likely to be achieved for the HepB3 administration which globally covered around 85% of infants by 2019 (de Villiers et al., 2021). However, rising the administration of HepB-BD to 90% results to be hard in many countries despite the recommendation (de Villiers et al., 2021). According to the available data, only 41% of the newborns received HepB-BD vaccine by 2020. This evaluation drops drastically to 6% in the case of WHO AFRO regions by 2020 (WHO, 2021).

In addition, according to prediction models, disruptions of vaccination efforts due to the COVID-19 pandemic may be translated in an increase of HBV-related deaths (de Villiers et al., 2021). Indeed, the drop in HepB-BD coverage due to COVID-19 disruption in 2020 may provoke an additional 15,000 HBV-related deaths (Fig. 2) (de Villiers et al., 2021). In a future vision, delays in boosting the HepB-BD coverage to up to 90% may provoke an additional 580,000 HBV-related deaths in the 2020-2030 birth cohorts (Fig. 2) (de Villiers et al., 2021).
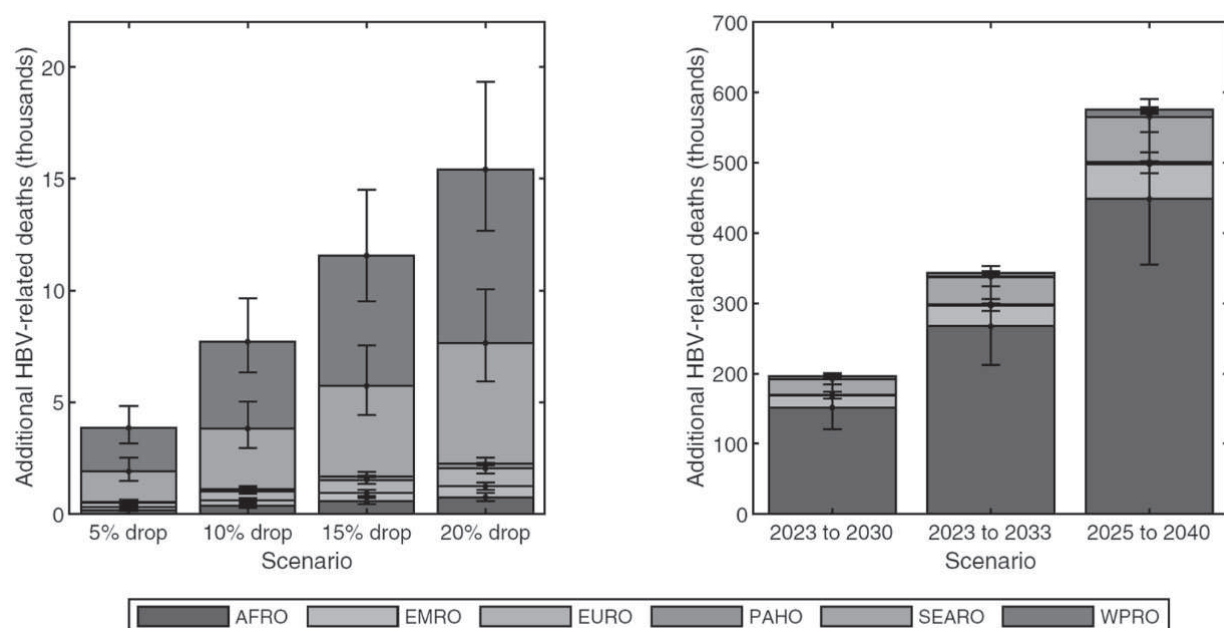
Figure 2. Prevision on disruption scenario regarding the scale-up of timely HepB-BD on HBV-related deaths. Left panel: prevision on HBV-related deaths in the birth cohorts in 2020-2030 due to a decline in the proportion of new-borns receiving timely HepB-BD in 2020. Right panel: prevision on HBV-related deaths in the birth cohorts in 2020-2030 due to delays in scaling up timely HepB-BD coverage to >90% between 2020 and 2030. AFRO, EMRO, EURO, PAHO, SEARO, and WPRO indicate WHO offices for African, Eastern Mediterranean, Pan American, South-East Asia and Western Pacific regions, respectively. Image obtained from (de Villiers, Nayagam, & Hallett, 2021).

HBV GENOME ORGANIZATION

HBV belongs to the family of *Hepadnaviridae*. An HBV virion contains one single copy of the partially double-stranded relaxed-circular DNA (rcDNA) (Summers, O'Connell, & Millman, 1975) which has a length of about 3.2 kilobase (kb) pairs, depending on the genotype. 9 different genotypes (from A to I) plus one putative genotype (J) have been identified by > 8% sequence divergence (Araujo, Teles, & Spitz, 2020; Kramvis, 2014). The 2 strands of HBV rcDNA overlap at the 5' ends and form a circular structure in which the viral DNA polymerase covalently binds the 5' of the negative (-) strand whereas the 5' of the positive (+) strand is attached covalently to a 19nt oligoribonucleotide primer. The HBV genome is characterized by a small and condensed organization therefore it displays a certain complexity towards its structure. It is composed by four overlapping and frame-shifted open reading frames (ORFs) (Fig. 3). Moreover, HBV genome includes different regulatory elements which are two transcriptional enhancers (EN1/2) and two direct repeats (DR1/2) (Fig. 3) (Asselah et al., 2019; Seeger, Ganem, & Varmus, 1986; Y. Wang & Tao, 1990). Viral replication and capsid assembly are described in the next section.
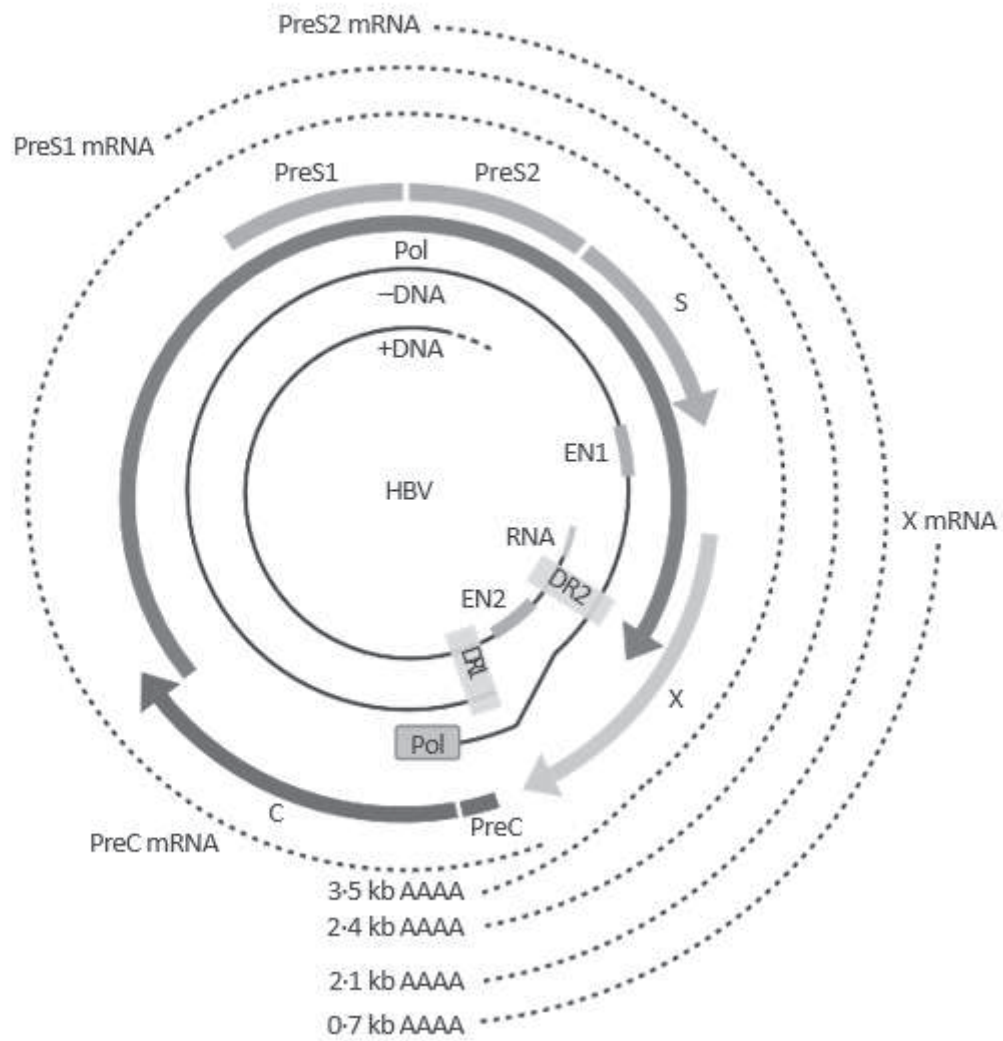
Figure 3. HBV genome. Organization of HBV regulatory elements and ORFs. PreC: pre-capsid, C: capsid, PreS1: pre-surface 1, PreS2: pre-surface 2. Image adapted from (Asselah et al., 2019).

DECIPHERING HBV LIFE

HBV entry, endocytosis and internalization

HBV entry into the host hepatocytes is a multi-step process which begins with the attachment of the particle to the cell surface (Fig. 4). As many other viruses such as herpes simplex virus (HSV), human papillomavirus (HPV), Dengue and SARS-CoV-2 (Y. Chen et al., 1997; Giroglou, Florin, Schafer, Streeck, & Sapp, 2001; X. Wang, Bie, & Gao, 2022; WuDunn & Spear, 1989), HBV exploits the heparan-sulfate proteoglycans (HSPGs) to attach to the cell surface. This low-affinity binding occurs via electrostatic interaction between negatively charged HSPG and the two positively charged residues of the antigenic loop in the S domain of the envelope proteins (Sureau & Salisse, 2013). Moreover, HBV recognizes glypican 5 (GPC5), a type of HSPG involved in the development of liver disease and cancer (Verrier, Colpitts, Bach, et al., 2016). The interaction between HBV and HSPG is possibly needed to stabilize the virus on cell surface promoting its consecutive binding, through its preS1 regions, to the sodium taurocholate co-transporting polypeptide (NTCP) receptor, which was identified in 2012 by Yan et al., as receptor for both HBV and HDV (H. Yan et al., 2012). NTCP is a carrier protein located mainly on the basolateral membrane of the hepatocytes and it serves to uptake the conjugated bile salts from plasma in a sodium-dependent way (Hagenbuch & Dawson, 2004). However, overexpression of NTCP is necessary but not sufficient to assure HBV infection, indicating that other coreceptors and host factors are required (Lempp et al., 2016; Meredith et al., 2016; Oswald et al., 2021). Recently, other factors involved in HBV internalization have been identified. Among them, E-cadherin appeared to influence the distribution of NTCP receptor in the basolateral membrane (Q. Hu et al., 2020). Moreover, it has been demonstrated that the epidermal growth factor receptor (EGFR) interacts with NTCP and its dissociation affects HBV internalization (Iwamoto et al., 2019). While EGFR downstream signaling has revealed to have no big impact on HBV infection, its endocytosis machinery is essential for HBV internalization. Indeed, HBV localization especially in late endosome seems to be crucial for the infection (Iwamoto et al., 2020).

Several studies have reported conflicting results regarding different mechanisms of HBV-associated endocytosis. Indeed, despite caveolin-1-mediated endocytosis seemed essential for HBV infection in HepaRG and COS-7 cells (Macovei et al., 2010), other studies using primary hepatocytes from the tree shrew (*tupaia belangeri*), a small animal related to primates which is susceptible for HBV infection (Ruan et al., 2013; Walter, Keist, Niederost, Pult, & Blum, 1996), reported that HBV infection was not impaired by genistein, an inhibitor of caveolae-mediated endocytosis (Bremer, Bung, Kott, Hardt, & Glebe, 2009). Moreover, caveolin-1 knockdown in HepG2-NTCP cells has not shown a reduction in HBV infection, which, on the other hand, was largely decreased by silencing of dynamin-2, one of the major components of the clathrin-mediated endocytosis machinery (Chakraborty et al., 2020; Herrscher et al., 2020). In addition to that, several other lines of evidence support the use of clathrin-mediated endocytosis by HBV for cell internalization rather than caveolin-related mechanisms (Chakraborty et al., 2020; A. Cooper & Shaul, 2006; Herrscher et al., 2020; H. C. Huang, Chen, Chang, Tao, & Huang, 2012). Next, the fusion of the viral envelope and the endosome, due to a decrease of pH within the late endosome, allows the nucleocapsid release (Hayes et al., 2016). Following this, remodeling of the actin cytoskeleton allows the nucleocapsid to reach the nucleus, accompanied by motor protein along the microtubules, in order to overcome the viscosity of the cytoplasm (Diogo Dias, Sarica, & Neuveut, 2021; Iwamoto et al., 2017; Osseman et al., 2018; Rabe, Glebe, & Kann, 2006). The entry of the HBV genome within the nucleus occurs via the nucleus pores complex. This process involves destabilization of the capsid which may occur at cytoplasmatic level or inside the nuclear basket, according to mechanisms which remain matter of debate (Diogo Dias et al., 2021). The HBV genome nuclear import may be mediated by the C-terminal domain (CTD) of HBc, which contains nuclear localization signal (NLS) sequences (Diogo Dias et al., 2021). Moreover, phosphorylation of HBc appeared necessary for nuclear pore binding (Kann, Sodeik, Vlachou, Gerlich, & Helenius, 1999). Besides that, the mechanism of capsid import inside the nucleus has shown to be additionally mediated by the cellular transport receptors importin α and β (Rabe, Vlachou, Pante, Helenius, & Kann, 2003). Moreover, the viral nuclear import may be

mediated by the presence of a bipartite NLS localized in the TP domain of viral polymerase (Lupberger, Schaedler, Peiran, & Hildt, 2013).

cccDNA formation

In the nucleus, rcDNA is modified by a series of cellular factors and converted in covalently closed circular DNA (cccDNA) (Diogo Dias et al., 2021). The multi-step process which characterized the formation of the cccDNA is not fully elucidated yet, but it is known to involve the flap structure-specific endonuclease 1 (FEN1) (Kitamura et al., 2018), the host cell polymerases, such as the DNA polymerase k (Qi et al., 2016), DNA ligases 1, 3 and 4 (Long et al., 2017) and a DNA repair enzyme, the tyrosyl-DNA-phosphodiesterase 2 (Koniger et al., 2014). Moreover, recently it has been suggested that cccDNA formation is the result of different repairing processes on the rcDNA (L. Wei & Ploss, 2021). In the nucleus, cccDNA takes a chromatin-like conformation, associating with both histones and non-histones proteins as described in the section Histone epigenetic modifications (page 43). This constitutes a stable minichromosome which displays a typical "beads-on-a string" appearance observed via electron microscopy (Bock, Schranz, Schroder, & Zentgraf, 1994; Bock et al., 2001). cccDNA is extremely stable within the nucleus, and it has a half-life of forty days in HepG2-NTCP cells (Ko et al., 2018), while a study reported that it is up to nine months in HBV patients during treatment (Boyd et al., 2016). Different host factors and viral proteins maintain cccDNA stability within the nucleus and some of them have been already identified and they are discussed in the section HBV modulation of host epigenetic program (page 41). Interestingly, it is known that Interferon (IFN)-γ and tumor necrosis factor alpha destabilize cccDNA (Xia et al., 2016). Moreover, high dose of IFN-α upregulates APOBEC3A cytidine deaminase which in turn causes cccDNA degradation via deamination processes (Lucifora et al., 2014). Standardized non-invasive procedures are urgently needed to measure cccDNA level in order to replace invasive methods such as liver biopsy (Asselah et al., 2019; X. Li, Zhao, Yuan, & Xia, 2017). Potential surrogate biomarkers for cccDNA have been suggested and they are introduced in the section HBV-specific biomarkers (page 8).

HBV transcription and translation

cccDNA is sequestrated within the nucleus of the hepatocytes allowing the persistence of the infection. cccDNA is transcribed into the 3.5 kb, 2.4 kb; 2.1 kb and 0.7 kb HBV RNAs transcripts (Fig. 3). Once that all the HBV RNAs translocate into the cytoplasm, they are translated into viral proteins (Tsukuda & Watashi, 2020). So far, two types of 3.5 kb HBV RNAs are known: the precore mRNA and the pre-genomic RNA (pgRNA). The precore mRNA translation and the consequent post-translational modification bring to the formation of the non-structural protein HBeAg that is released into the blood (Deroubaix & Kramvis, 2021). The pgRNA serves for the translation of Pol and HBV core antigen (HBcAg). In addition, it is encapsulated and reverse transcribed (Quarleri, 2014). The 2.4 kb (PreS mRNA) and the 2.1 kb HBV RNAs (S mRNA) are respectively translated into large (L-HBsAg) and medium (M-HBsAg) or small (S-HBsAg) HBsAg, depending on the AUG start codon. These proteins are further processed in the Golgi apparatus. Furthermore, the 0.7 kb HBV RNA is translated into HBx. To our knowledge, the integration of the HBV DNA into the host genome does not contribute to the transcription of the 3.5 kb pgRNA and precore mRNA. However, both the 2.4 kb and the 2.1 kb RNAs are transcribed from integrated HBV DNA (Moolla, Kew, & Arbuthnot, 2002).

Reverse transcription and rcDNA formation

To our current knowledge, only pgRNA is required for genome replication. The recognition of the pgRNA by the Pol is attributed to one part of the secondary structure (ε encapsidation signal) at 5′ end of the pgRNA. The so-called ribonucleoprotein (RNP) complex is assisted by the chaperones which cover a key role in the stabilization and encapsidation. Moreover, the RNP complex is additionally agglomerated by the core dimers (A. Chen & Brown, 2012; Jones & Hu, 2013b; Seifer & Standring, 1995; J. Wang et al., 2020). Inside this structure, the reverse transcription process can start. The Pol catalyzes the DNA synthesis by reverse transcription, causing high error rate and a large repertory of genetic variants due to the absence of proofreading activity (Caligiuri, Cerruti, Icardi, & Bruzzone, 2016; W. L. Tsai &

Chung, 2010; H. I. Yang et al., 2008). Therefore, the rcDNA is formed (Jones & Hu, 2013a; L. Tang, Sheraz, McGrane, Chang, & Guo, 2019). The nucleocapsid containing the core particles and the genetic material is hereby created and it is destinated to two roads: go back to the nucleus to constitute a pool for the cccDNA, or assemble with the envelopes proteins and be expulsed from the cell (Beck & Nassal, 2007).

Release of the new particles

Various particles and intermediates can be detected in the serum of HBV-infected patients. However, the role of some of them is partially unknown (Bardens, Doring, Stieler, & Prange, 2011; J. Hu & Liu, 2017). So far, at least three forms of infectious and non-infectious particles have been identified. The Dane particle is the bigger one with a diameter of 42 nm and it is infectious. The outside capsid of the Dane particle consists of a lipid membrane and the three forms of HBsAg. This layer surrounds a an icosahedral nucleocapsid constituted by the HBcAg (Heermann et al., 1984; Marion & Robinson, 1983). This in turn contains the viral Pol which is covalently attached rcDNA (Gilbert et al., 2005).

Importantly, the L, M, and S surface proteins produced in excess self-assemble forming empty subviral particles (SVPs) which can be released in large excess into the blood. SVPs can be spherical or filamentous and have a diameter/length of 22 nm (Tsukuda & Watashi, 2020). Since they only contain the envelope proteins and lack of the nucleocapsid (naked particles), they are not infectious (J. Hu & Liu, 2017). The relevance of the SVPs for HBV life cycle is not totally clear so far. Probably, they may act as decoy for the immune system, increasing the possibility for the infectious particles to reach undisturbed the hepatocytes (Ganem, 1991). Moreover, HBV SVPs are immunogenic and used for the development of an effective vaccine (Blumberg, 1984).
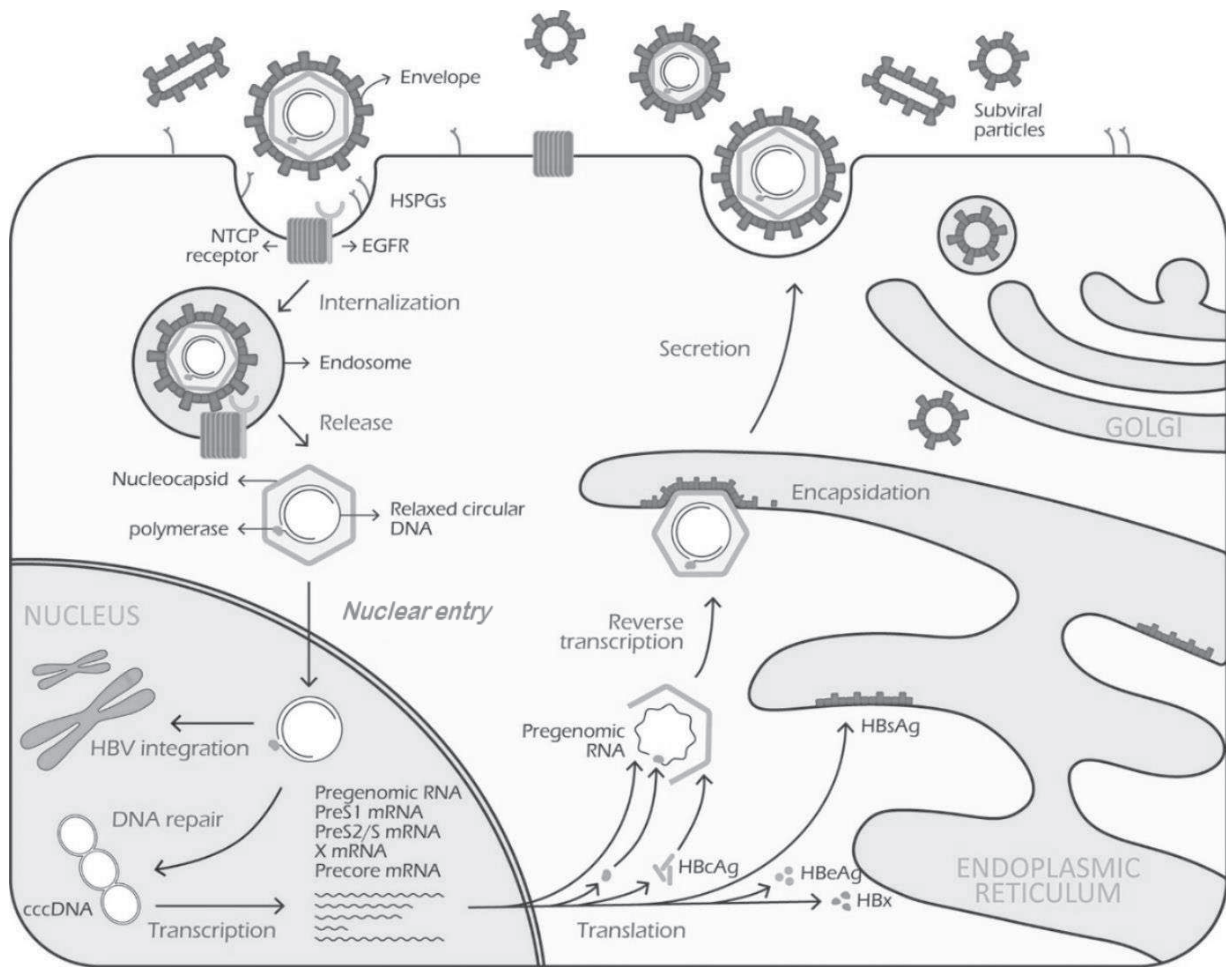
Figure 4. HBV life cycle. Image modified from (F. Zhao et al., 2021).

NATURAL AND IN VITRO MODELS FOR HBV REPLICATION STUDIES

Natural infection models

Since HBV discovery, a series of models for the study of the viral life cycle have been emerging. However, it became immediately clear that HBV models are limited by the strict species specificity. Chimpanzee (*pan troglodyte*) was the only model suitable for the study of the immune response to HBV infection, given the similarity of the genetic background and immune system with *homo sapiens*. Indeed, it has been shown that chimpanzees are susceptible to the infection using human plasma of HBV infected individuals (Maynard, Berquist, Krushak, & Purcell, 1972). Since this observation, this animal has been used as a model for testing safety and efficacy of the first-generation vaccines from HBV-infected human sera (Buynak et al., 1976; Hilleman et al., 1975; Purcell & Gerin, 1975) and later for the development of alternative vaccines using synthetic peptides (Itoh et al., 1986) or viral proteins as immunogens (Itoh et al., 1986). Importantly, chimpanzee model allowed to understand the "stealth" behavior of HBV, which, differently from HCV, does not induce (or immediately counteracts) the upregulation of genes related to innate immune response during acute phase (few weeks after the infection) (Thimme et al., 2003; Wieland, Thimme, Purcell, & Chisari, 2004). This was later confirmed in HBV-infected patients (Dunn et al., 2009). However, ethics concerns and the need of big infrastructures bring to the interruption of the chimpanzee as model of study (Dandri & Petersen, 2017).

The identification of NTCP as functional receptor for HBV entry within the hepatocytes (H. Yan et al., 2012) was possible thanks to the recognition of the tree shrews (*Tupais belangeri)* as *in vivo* model. These little animals, closely related to primates, well recapitulate different aspects of HBV infection in humans and can develop HCC as late-stage consequence of HBV chronic infection (Ruan et al., 2013; C. Yang et al., 2015). Other surrogate models for HBV infection are represented by woodchucks infected by the woodchuck hepatitis virus (WHV) and by pekin ducks. While the first model has been useful for the development of antivirals and vaccines

(M. Lu et al., 2008; Roggendorf, Schulte, Xu, & Lu, 2007), the second one has revealed the presence of a nuclear cccDNA as viral pool to produce infected particles, even in the absence of a stable genome integration (Tuttleman, Pourcel, & Summers, 1986). However, the use of these animal models presents several limitations, such as limited supply and the difficulties in their domestication, as well as the evolutionary differences of the immune system between ducks and humans (X. Zhang, Wang, Wu, Ghildyal, & Yuan, 2021). For these reasons, transgenic and transfected mice have been developed. Mouse hepatocytes expressing NTCP support HBV entry, while they are not suitable for study on cccDNA (H. Li et al., 2014). Adeno-associated virus (AAV)-HBV-transduced mice have demonstrated to support the cccDNA formation. This model allows the test of compounds targeting cccDNA pool *in vivo* (Lucifora et al., 2017).

In vitro infection models

A huge amount of *in vitro* infection models has been developed in order to provide reliable and feasible tools for the study of HBV infection and host-virus interactions and cccDNA persistence, as well as to allow the screening of promising antiviral molecules (Verrier, Colpitts, Schuster, Zeisel, & Baumert, 2016). The different types of *in vitro* infection model systems can be schematically summarized as follows:

HBV system based on hepatoma cell lines (HepG2.2.15 and HepAD38)

Huh7 and HepG2 cell lines are hepatoma-derived cells widely used as a model for hepatocytes (Verrier, Colpitts, Schuster, et al., 2016). These cell types are not susceptible for HBV infection. This is probably due to their lack of NTCP and other cellular surface receptors (Clayton et al., 2005). However, the transfection of hepatoma cells with cloned circular HBV DNA is capable to stimulate the production of HBV particles (Sureau, Romet-Lemonne, Mullins, & Essex, 1986; Verrier, Colpitts, Schuster, et al., 2016). Today, HepG2.2.15 and HepAD38, two HepG2-derived cell lines stably transfected with HBV genome (Ladner et al., 1997; Sells, Chen, & Acs, 1987), are widely used as source of HBV infectious particles and for testing potential antiviral compounds (van de Klundert, Zaaijer, & Kootstra, 2016). However, they are not suitable to

study the early host-virus interactions which are involved in entry, attachment and uncoating processes (Wose Kinge, Bhoola, & Kramvis, 2020).

Hepatic stem cell line (HepaRG)

HepaRG is a human progenitor liver cell line which derived from the tissue of a female with liver cancer associated with chronic hepatitis C (CHC) infection (Parent, Marion, Furio, Trepo, & Petit, 2004). Differentiated HepaRG cells express NTCP receptor and therefore support HBV infection. In addition, they are considered morphologically and functionally similar to PHHs (Gripon, Diot, & Guguen-Guillouzo, 1993). Moreover, this model allows cccDNA formation and secretion of infectious viral particles (Hantz et al., 2009). However, their long differentiation process and the delicate culture condition, together with the low or variable infection rate, limited their use. Interestingly, a new and fast method for HepaRG differentiation has been recently established, allowing a fast differentiation and subsequent HBV and HDV infection (Lucifora, Michelet, Salvetti, & Durantel, 2020).

NTCP-expressing cell lines

The discovery of NTCP receptor for HBV and HDV entry was determinant for the development of cell lines sustaining efficient and robust viral infection. NTCP-overexpressing cell lines, such as Huh7-NTCP and HepG2-NTCP, finally allowed the possibility to effectuate screening of antiviral compounds and the identification of key steps of viral life cycle. However, the infection does not result in a significant viral spreading and cccDNA is detected in modest amounts (Verrier, Colpitts, Schuster, et al., 2016). Still, the infection of NTCP-overexpressing cell lines requires a high multiplicity of infection (MOI) and the use of polyethilen glycol 8000 (PEG) for better absorption and penetration of the viral particles on cell surface (Gripon et al., 1993), which may restrict the understanding on viral entry pathways.

Primary human hepatocytes

PHHs is the most physiological model to study HBV infection *in vitro* therefore useful for experimental validation (J. Hu, Lin, Chen, Watashi, & Wakita, 2019) and allows the study of

the complete viral life cycle (Gripon et al., 1988; Rumin, Gripon, Le Seyec, Corral-Debrinski, & Guguen-Guillouzo, 1996). The infection of PHHs requires a very high MOI, probably due to the loss of expression of the NTCP receptor few days after isolation. However, donor-donor variability, which may influence the efficacy of the infection (H. Yan et al., 2012) and the limited supply, discourage the use especially for large-scale screening.

SIGNALING PATHWAYS AND HOST-VIRUS INTERACTION

Communication is indispensable for any organism in order to survive. Within the cell, the responses to stimuli are mediated by a myriad of finely tuned signaling pathways. At first correlated with endocrinologic studies (Rodbell, 1980), the discovery of signaling pathways is now considered crucial for many physiological and pathological processes (H. Tang & Xue, 2017). Signal transduction is a directed cascade of events, which comprises the binding of a ligand to a receptor, the activation of a second messenger and the final effect (Nair, Chauhan, Saha, & Kubatzky, 2019). However, the pattern of cellular transduction is susceptible to signal duration, cellular context and crosstalk with other pathways, which results in a highly intricate and tightly regulated phenotypic response. This high level of complexity associated with the signaling pathways may be due to several factors, such as mutations and selective pressure (Soyer & Bonhoeffer, 2006). Phosphorylation is the most abundant and extensively studied post-translational modification (PTM). This represents a reversible and dynamic process regulated by kinases and phosphatases acting as molecular switch of signal transduction (Fig. 5) (Ardito, Giuliani, Perrone, Troiano, & Lo Muzio, 2017).
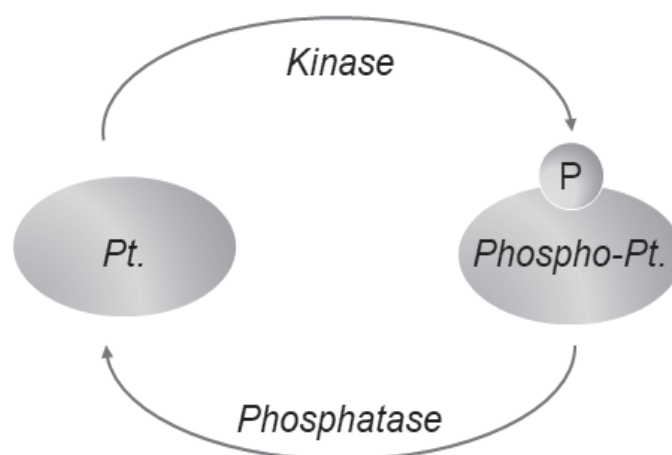

Figure 5. Cartoon showing the dynamic of protein phosphorylation.

While protein phosphorylation may occur on nine known amino acids residues, serine, threonine and tyrosine phosphorylation are the most abundant ones, probably due to their stability in acidic conditions during the analysis (Makwana, Muimo, & Jackson, 2018). These PTMs represent a critical step for a myriad of events involved in oxidative stress, inflammation, apoptosis and proliferation (Ardito et al., 2017). A dysregulation of signaling is an important cause of liver disease imposing an oncogenic pressure, which may persist even after that the underlying cause has been removed (Andrisani, 2021; Hamdane et al., 2019; Juhling et al., 2021). Importantly, signaling pathways are established drug targets. The understanding of signaling pathways that impact tumor cell proliferation, angiogenesis and invasion has led to the identification of novel therapeutics to treat HCC (Whittaker, Marais, & Zhu, 2010). Among them, Sorafenib, a multi-kinase inhibitor, has been approved for patients with advanced HCC (Llovet et al., 2008). In addition, a growing number of studies conducted on EGFR contributed to understand its pathological role especially in the context of virus-induced liver disease progression. Tyrosine phosphorylation-induced activation of EGFR by HCV triggers several downstream pathways such as MAPK and signal transducer and activator of transcription 3 (STAT3) with consequent impaired antiviral activity (Lupberger, Duong, et al., 2013; Lupberger et al., 2011). Moreover, EGFR is involved in HBV internalization (Iwamoto et al., 2019). In the context of fatty liver diseases, different dietary protective agents may be potentially useful to prevent the development of fatty liver diseases for their ability to modulate and restore PI3K/Akt/PTEN axis (Ikeda et al., 2020). Studying the signaling pathways gives important insight into the regulation of the viral life cycle and highlight novel therapeutic strategies to target liver disease progression and potential biomarkers to control or predict the degree of the disease.

Viral hepatitis (HCV, HBV, HDV) is known to alter the signaling circuits to their own benefit, e.g., by preventing apoptosis and by assuring the release of the infectious virions (Arzberger, Hosel, & Protzer, 2010). Moreover, by exploiting the regulatory pathways of the host cell, hepatitis viruses evolved strategies to subvert host signal transduction and by such evade the host antiviral response (Chathuranga, Weerawardhana, Dodantenna, & Lee, 2021; Xu, Chen,

& Chen, 2021). Despite their fundamental differences, these viruses hijack common signaling pathways using possibly different strategies which converge in the dysregulation of oxidative stress, metabolic pathways, inflammation processes, and establishment of oncogenic signatures (Boulahtouf et al., 2022). Based on previous studies focusing on individual canonical pathways, common dysregulated and disease-relevant signaling pathways involve TGF-β, β-catenin, PI3K/Akt/PTEN and STAT3 (Fig. 6). These pathways and others, such as Hippo, Hedgehog and MAPK, presumably ad to the pro-oncogenic pressure of chronic infection (Dimri & Satyanarayana, 2020). Given the strong crosstalk of host signaling, these examples highlight the need for an unbiased dissection of global phosphoproteome, which will contribute to the understanding of disease-relevant virus-host interactions and to identify druggable targets that hold the potential to reverse the pathological consequences of infection. More detail about the role of signaling pathways in liver pathogenesis can be retrieved from the reviews and book chapter which I first- or co-authored, which are presented in the ANNEX (See Annex IV, V, VI, VIII).
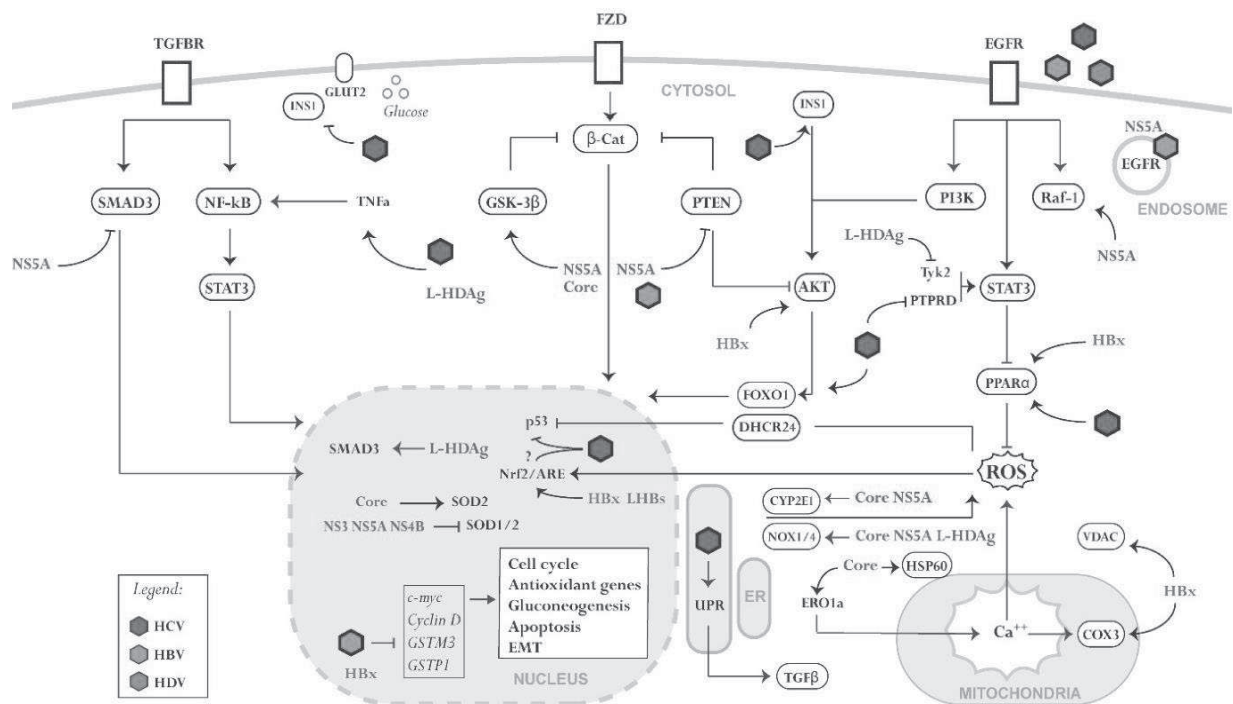
Figure 6. **Examples of signaling pathways perturbed by viral hepatitis**. NS3, NS4B, NS5A

(non-structural protein 3/4B/5A). Image by Alessia Virzì from (Boulahtouf, Virzi, Baumert, Verrier,

& Lupberger, 2022).

SIGNALING DEREGULATION IN HBV INFECTION

As emerged from the previous section, viruses exploit signaling pathways to sustain different aspects of their life cycle and promote malignancy. For many years, studies on HBV-associated signaling pathways have been hampered by the absence of a robust infection model. Therefore, a large number of studies using HBV proteins, such as HBx, has been conducted (Nikawa, Towatari, Ike, & Katunuma, 1989; Rawat & Bouchard, 2015). Recently, the increasing understanding of the multifactorial mechanisms of HBV-associated liver pathogenesis led to the identification of potential druggable targets. So far, evidence led to the identification of some signaling pathways molecules against HBV, which are in preclinical or early clinical trials (Torresi et al., 2019; Y. Yan, Qiu, Davgadorj, & Zheng, 2022). In addition to the TLR agonists and the modulators of RIG-I, which were discussed in the section "new drug development for HBV therapy", other potential therapeutic molecules acting on the modulation of signaling pathways such as Wnt/β-catenin, PI3K/Akt, TGF-β and JAK/STAT, have been discovered (Y. Yan et al., 2022). Wnt signaling is negatively regulated by the RNA helicase DEAD-Box Helicase 5 (DDX5). Therefore, decreased level of DDX5 promotes activation of Wnt signaling and cancer development (Mani et al., 2020). DDX5 is downregulated during HBV replication and it is an indicator of poor prognosis for HBV-induced HCC. The downregulation of DDX5 is caused by the expression of the protooncogenes miR17~92 and miR106b~25 and therefore may be restored by miR17~92/miR106b~25 antagomirs (Mani et al., 2020). PI3K/Akt signaling pathway has been identified as negative regulator of HBV replication. Indeed, treatment with PI3K/Akt inhibitors, such as Rapamycin and LY294002, induces higher HBV replication (W. Huang et al., 2014; Xiang & Wang, 2018). Interestingly, PI3K/Akt signaling has been studied also for its role in autophagy regulation and it has been recently shown that trypeptidil peptidase II, a molecule possibly related to this pathway, may restore autophagy in CD8+ T cells, inhibit HBV DNA replication and decrease HBsAg level (Q. Tan et al., 2022). In order to modulate TGF-β/SMAD-induced fibrogenesis in CHB patients, activators of SMAD7 may also constitute therapeutic candidates (Argentou et al., 2016). Moreover, JAK/STAT pathways may be controlled by different molecules to hamper HBV replication. HBV replication

is facilitated by MMP-9 via a reduction of JAK/STAT pathway (J. Chen et al., 2017). Compounds such as CDM-3008 (Furutani et al., 2019), Tapasin (Wu et al., 2018) and HBV-miRNA-3 (X. Zhao et al., 2020) have been proposed as therapeutic candidates for their ability to restore JAK/STAT pathways reducing HBV replication. Importantly, sustained HBV replication has been linked also to the ability of the virus to hijack the cell cycle machinery (Bagga & Bouchard, 2014; Xia et al., 2018). Indeed, it has been suggested that HBV exploits cyclin/cyclin-dependent kinases (CDKs) and cell proliferation pathways such as MAPK to hijack the cell cycle machinery and revert the cycle checkpoint to facilitate viral replication and persistence (Bagga & Bouchard, 2014). The understanding of the HBV-induced dysregulation of the cell cycle program assumes particular relevance considering that facilitating hepatocyte regeneration and proliferation may affect cccDNA metabolism and contribute to viral eradication (Allweiss et al., 2018; C. L. Chong et al., 2011).

HBV MODULATION OF HOST EPIGENETIC PROGRAM

Epigenetic modulation by HBV infection is especially interesting considering that cccDNA minichormosome regulation may follow the same mechanisms established for cellular chromatin (Tropberger et al., 2015). The minichromosome is extremely dynamic and believed to be highly regulated, switching from a closed to an opened conformation under different epigenetic modifications (Fig. 7) therefore driving a continuous production of HBV antigens (Z. Wang, Wang, & Wang, 2020). Different types of cccDNA-associated epigenetic regulation have been identified which can be triggered by DNA methylation, chromatin remodeling and histones modification. Many other epigenetic modifications on HBV minicromosome are possibly still unknown.

HBV DNA methylation

DNA methylation is one of the main post-translational modifications in cells and it generally provokes the inhibition of gene expression in mammalian cells (Turker, 2002). This epigenetic modification occurs when DNA methyltransferase (DNMT) adds methyl groups to

the CpG island of the DNA sequence (Schubeler, 2015). In the cccDNA, three CpG islands have been identified and different studies shown that their methylation is correlated with HBV replication. *In vitro* data on HepG2 cells demonstrated that the transfection of methylated HBV DNA reduces HBV mRNA level (Vivekanandan, Thomas, & Torbenson, 2009). Moreover, HBV infection leads to an upregulation of DNMTs in hepatocytes which in turn methylate viral DNA, causing a decrease of viral replication (Vivekanandan, Daniel, Kannangai, Martinez-Murillo, & Torbenson, 2010). In line with that, the analysis of cccDNA of HBV chronic infected patients, demonstrated a negative correlation between HBsAg and CpG island III methylation level, whereas CpG island II methylation was more correlated to low HBV DNA serum level (Y. Zhang et al., 2014).

HBV proteins and epigenetic modulation

The epigenetic control of cccDNA is especially driven by HBx, a regulatory protein recruited in the minichromosome complex (Belloni et al., 2009) and required for cccDNA transcription control. Nuclear HBx enrolls a large amount of transcription factors and co-activators, such as acetyltransferases CBP, p300, PCAF/GCN5, and the histone deacetylases HDAC1 and SIRT1, orchestrating finely cccDNA remodeling (Belloni et al., 2009). It has been demonstrated that cells treated with HBx-deficient HBV do not lead to productive HBV infection compared to the wild-type (Lucifora et al., 2011). In line with this, silencing of HBx showed to decrease the episomal HBV stability (Hensel, Cantner, Bangert, Wirth, & Postberg, 2018). Moreover, HBx triggers the degradation of the structural maintenance of chromosome 5/6 (SMC5/6) complex, promoting its interaction with the E3 ubiquitin ligase proteosome system thus enhancing HBV gene expression relieving the inhibition maintained by the complex (Allweiss et al., 2022; Decorsiere et al., 2016; Murphy et al., 2016). The reduction of HBx levels via siRNA and peg-IFNα has demonstrated to enable the expression of SMC5/6 with consequent epigenetic suppression of the minichromosome. Moreover, antiviral interruption reduces the activity of SMC5/6 inducing HBV rebound (Allweiss et al., 2022).

In addition to HBx, also HBc is present in the nucleus and associates with the cccDNA. HBc is gaining more and more importance as epigenetic regulator, acting as positive effector for viral transcription and replication (Guo, Li, Zhao, Zhang, & Yan, 2011). It has been shown that HBc preferentially binds at CpG island II, promoting an epigenetic permissive state for the cccDNA. In particular, the level of HBc binding to CpG island II resulted to be associated with hypomethylation in CpG island II of HBV cccDNA minichromosome (Guo et al., 2011). Although previous studies showed that newly synthetized HBc protein is not required for the maintenance and transcription of the cccDNA (Qi et al., 2016; Tu, Zehnder, Qu, & Urban, 2021), recently, chromatin immunoprecipitation assay has shown that HBc binds to the cccDNA in the absence of viral transcription and *de novo* protein production. Thus, virions-delivery HBc is able to associate with cccDNA independently from *de novo* protein synthesis. This complex stays stable for at least six weeks within the nucleus of HepaRG cells (Lucifora et al., 2021). Importantly, cccDNA ChIP approaches revealed that multiple enzymes and factors are associated with HBc. Indeed, HBc, which does not have any enzymatic activity, attracts histone acetyltrasferase (HAT) to cccDNA, whereas mutant forms of HBc cause a loss of interaction between HAT and cccDNA (C. K. Chong et al., 2017).

Histone epigenetic modifications

Histone proteins have essential roles in chromatin remodeling and therefore are strictly implicated in the regulation of cell cycle events. The first level of chromatin compaction is constituted by the nucleosome core particle. DNA is tightly packaged around an octameric core formed two copies of each core histone named H2A, H2B, H3 and H4. A short stretch of linker DNA connects the two adjacent nucleosomes, associating with the linker histone (H1) forming the chromatosome (Davey, Sargent, Luger, Maeder, & Richmond, 2002; Hergeth & Schneider, 2015; Luger, Mader, Richmond, Sargent, & Richmond, 1997). Histones are very mobile structures, with a residence time on chromatin between exchange events of hours for core histones or minutes for linker histones (Lever, Th'ng, Sun, & Hendzel, 2000; Misteli, Gunjan, Hock, Bustin, & Brown, 2000). Therefore, these proteins probably bind chromatin

regions in a "stop and go" fashion (Misteli et al., 2000). During gene transcription, chromatin remodeling mechanisms displace histones to increase the accessibility to the underlying DNA sequences. Especially linker histones are considered to be rapidly shuffled between different chromatin regions (Lever et al., 2000; Misteli et al., 2000).

In the minichromosome, histone proteins may undergo different types of epigenetic modification, such as acetylation, methylation and phosphorylation. Acetylation of cccDNA-associated H3 and H4 promotes HBV viral replication (Gong et al., 2011; Pollicino et al., 2006; Z. Q. Wei et al., 2017), which is also promoted by histone deacetylase (HDAC) inhibitors. In line with that, the downregulation of HAT1 hampers HBV replication and minichromosome formation, impeding H3 and H4 assembly, as well as the recruitment of HBx and p300, on the cccDNA (G. Yang et al., 2019). Moreover, H3 histones associated with cccDNA have found to be phosphorylated (Gong et al., 2011). Among the methylation events which have been identified to play a role in cccDNA chromatin remodeling, evidence suggests that trimethylation of lysine 4 on histone H3 (H3K4me3) is associated with active transcription, whereas H3K9me3 is associated with silencing transcription (Tropberger et al., 2015) (Fig. 7).
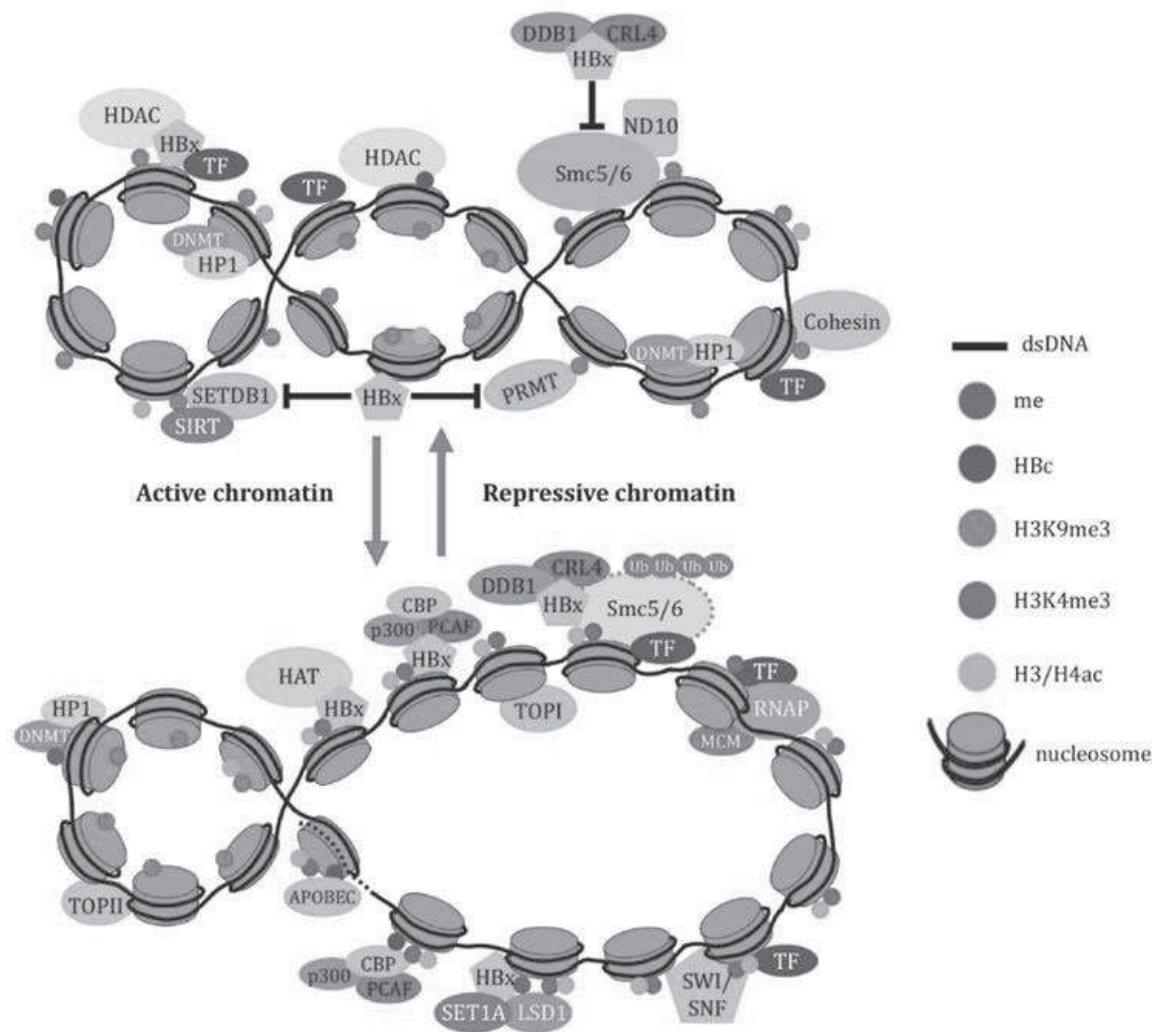
Figure 7. HBV cccDNA minichromosome under different epigenetic modifications.
Image from (Y. Wang et al., 2021).

Linker histones

Liker histones family is characterized by higher sequence variability than the core histones. This is revealed by existence of eleven linker histone variants expressed by the human genome (Happel & Doenecke, 2009; Hergeth & Schneider, 2015). Linker histones are categorized according to their different cell-type expression. Seven members of linker histones (H1.0 to H1.5 and H1.x) are present in somatic cells. The linker histones H1.1 to H1.5 reach a peak of expression at the S phase of the cell cycle, whereas H1.0 and H1.x expression is

generally replication-independent (Happel & Doenecke, 2009; Prendergast & Reinberg, 2021). Moreover, different types of linker histone proteins (H1t, H1T2, H1LS1 and H1oo) are present exclusively in germline cells (Perez-Montero, Carbonell, & Azorin, 2016). The somatic linker histones variants share a common structural organization constituted by a short N-terminal domain, a central globular domain and a long C-terminal domain. The C-terminal domain of H1 is important for chromatin compaction together with the globular part (Bednar et al., 2017; Prendergast & Reinberg, 2021; Turner et al., 2018). H1.1 and H1.2 variants have a short C-terminal tail and weekly compact the chromatin (Clausell, Happel, Hale, Doenecke, & Beato, 2009; Prendergast & Reinberg, 2021). Both the terminal domains are intrinsically disordered and rich of lysine residues which confer a positive net charge to the histone (Bradbury et al., 1975; X. Lu & Hansen, 2004). Considering that lysine amino acid is susceptible of multiple PTMs, all H1 subtypes are profoundly post-translationally modified (Andres, Garcia-Gomis, Ponte, Suau, & Roque, 2020; A. Lu et al., 2009). Besides other PTMs (Andres et al., 2020), phosphorylation events associated with H1 have previously been identified (Balhorn, Chalkley, & Granner, 1972). However, due to the lack of developed specific tools for H1, linker histone modifications are currently less studied than core histones. Due to their tendency to quickly dissociate from the nucleosome, the acquisition of H1 proteins in high-resolution structure is challenging. *Zhou et al*., solved the crystal structures of H1.0, H1.4 and H1.x using single-chain antibody fragment (scFv) to prevent dissociation (B. R. Zhou et al., 2021).

As mentioned before, linker histones are mainly phosphorylated at the tail region level. Especially, the C-terminal tail contains several (S/T)-P-X-(K/R) motifs which are recognized by cyclin-CDKs (Hergeth & Schneider, 2015).

Several studies underlined the link between H1 phosphorylation and replication initiation. In different models, phosphorylation of H1 caused a reduction of its binding affinity to chromatin with consequent detachment of H1 from active promoter regions (Fig. 8) (Belikov, Astrand, & Wrange, 2007; Bhattacharjee, Banks, Trotter, Lee, & Archer, 2001; Hergeth & Schneider, 2015; Koop, Di Croce, & Beato, 2003; Vicent et al., 2011). In an *in vivo* model of *Tetrahymena*,

phosphorylation of Serine (S) or Threonine (T) of H1 causes electrostatic charges with consequent repulsion of DNA chain and transcription modulation (Dou & Gorovsky, 2000). Based on current evidence, phosphorylation of H1 during S phase allows chromatin unfolding for DNA replication (Alexandrow & Hamlin, 2005; Contreras et al., 2003). Combining site-directed mutagenesis and knock-out methods, *Dou et al.,* demonstrated that H1 phosphorylation regulates expression of specific genes, which resulted to be similarly dysregulated after mimicking H1 knock-out (Dou, Mizzen, Abrams, Allis, & Gorovsky, 1999)**.**
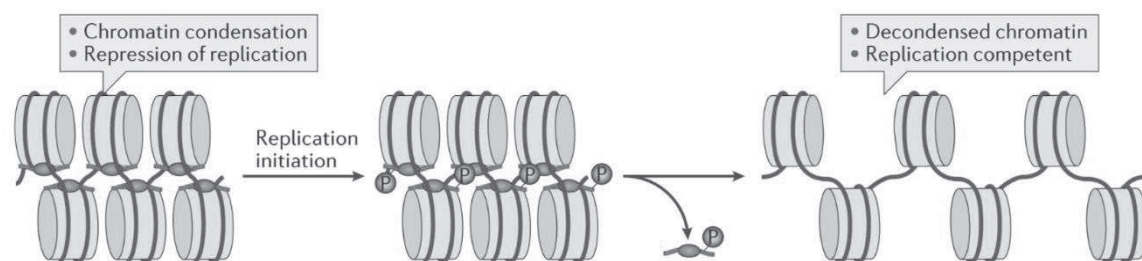
Figure 8.   Phosphorylation   of   linker   histones   associated   with   chromatin decondensation. Image extracted from (Fyodorov, Zhou, Skoultchi, & Bai, 2018).

Surprisingly, additional H1 phosphorylation states during mitosis have been associated with heterochromatin formation. These phosphorylation events on H1 are known to be caused by Aurora B, CDK1 and GSK-3β (Chu et al., 2011; Happel, Stoldt, Schmidt, & Doenecke, 2009; Hergeth et al., 2011). During G1 phase, H1 resulted generally less phosphorylated although no phosphatases have been identified so far. It is possible that phosphorylation of H1, beyond mitosis, occurs also in G1 and S phase for events which require chromatin relaxation. In the context of HBV infection, cccDNA transcription may be one hypothesis. Furthermore, beside cell cycle, it is conceivable that H1 are involved in other chromatin reorganization events, such as DNA repair, which requires chromatin relaxation (Whitefield, Spagnol, Armiger, Lan, & Dahl, 2018). Interestingly, phosphorylation of H1 may act as indicator of DNA damage, allowing

chromatin decondensation and the recruitment of proteins involved in DNA repair. If DNA damage increases, more H1 proteins are phosphorylated and released from the chromatin region which is beyond repair. This may also cause release of cytochrome C and apoptosis (Chubb & Rea, 2010; Kysela, Chovanec, & Jeggo, 2005).

H1 phosphorylation is currently a complex event difficult to comprehensively analyze. As mentioned before, it may mediate both chromatin condensation and relaxation in a still unidentified mechanism. Structural data suggest that different phosphorylated sites of H1 bring different structural changes when bound the DNA, presumably leading to different fates (Roque, Ponte, Arrondo, & Suau, 2008). Another study underlines that phosphorylation on serine residues of linker histones may occur prevalently throughout G1 and G2 phases, whereas phosphorylation events on threonine are more common during mitosis (Sarg, Helliger, Talasz, Forg, & Lindner, 2006). Interestingly, H1 phosphorylation and its consequent detachment from chromatin seems to induce HIV-1 gene transcription (O'Brien, Cao, Nathans, Ali, & Rana, 2010).

H1.4 is one of the most phosphorylated linker histone variants. It has been proposed a model in which H1.4 phosphorylation status on different aminoacids sites may be related to cell cycle progression events (Hergeth & Schneider, 2015) (Figure 9). Phosphorylation at the Ser172 and Ser187 present at the C-terminal domain of H1.4 have been detected during interphase in of H1.4 in human lymphoblastic T-cells and HeLa cells and associated with transcription activation (Sarg et al., 2006; Zheng et al., 2010) (Figure 9). Beside chromatin condensation, specific phosphorylation at Ser187 of H1.4 has been also identified during interphase and correlated with ribosomal genes transcription. Moreover, phosphorylation at Ser187 of H1.4 is induced at steroid hormone response elements following hormone treatment (Zheng et al., 2010). Phosphorylation of Serine on these residues may occur via CDK2. However, there is no evidence on specificity for H1.4 (Bhattacharjee et al., 2001; Contreras et al., 2003; Vicent et al., 2011). On the other hand, phosphorylation of Ser187 studying specifically H1.4 has found to be linked to CDK9 activity and it is associated with transcription of specific genes

involved in pluripotent cell differentiation processes (Liao & Mizzen, 2017). Moreover, phosphorylation of Ser187 in H1.4 by CDK9 is enriched primarily at the transcription start sites of genes related to estrogen response (Saha, Seward, Stubbs, & Mizzen, 2020). Besides that, the other possible specific functions related to H1.4 variant remain to be revealed. During mitosis, the phosphorylation status is the highest, and up to six phosphorylation sites in both N- and C- domains have been reported in HeLa cells (Figure 9) (Hergeth & Schneider, 2015; Zheng et al., 2010). Here, phosphorylation occurs at both Serine and Threonine residues, and it may be mediated by CDK1 and Aurora B (Baatout & Derradji, 2006). Schneider et al., reported phosphorylation of H1.4 at Ser27 during mitosis by Aurora B kinase in mouse fibroblasts and HeLa cells (Daujat, Zeissler, Waldmann, Happel, & Schneider, 2005; Hergeth et al., 2011). In addition to the ones shown in figure 9, another H1.4-specific phosphorylation has been identified by Chu et al. By *in vitro* kinase assay. They demonstrated that H1.4 can be phosphorylated at Ser35 by PKA during mitosis and it accumulates after phosphorylation of H3 at ser10, but it cannot be phosphorylated by Aurora kinase, revealing a site-specific phosphorylation event. Importantly, this phosphorylation causes the dissociation of H1.4 from the mitotic chromatin (Chu et al., 2011; Prendergast & Reinberg, 2021).

Generally, few studies have been performed so far focusing on the different linker histones variants. Moreover, studies on H1 have been performed mainly using HeLa cells, which possess restricted heterogeneity of H1 variants (Zheng et al., 2010). Generally, it is emerging that global histone H1 phosphorylation is associated with cell cycle progression but the functions of phosphorylation on the site-specific H1 variants remain largely unknown (Prendergast & Reinberg, 2021).
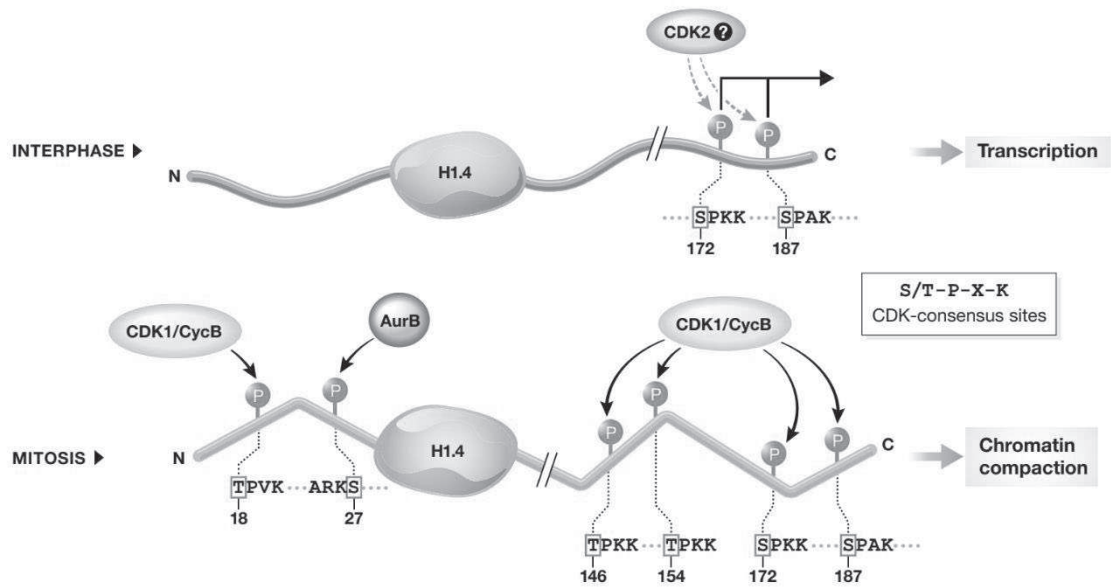
Figure 9. Scheme of possible H1.4 phosphorylation status associated with cell cycle phases. From (Hergeth & Schneider, 2015).

Cell cycle and HBV

Generally, cell cycle is a precisely regulated process which contains multiple checkpoints and can be divided into two big phases: interphase and mitosis (M). Normally, cells spend 95% of the time in interphase, which includes gap 1 (G1), DNA synthesis (S) and gap 2 (G2) phases (G. M. Cooper, 2000). During G1, the cells actively growth but they do not replicate their DNA. In this phase, cells are diploids (2*n*), therefore contain two copies of each chromosome. During this step, the cell carried out a series of processes necessary for DNA synthesis which occurs in the S phase. Moreover, the cell can skip the S phase and enter in a quiescent state, called G0. Following S phase, phase G2 is propaedeutic for entering in mitosis (Vermeulen, Van Bockstaele, & Berneman, 2003). The existence of checkpoints is crucial for cell cycle regulation. The cell cycle is transcriptionally regulated by CDKs which control cell cycle progression by the phosphorylation of protein targets. Among them, retinoblastoma-associated protein (pRb) is a major G1 checkpoint (Bertoli, Skotheim, & de Bruin, 2013; Giacinti & Giordano, 2006). When it is not phosphorylated, pRb represses the transcription of genes involved in the transition between G1 and S phase, by both binding to the transactivation domain of the transcription factor E2F and to the promoter regions of the genes (Adams & Kaelin, 1995; Giacinti & Giordano, 2006). Thus, pRb appears hypophosphorylated in G0 and early G1 phase (Giacinti & Giordano, 2006; Mihara et al., 1989), often leading to G1 arrest (Rigberg, Kim, Sebastian, Kazanjian, & McFadden, 1999). When it is phosphorylated, pRb conformational changes provoke a decrease in affinity for E2F, which is therefore active (Fig. 10) (Giacinti & Giordano, 2006).
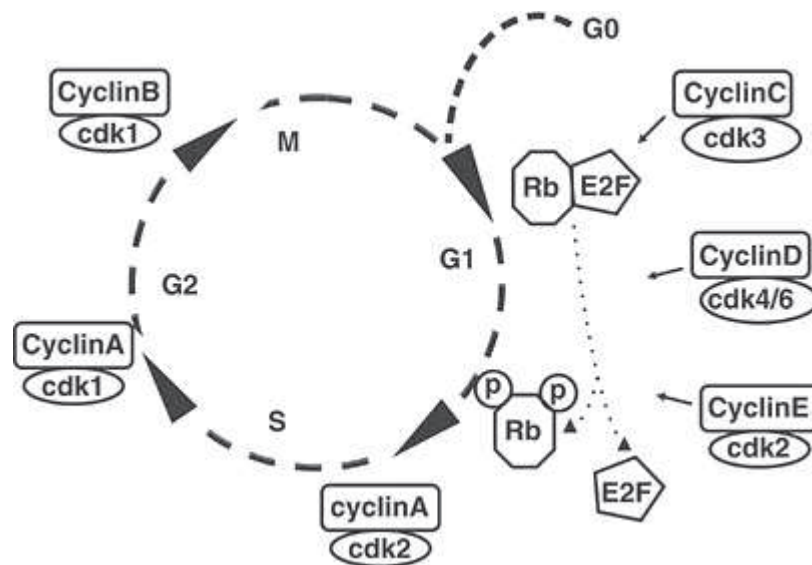
Figure 10. pRb phosphorylation and cell cycle progression events. Image from (Giacinti & Giordano, 2006).

In the context of HBV, evidence suggests that HBV induces a cell cycle arrest in G1 phase with consequent increase of infection rate (Eller et al., 2020; T. Wang et al., 2011). A recent study identified cyclin-dependent kinase 4 inhibitor C (CDKN2C) as an important host factor for HBV replication, acting through inhibition of CDK4/6, impairs pRb phosphorylation with consequent G1 arrest. At the same time, Palbociclib, is likely to act following the same mechanism (Fig. 11) (Eller et al., 2020). On the other hand, studies in HBV-infected PHHs indicate that HBV prevalently replicates in G2/M (Xia et al., 2018). Interestingly, a study on PHHs demonstrated that hepatocytes division triggers significative cccDNA loss (Allweiss et al., 2018). Together, these results suggest that perturbation of cell cycle promotes HBV infection/replication and impacts cccDNA stability. However, the exact mechanism of HBV-induced cell cycle arrest remains unclear. Moreover, it needs to be clarified if HBV strategically inhibits cell division to avoid cccDNA loss or whether HBV better replicates in subpopulation of cells refractory to cell division (Allweiss & Dandri, 2017).
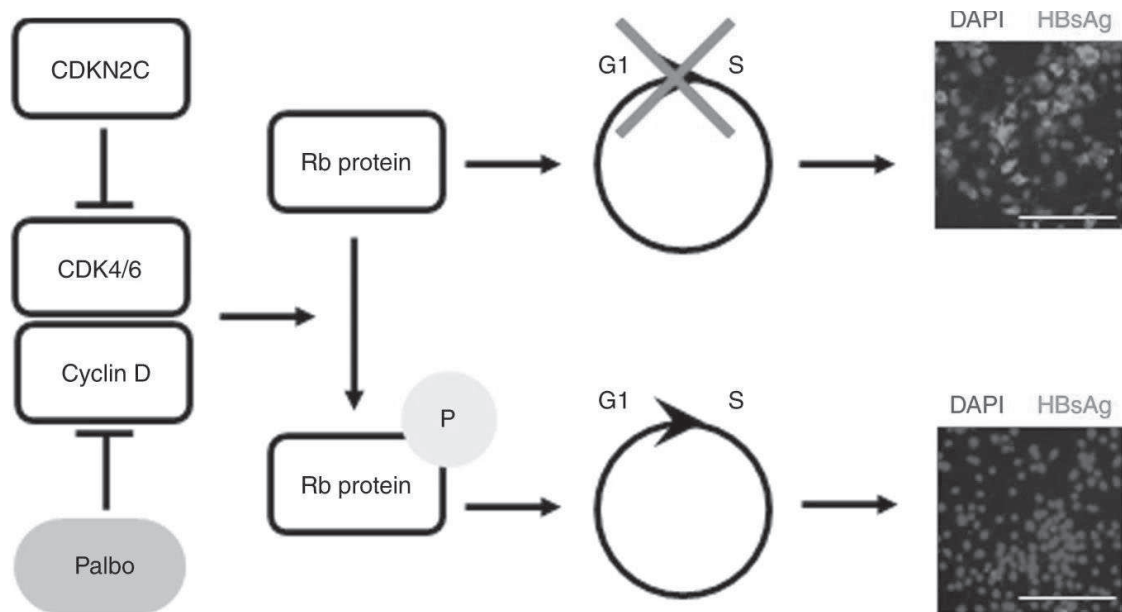
Figure 11. pRb protein phosphorylation induces cell cycle progression. Image from (Eller et al., 2020).

CHAPTER 3

"Wherever the art of medicine is loved, there is also a love of humanity"

"Cure sometimes, treat often, comfort always"

Hippocrates

HBV-INDUCED LIVER DISEASE

HBV infection is highly dynamic and it is characterized by replicative and non-replicative phases. If the infection occurs in adults, it is usually self-limited with a small probability (3-5%) to become persistent. However, this number rises to 95% if the infection occurs during childhood (Fig. 12) (CADTH, 2018). Nevertheless, young adults remain at higher risk of fulminant HBV (Ichai & Samuel, 2019). Chronic infection, as well as the acute one, can be symptomatic or asymptomatic. It has been estimated that one third of HBV infected individuals develops cirrhosis, liver failure and HCC if left untreated. Especially, the cumulative incidence at 5 years of cirrhosis in untreated CHB individuals, after diagnosis, ranges between the 8% and 20% (Fig. 12) (EASL, 2012; Tao et al., 2020). In turn, the 5-year cumulative incidence of hepatic decompensation in cirrhotic patients is 20%. Moreover, 2-5% of cirrhotic patients develop HCC every year (Fig. 12) (EASL, 2017; Raffetti, Fattovich, & Donato, 2016; Tao et al., 2020). HBV is known to cause HCC also through direct mechanisms of insertional mutagenesis, genomic instability and through the oncogenic role of its proteins (Levrero & Zucman-Rossi, 2016).

Figure 12. HBV-associated liver disease progression. Figure modified from (Fricker & Reddy, 2019).

Other etiologies contribute to liver cancer death worldwide, therefore the risk associated to HBV-associated liver cancer widely ranges among different geographic areas (Global Burden of Disease Liver Cancer et al., 2017). For instance, HBV caused 45% of liver cancer deaths in Wester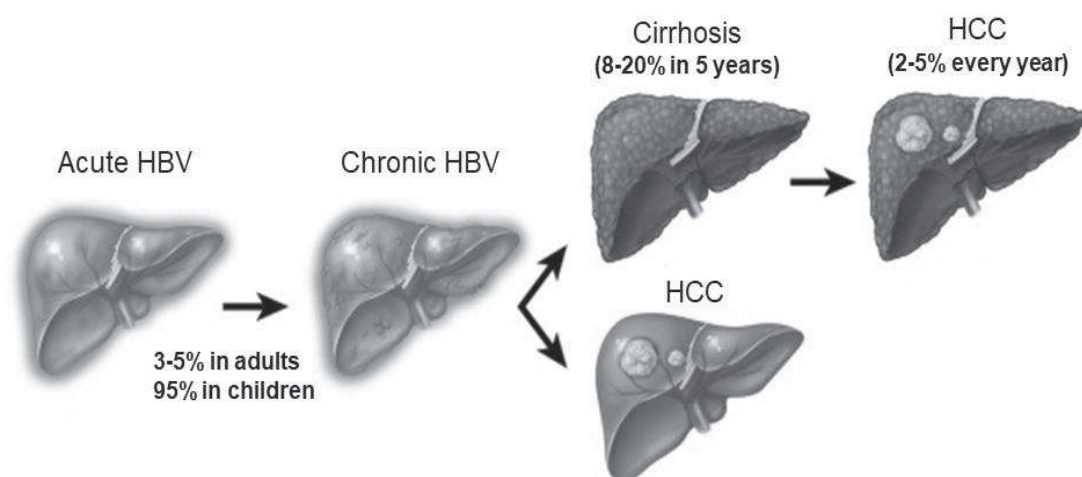n sub-Saharan Africa and Andean Latin America, whereas only 4% in Mexico by 2015 (Global Burden of Disease Liver Cancer et al., 2017), where other etiologies such as alcohol consumption heavily contribute to the liver disease development (Yeverino-Gutierrez, Gonzalez-Gonzalez, & Gonzalez-Santiago, 2020). Moreover, it is important to consider that in 2015, only 9% of HBV infected individuals were aware of their diagnosis. Among them, only 8% were receiving an antiviral therapy (Asselah et al., 2019). Moreover, HBV causes several extrahepatic manifestations which are possibly immune mediated, such as polyarteritis nodosa, arthritis-dermatitis syndrome and neurological disorders (S. H. Han, 2004).

NATURAL HISTORY OF THE DISEASE

The National Institutes of Health supported two research workshops on the management of CHB in 2000 and 2006 (Hoofnagle, Doo, Liang, Fleischer, & Lok, 2007; Lok, Heathcote, & Hoofnagle, 2001). In these occasions, three phases of CHB infection have been defined by the participants, considering the level of HBeAg, HBV DNA, alanine aminotransferase (ALT) and the grade of liver inflammation. These phases can be schematically summarized in immune tolerant phase, immune active phase and inactive carrier phase. Today, two additional phases can be described. Altogether, this summarizes the natural history of HBV infection (Fig. 13) (CADTH, 2018; Fanning et al., 2019; McMahon, 2009).

Phase I or immune-tolerant phase. It is characterized by the presence of HBeAg, HBsAg and antibodies against HBc (anti-HBc) in serum and very high level of HBV DNA (10^9 to 10^{10} IU/mL), which confers high contagiousness and a high frequency of integration event, suggesting that hepatocarcinogenesis may start already at early phase (CADTH, 2018). Spontaneous HBeAg rate loss is very low and there is no or low liver necroinflammation and

normal ALT (ULN <40 IU/L). This phase typically is short but may persist for years in the case of vertical transmission (McMahon, 2009).

Phase II or HBeAg-positive immune-active phase. This phase is characterized by HBeAg presence in the serum, high levels of HBV DNA (above 2000 IU/mL), elevated ALT level, active liver necroinflammation with fibrosis (but not always) (Ezzikouri, Kayesh, Benjelloun, Kohara, & Tsukiyama-Kohara, 2020). It is more common among individuals which get infected during adulthood and the transition from the immune-active to the inactive phase occurs normally in 67% to 80% of patients. In these individuals, the transition to the inactive phase is characterized by spontaneous HBeAg seroconversion to antibodies against HBeAg (anti-HBe) and HBV viral suppression (CADTH, 2018; McMahon, 2009).

Phase III or HBeAg-negative chronic infection (historically termed as ''inactive-carrier phase''). This phase is characterized by the absence of HBeAg but the presence of anti-HBe. HBV DNA level is low or no detectable (< 2,000 IU/mL), and ALT level is normal. Approximately 1% to 3% of patients per year undergo spontaneous HBsAg loss and/or seroconversion. Nevertheless, some patients (approximately 4% to 20%) can experience reversions back to an HBeAg-positive state. Moreover, minimal liver necroinflammation and fibrosis can be present, resulting from previous liver injury during the immune-active phase (CADTH, 2018; EASL, 2017; McMahon, 2009).

Phase IV or advanced HBeAg-negative chronic hepatitis. This phase is characterized by the absence of serum HBeAg, accompanied by detectable anti-HBe and unstable moderate to high levels of serum HBV DNA in 10% to 30% of seroconverted patients. In this step, the rate of spontaneous disease remission is low (EASL, 2017; McMahon, 2009).

Phase V or resolved CHB infection categorized by the European Association for the Study of the Liver (EASL) is characterized by the absence of HBsAg and the presence of anti-HBs and anti-HBc in the serum. Also known as occult HBV infection, patients in this phase have normal ALT values and usually undetectable serum HBV DNA. If cirrhosis has developed before

HBsAg loss, patients remain at risk of HCC, therefore the surveillance is important (CADTH, 2018; EASL, 2017; Ezzikouri et al., 2020; McMahon, 2009).

	HBeAg		anti-HBe		
	HBeAg⁺ chronic infection	HBeAg⁺ chronic hepatitis	HBeAg⁻ chronic infection	HBeAg⁻ chronic hepatitis	Functional cure
	Phase I	Phase II	Phase III	Phase IV	Phase V
Serum HBsAg	+++++	++++	+	++	−
cccDNA	+++++	+++++	++	+++	+
Fibrosis	Minimal fibrosis	Variable degree of fibrosis (progressing)	Variable degree of fibrosis (progressing)	Moderate to severe fibrosis (resolving)	Variable fibrosis (sequelae of previous phases)

Figure 13. **Natural history of chronic hepatitis B**. Clinical and virological phases of HBV infection. Image modified from (Fanning, Zoulim, Hou, & Bertoletti, 2019).

DEFINITION OF CURE FOR CHRONIC HEPATITIS B

The development of drug-acting antivirals (DAAs) as a remarkable treatment for CHC opened a new era in the history of antiviral therapy and renewed the search for a cure against CHB. Although many progresses have been done in the pipeline of drug development (Lok, Zoulim, Dusheiko, & Ghany, 2017a), the approval of an optimal treatment against HBV is still pending and HBV patients often required lifelong therapy (Lok, Zoulim, Dusheiko, & Ghany, 2017b). Three different levels of HBV cure have been defined so far by the scientific community

(H. W. Lee, Lee, & Ahn, 2020; Lok et al., 2017b; Tsounis, Tourkochristou, Mouzaki, & Triantos, 2021):

1) **Partial cure** is characterized by undetectable serum level of HBV DNA and normal ALT level but detectable HBsAg. It is considered the acceptable intermediary step since it has been associated with a reduction in liver disease progression after short-term treatment (Liaw et al., 2004; Lok et al., 2017b).

2) A sustained loss of serum HBsAg, called **functional cure**, is assumed to indicate an efficient immune response against the HBV infection and improves long-term outcomes. For this reason, it is a better endpoint to the partial cure. Nevertheless, permanent HBsAg loss is not always achieved, or it can take many years. Today, it is considered to be the most realistic therapeutic goal (Cornberg, Lok, Terrault, Zoulim, & Faculty, 2019; EASL, 2017).

3) **Virological cure** also known as complete sterilizing cure, is defined as the total eradication of the virus from the organism. It is characterized by the absence of HBsAg in the serum and the eradication of HBV DNA from both serum and liver. However, due to the integration of HBV DNA into the host genome and the persistence of the cccDNA within the hepatocytes, this does not represent yet a realistic therapeutic aim. (Lok et al., 2017b; Virzi et al., 2021).

ANTIVIRAL THERAPY

HBV DNA levels and cirrhosis are strong indicators for antiviral therapy. Indeed, high serum level of HBV DNA has been correlated to increased risk of developing cirrhosis and HCC (C. F. Chen et al., 2011; C. Liu et al., 2018). Moreover, also elevated levels of HBV core-related antigen (HBcrAg) reflected an increased risk of cirrhosis progression (Tada et al., 2018) and correlates with cccDNA amount (Suzuki, Miyakoshi, Kobayashi, & Kumada, 2009), as well as HBsAg (Tantiwetrueangdet, Panvichian, Sornmayura, Sueangoen, & Leelaudomlipi, 2018). The decision to begin the antiviral treatment as well as length and combination of drugs, is based on several factors. Strong indicators for antiviral therapy are HBV DNA, ALT levels and

the grade of liver necroinflammation or fibrosis, measured by liver biopsies or non-invasive biomarkers. Moreover, age, disease history, other diseases and patient's characteristics are considered. Importantly, drug resistance strongly impairs the efficacy of the antiviral therapy against HBV (Dogan, Ozturk, Akin, Yalaki, & Sayan, 2014; EASL, 2017; Tao et al., 2020). Generally, the current antiviral therapy is recommended for CHB patients in immune-active phase, whereas patients with normal ALT level and low or undetectable HBV DNA, especially if of young age (< 30 years old) have good prognosis and are not candidates for antiviral treatment, in particular to avoid the risk of drug resistance development (G. L. Wong, 2018). Nevertheless, HBV infection is dynamic and immune tolerant-phase individuals may reach the immune active phase therefore it is important to constantly monitor the disease course to guide the treatment (Terrault et al., 2018). In the case of compensated cirrhosis and detectable HBV DNA level, indefinite therapy has to be followed to decrease the risk of decompensation and HCC (EASL, 2017). Moreover, patients with extrahepatic manifestations which are associated with increased morbidity and mortality, such as vasculitis and neurological complications, as well as patients with comorbidities, are possible candidates for antiviral therapy (Asselah et al., 2019). The goal of the therapy is to reach undetectable levels of HBV DNA, seroconversion of HBeAg and normalization of ALT level. However, the optimal endpoint consists in the HBsAg loss, which occurs rarely (Tout et al., 2020), and cccDNA remains the main obstacle for permanent HBV eradication. Currently, the main purpose of antiviral treatment against HBV is the increment of survival rate and the decrease of risk of liver disease complication (Fig. 14). Antiviral drugs consist of nucleoside/nucleotide analogues (NAs) and polyethylene glycol interferon-alpha (Peg-IFN-α).
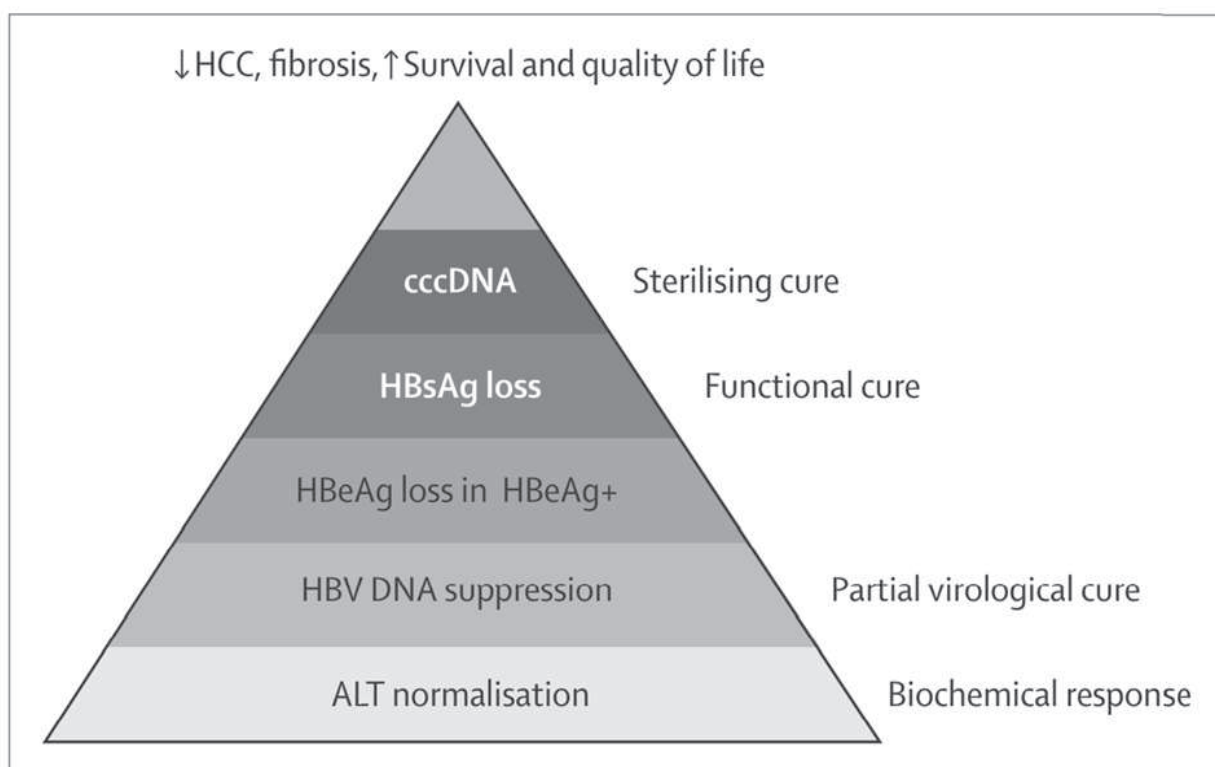
Figure 14. Therapeutic goals for HBV patients. Image from (Asselah, Loureiro, Boyer, & Mansouri, 2019).

Peg-IFN-α

The role of the immune system against HBV infection is controversial as HBV seems to counteract the immune response and IFN activation acting as "stealth" virus (Cheng et al., 2017; Durantel & Zoulim, 2009; Suslov, Boldanova, Wang, Wieland, & Heim, 2018; Verrier et al., 2018). Pegylated IFN-α (Peg-IFN-α) has better pharmacokinetics proprieties than the non-pegylated form and it is used against HBV infection (Asselah et al., 2007). IFN-α exerts antiviral, antiproliferative and immunomodulatory activities on both innate and adaptive immune response. However, the efficacy of IFN-α is limited and several factors such as HBV genotype, viral load and host factors, influence the therapeutic response to IFN-α treatment (Tao et al., 2020; Ye & Chen, 2021). In addition to that, side effects are common, and the administration requires injection. It is contraindicated for pregnant women, patients with decompensated cirrhosis or autoimmune diseases. Despite that, IFN-α therapy has some advantages, such as the absence of drug resistance and the treatment duration that it is

relatively definite (Tao et al., 2020). Even if this therapy induces high rate of HBsAg loss, it is usually effective in a minority of patients (D. K. Wong et al., 1993).

Nucleos(t)ide analogues (NAs)

Long-term NAs treatment represents the first line therapy for patients with CHB (Tout, Lampertico, Berg, & Asselah, 2021). NAs therapy is preferred by the patients in comparison with IFN for its easier oral administration and better tolerability. However, patients who skip the doses increase the risk of drug-resistant variants development (Marcellin, Sung, & Piratvisuth, 2010). The mechanism of action of NAs consists in the inhibition of HBV reverse transcriptase activity (Menendez-Arias, Alvarez, & Pacheco, 2014). For this reason, some NAs are additionally used against HIV (Kammarabutr, Mahalapbutr, Okumura, Wolschann, & Rungrotmongkol, 2021). The NAs approved as therapy for CHB infection can be classified as followed:

- Low barrier to HBV resistance: Lamivudine (LAM), Adefovir dipivoxil (ADV) and Telbivudine (LdT) which represents the first-generation NAs.
- High barrier to HBV resistance: Entecavir (ETV), Tenofovir disoproxil fumarate (TDF) and Tenofovir alafenamide fumarate (TAF).

The NAs with high barrier to HBV resistance are recommended as first line oral anti-HBV treatment and currently they are the most potent drugs against HBV. In general, they are characterized by poor side effects and little need of monitoring. ETV is widely used. However, since ETV has low antiviral activity against HIV, it should not be used alone in patients with HBV-HIV co-infection to avoid antiviral resistance (LiverTox, 2012). Tenofovir is poorly absorbed orally and therefore the prodrugs TDF and TAF have been developed and currently are used. After entering the cell, tenofovir is phosphorylated into its active form, tenofovir diphosphate. TAF is considered more secure in term of side effects, especially regarding phosphate metabolism, bone, and kidney toxicity (LiverTox, 2012) therefore patients monitoring is essential (Tao et al., 2020).

New drug development for HBV therapy

Nowadays, the antiviral treatments for CHB can ensure partial cure in most patients and reduce morbidity and mortality due to HBV-associated liver diseases. However, functional cure is achieved in a small portion of patients (10%) and requires treatment for a long or indeterminate period of time (Tao et al., 2020). Moreover, though NAs may reduce cccDNA level, its eradication is hardly achievable. For these reasons, novel therapeutic drugs are under development with the objective to induce HBsAg loss and ultimately cccDNA eradication, with the possibility of immune restoration (Asselah et al., 2019).The new classes of antiviral drugs acting against HBV can be summarized in Table 1. They can be schematically divided into molecules which target different steps of HBV life cycle (direct-acting antivirals) and immune modulators.

Direct-acting antivirals targeting HBV life cycle

This new category of drugs therapeutically targets different steps of HBV life cycle, such as entry, HBsAg secretion, capsid assembly and cccDNA formation (Alexopoulou, Vasilieva, & Karayiannis, 2020; E. Tsai, 2021). In 2020, Bulevirtide (previously Myrcludex), an HBV/HDV entry inhibitor, has been approved for chronic HDV infection in HDV RNA positive adults with compensated liver disease (H. W. Lee et al., 2020; Loureiro, Castelnau, Bed, & Asselah, 2022; Yardeni & Koh, 2021). Other entry inhibitors are constituted by cyclosporine (CsA) derivatives such as SCY450 and SCY995 which reduce the adverse effect of cyclosporine on bile acid transport (Shimura et al., 2017). JNJ-56136379 and ABI-H0731 belong to the capsid assembly modulators and bind/inhibit core protein preventing the formation of the viral capsid (Q. Huang et al., 2020; Zoulim, Lenz, et al., 2020). JNJ-56136379 (JNJ-6379) showed a good tolerability and antiviral activity. However, decrease in HBcrAg were not accompanied by relevant changes in HBsAg levels as been estimated after short-term treatment (Zoulim, Lenz, et al., 2020). Among the HBsAg secretion inhibitors, REP 2139, REP 2055 and REP 2165 are nucleic acid polymers with the ability to clear circulating HBsAg by blocking the releasing of SVPs, widely produced during the infection (Al-Mahtab, Bazinet, & Vaillant, 2016; Bazinet et al.,

2017). Clinical trials on REP 2139 showed a substantial attenuation of HBsAg and seroconversion to anti-HBsAg after a relatively short period of time by intravenous administration (Bazinet et al., 2017). The ultimate approach toward HBV cure is targeting cccDNA. Several strategies are in course of development. Partial elimination of cccDNA can be obtained by deamination, for example via APOBEC3A/3B cytidine deaminase (Lucifora et al., 2014). Moreover, progresses in genetic editing techniques made possible the establishment of direct approaches to specifically target cccDNA (Kayesh et al., 2020; van den Berg et al., 2020). However, despite the encouraging results obtained, gene-editing approaches need to be better studied to address off-target effects before reaching the clinical application (Anderson et al., 2018). On the other hand, the regulation of cccDNA transcription by epidrugs may represent a valuable therapeutic option in the next future (Zeisel, Guerrieri, & Levrero, 2021).

Immune response modulation

Some of the immune modulators under development are the TLR-7 and TLR-8 agonists, retinoic acid-inducible gene 1 (RIG-I)/nucleotide-binding oligomerization domain protein 2 (NOD-2) agonists, programmed death-1 (PD-1) inhibitors and therapeutic vaccines. The mechanism of action of TLR agonists consists mainly of the induction of IFNs and the activation of natural killer and T cells (Amin et al., 2021; Jo et al., 2014; Luk et al., 2020; Niu et al., 2018). An example is constituted by GS-9620 (Vesatolimod), a TLR-7 agonist (K. Agarwal et al., 2018). Targeting of RIG-I signaling enhanced antiviral defenses against HBV (S. Lee et al., 2021). The RIG-I agonist SB 9200 (Inarigivir) caused a reduction of HBsAg in the 22% of patients (Smolders, Burger, Feld, & Kiser, 2020). However, this drug has been withdrawn due to liver toxicity (Kosh Agarwal et al., 2020).

During CHB infection, T-cells express PD-1 which contributes to T cell exhaustion. Therefore, molecular inhibition of PD- 1 pathway may promote the restoration and proliferation of HBV-specific T-cells (Feray & Lopez-Labrador, 2019). Nivolumab, an anti-PD-1 mAb, has been recently investigated (Gane et al., 2019).

Another method to boost HBV-specific immune responses can be the administration of therapeutic vaccines against HBV. Several compounds have been proposed using different strategies for development of therapeutic vaccines (Cargill & Barnes, 2021; D. T. Jansen, Dou, de Wilde, Woltman, & Buschow, 2021). Among the many, GS-4774 (Boni et al., 2019), TG1050 (Zoulim, Fournier, et al., 2020) and GSK3528869A (Knolle, Huang, Kosinska, Wohlleber, & Protzer, 2021) are currently under investigation.

Category	Name	Action	Reference
HBV life cycle			
Entry inhibitor	Bulevirtide (Hepcludex, previously Myrcludex)	NTCP competitive binding	(Yardeni & Koh, 2021)
	SCY450 (CsA derivative)	NTCP inhibition	(Shimura et al., 2017)
	SCY995 (CsA derivative)		
Capsid assembly modulator (CAM)	JNJ-56136379 (JNJ-6379)	HBV core binding/inhibitor	(Zoulim, Lenz, et al., 2020)
	ABI-H0731		(Q. Huang et al., 2020)
HBsAg secretion inhibitor	REP 2139	Blockage of SVPs assembly	(Bazinet et al., 2017) (Al-Mahtab et al., 2016)
	REP 2055		
	REP 2165		
cccDNA inhibitor	APOBEC3A	cccDNA deamination	(Lucifora et al., 2014)
	APOBEC3B		
Immune response modulation			
TLR agonist	GS-9620 (Vesatolimod)	TLR-7 agonist	(K. Agarwal et al., 2018)
RIG-I/NOD-2 agonist	SB 9200 (Inarigivir)	RIG-I agonist	(Kosh Agarwal et al., 2020)
Immune checkpoint	Nivolumab	PD-1 inhibitor	(Gane et al., 2019)
Therapeutic vaccine	GSK3528869A	Viral vector and adjuvanted proteins as immunogens	(Knolle et al., 2021)
	GS-4774	Vector-based	(Boni et al., 2019)
	TG1050	Adenovirus-based	(Zoulim, Fournier, et al., 2020)

Table 1. New therapeutic strategies proposed to cure HBV infection.

CHAPTER 4

''Many diseases are the result of bad collagens../.. If we could understand those signals of regeneration we could cure most diseases, renew the body's organs, reverse chronic diseases"

Morten Asser Karsdal from TEDx talk, February 2021

FIBROSIS AS IMPORTANT INDICATOR OF LIVER DISEASE SEVERITY

The liver, more than any other organ of the human body, has a remarkable ability to adapt to injury through regeneration. For this reason, wound healing and liver fibrosis can be considered for many aspects highly conserved reparative mechanisms against tissue damage. If fibroblast's activation and collagen production can be balanced by scar-resolving mechanisms upon acute or short-term liver injury, fibrosis brings to an excessive accumulation of ECM proteins, leading to distortion of the normal liver architecture and cirrhosis. Currently, approximately 5% of the global population has liver fibrosis, evaluated by transient elastography (Caballeria et al., 2018). The development of fibrosis with consequent cirrhosis of the liver causes *septae* and nodule formation, altering the blood flow and finally compromising the organ functions (Pellicoro, Ramachandran, Iredale, & Fallowfield, 2014). Especially, chronic portal hypertension is the major consequence of clinical complications of liver fibrosis (D'Amico et al., 2018). For these reasons, the assessment of liver fibrosis has an important prognostic value for liver disease progression and survival (Berumen, Baglieri, Kisseleva, & Mekeel, 2021). Although a robust series of data demonstrated that the elimination of the underlying etiology may stop fibrosis progression (Rockey, 2016), the risk of HCC still occurs, especially in the case of CHB infection where cccDNA persists and HBsAg seroconversion is rare. Moreover, low but persistent level of residual HBV may still promote fibrosis progression (Y. Sun et al., 2020).

At cellular level, the classical hallmark of liver fibrosis is the activation of the HSCs, which secrete ECM proteins, such as collagen I and III, predominant in the fibrotic scar. Moreover, liver fibrosis is associated with release of pro-inflammatory cytokines, chronic inflammation and hepatocytes death (Berumen et al., 2021). All these aspects, then crucial for tumor microenvironment formation and hepatocarcinogenesis development, are modulated by the innate and adaptive immune system (Saviano et al., 2019). This topic was elaborated in

more details in a review which I co-authored and it is provided in the ANNEX: <u>Annex VII:</u>

<u>Stromal and Immune Drivers of Hepatocarcinogenesis</u>.

ECM REMODELING: FROM MECHANICAL TENSION TO PATHOLOGICAL FUNCTION

The ECM homeostasis is crucial for the physiological development of tissues and organs, and mutations in the associated genes expression are the cause of connective tissues diseases (Poltavets, Kochetkova, Pitson, & Samuel, 2018). Besides providing structural support, the ECM regulates cellular behavior representing a reservoir of several different proteins and molecules involved in a wide range of signaling pathways and mechanotransduction events. A reciprocal influence exists between the biochemical and biomechanical changes of the ECM and disease development, as well as cancer cell plasticity (Poltavets et al., 2018). For this reason, understanding the behavior of the dynamicity of the ECM and its composition is required for evaluation of prognosis in liver disease as well as for the development of valuable biomarkers and the identification of druggable targets. ECM is represented by a precise and complex assortment of molecules which are flexible or extensible such as collagens, glycoproteins, glycosaminoglycans and proteoglycans and influences saliently the intra-cellular signaling of different cell types residing within the tissue. Alteration of ECM composition arises from aberrant expression or turnover of these components, as well as altered post-translational modifications (Chang et al., 2017; Hastings, Skhinas, Fey, Croucher, & Cox, 2019; Karsdal et al., 2013). In physiological condition, the epithelial cells normally interact with the components of the basement membranes of the ECM, such as laminin (LN) and collagen IV. However, tissue injury and disruption of the basement membrane cause a rearrangement of ECM proteins which emerge from the sub-epithelium to the basement and are recognized by cells surface receptors with consequent signaling pathways deregulation (Yokosaki & Nishimichi, 2021). Interestingly, proteomic studies on lung cell model suggested that the excess of secreted components saturates the ECM and therefore provokes an increased turnover. Moreover, crosslinking of ECM proteins may influence their solubility and function and impacts cell signaling (Decaris et al., 2014; Schiller et al., 2015). The ECM homeostasis is tightly regulated by matrix metalloproteinases (MMPs) and their specific regulators tissue inhibitors of the metalloproteinases (TIMPs) (Arpino, Brock, & Gill, 2015).

Discoordination in the activity of these enzymes has demonstrated to impar the normal liver function. MMP1 has been associated with decrease of liver fibrosis (Iimuro et al., 2003), whereas MMP2 levels tend to rise with fibrosis progression (Takahara et al., 1997). Moreover, TIMPs inhibit degradation activity of MMPs, therefore augmenting ECM deposition (Cabral-Pacheco et al., 2020).

Mechanisms of hepatic stellate cells activation

Quiescent HSCs represents 3-5% of all resident liver cells and are characterized by the storage of retinyl esters in lipid droplets which allow their isolation by density gradient centrifugation (Geerts, 2001; Wake, 1971; Weiskirchen & Gressner, 2005). HSCs activation is a transdifferentiation process from vitamin A-storing cells to myofibroblasts, which become more proliferative and enhance ECM components production (Puche, Saiman, & Friedman, 2013) (Fig. 15). Different cell types contribute to HSCs activation, among them, the hepatocytes (J. Han et al., 2019; Y. S. Lee et al., 2017; Z. Tan et al., 2018; Wagai, Yamaguchi, Sekiguchi, & Tawara, 1990). Moreover, HSCs surface receptors interact with a plethora of ECM molecules which allow the cell to adapt according to the surrounding microenvironment (Poltavets et al., 2018).
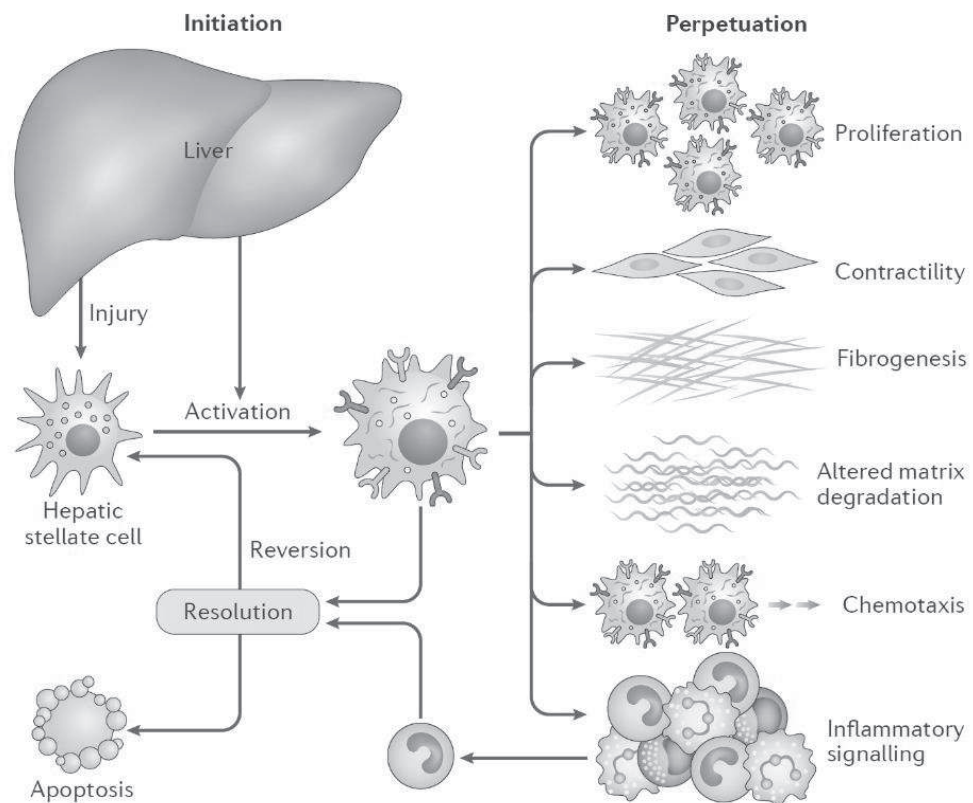
Figure 15. Activation and resolution mechanisms of HSCs after liver injury. Image from (Tsuchida & Friedman, 2017).

HSCs activation: interplay with hepatocytes and signaling pathways involved

Liver injury affects the gene expression and secretome profile of hepatocytes and other cell types. Injured hepatocytes release a wide list of mediators which influence inflammation and fibrosis. Among them, damage-associated molecular patterns (DAMPs), exosomes containing microRNAs, IL-33 and other cytokines contribute to HSCs activation (Y. S. Lee et al., 2017; Roehlen, Crouchet, & Baumert, 2020; Z. Tan et al., 2018) (Fig. 16). The release of transforming growth factor-β (TGF-β), platelet derived growth factor (PDGF), vascular endothelial growth factor (VEGF) and connective tissue growth factor (CTGF) by hepatocytes, macrophages, Kupffer cells and biliary epithelial cells also have a crucial role in HSCs activation (Kikuchi et al., 2017; Tsuchida & Friedman, 2017; Ying et al., 2017). TGF-β, which constitutes the most studied and potent HSCs activator, regulates SMAD signaling pathway via the binding and consequent phosphorylation of the TGF-beta receptor type-1 (TGFβR1). Phosphorylation of SMAD3 predominantly promotes the transcription of type I and III collagens. In addition, TGF-β promotes HSCs activation by the initiation of non-canonical signaling pathway which includes the mitogen-activated protein kinase (MAPK), PI3K/Akt, mammalian target of rapamycin (mTOR) and Rho/GTPase (Dewidar, Meyer, Dooley, & Meindl-Beinker, 2019). PDGF and VEGF are factors mainly responsible for liver regeneration and repair and play a key role in angiogenesis, proliferation and migration of HSCs (Kantari-Mimoun et al., 2015; Kocabayoglu et al., 2015; L. Yang et al., 2014). In addition, CTGF is implicated in ECM production and cells proliferation, migration and survival (G. Huang & Brigstock, 2012).
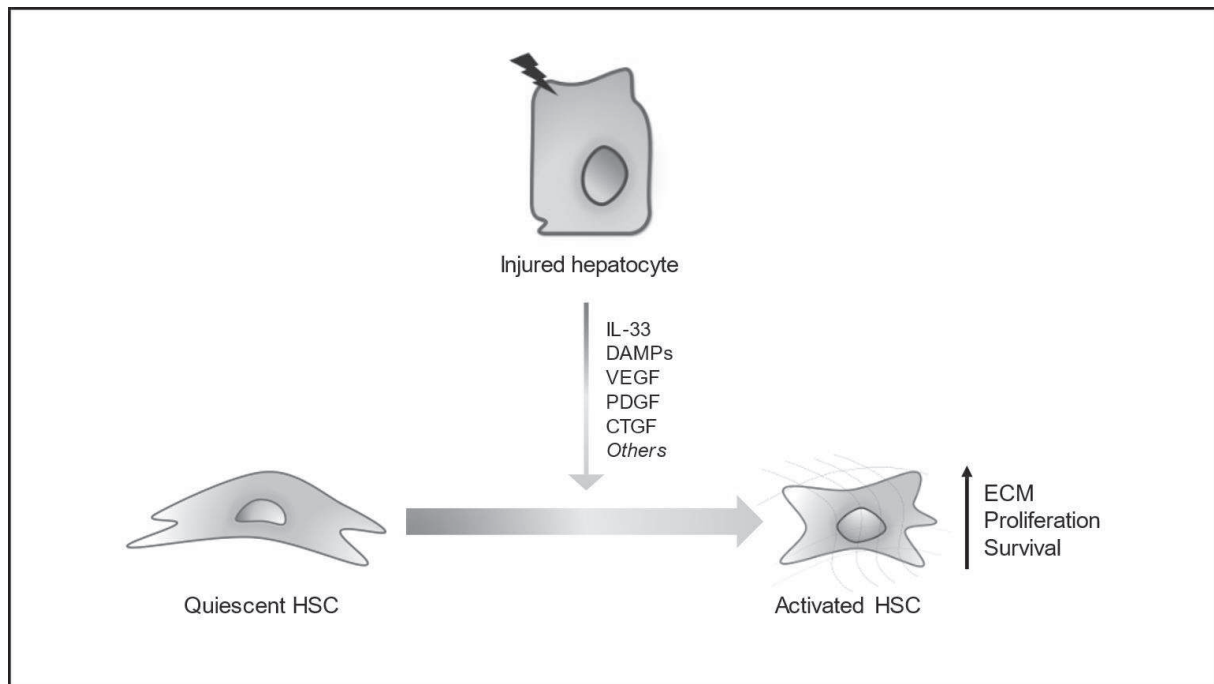
Figure 16. Cartoon showing some of the known activators of HSCs released by injuried hepatocytes.

The leading players of hepatic fibrosis represented by TGF-β, ECM molecules and HSCs, depend on integrin proteins to exert their functions (Henderson & Sheppard, 2013; Nishimura, 2009; Yokosaki & Nishimichi, 2021). Integrins constitute heterodimeric transmembrane receptors formed by 18 alpha subunits and 8 beta subunits which can assemble in 24 different combinations, dictating the binding affinity to specific ECM ligands (Alam et al., 2007). At molecular level, integrins represent the most studied family of proteins involved in the ECM signal transduction and osmosignaling (Bonus, Haussinger, & Gohlke, 2021). Integrins are strongly involved in cell survival, spreading and migration (Harburger & Calderwood, 2009). In the liver, one of the most important mediators of integrin downstream effects is constituted by focal adhesion kinase (FAK) (Weng et al., 2020). FAK undergoes autophosphorylation in response to integrin clustering. This activates Src family kinases, which in turn phosphorylate FAK, promoting its kinase activity. The activation of FAK signaling induces multiple downstream pathways to regulate different cellular functions such as survival,

proliferation, migration and invasion. (Guan, 2010; Harburger & Calderwood, 2009). In addition to FAK signaling, integrin mediates the activation of other signaling pathways such as serine/threonine-protein kinase PAK1 and yes-associated protein 1 (YAP1) (Martin et al., 2016).

The different expression of the integrin subunits in diverse tissue and disease, make them interesting therapeutic targets (Yokosaki & Nishimichi, 2021). Target therapies on integrins have led to the approval of six integrin inhibitors (Slack, Macdonald, Roper, Jenkins, & Hatley, 2022). Among them, Natalizumab is an FDA approved monoclonal antibody against α4-integrin used in multiple sclerosis and for refractory moderate-to-severe Crohn's disease (Brandstadter & Katz Sand, 2017; MacDonald & McDonald, 2007). For liver fibrosis treatment, few clinical trials have been reached so far. A small molecule which targets αvβ1 may be indicated for end-stage liver fibrosis in NASH (Slack et al., 2022). Remarkably, (ITGβ1) has recently been identified as key regulator of fibrosis development in HBV-fibrotic patients, promoting HSCs activation (M. Wang et al., 2017). One of the substrates of integrin is represented by collagen VI (ColVI) (Doane, Howell, & Birk, 1998), a remarkable ECM protein which exerts mechanical and intracellular functions in different tissues and organs (Cescon et al., 2015). The physiological and pathological roles of this molecule will be analyzed in the next section.

COLLAGEN VI: STRUCTURAL ROLE AND BEYOND

ColVI is expressed in different tissues and it exerts a broad spectrum of roles, both mechanical and intracellular. It is encoded by 6 different genes (from *COL6A1* to *COL6A6)* and is secreted via a multi-step process which involves different intracellular assembly steps and establishment of large aggregates before secretion of the formed tetrameric structure in the ECM (Fig. 17) (Knupp et al., 2006).
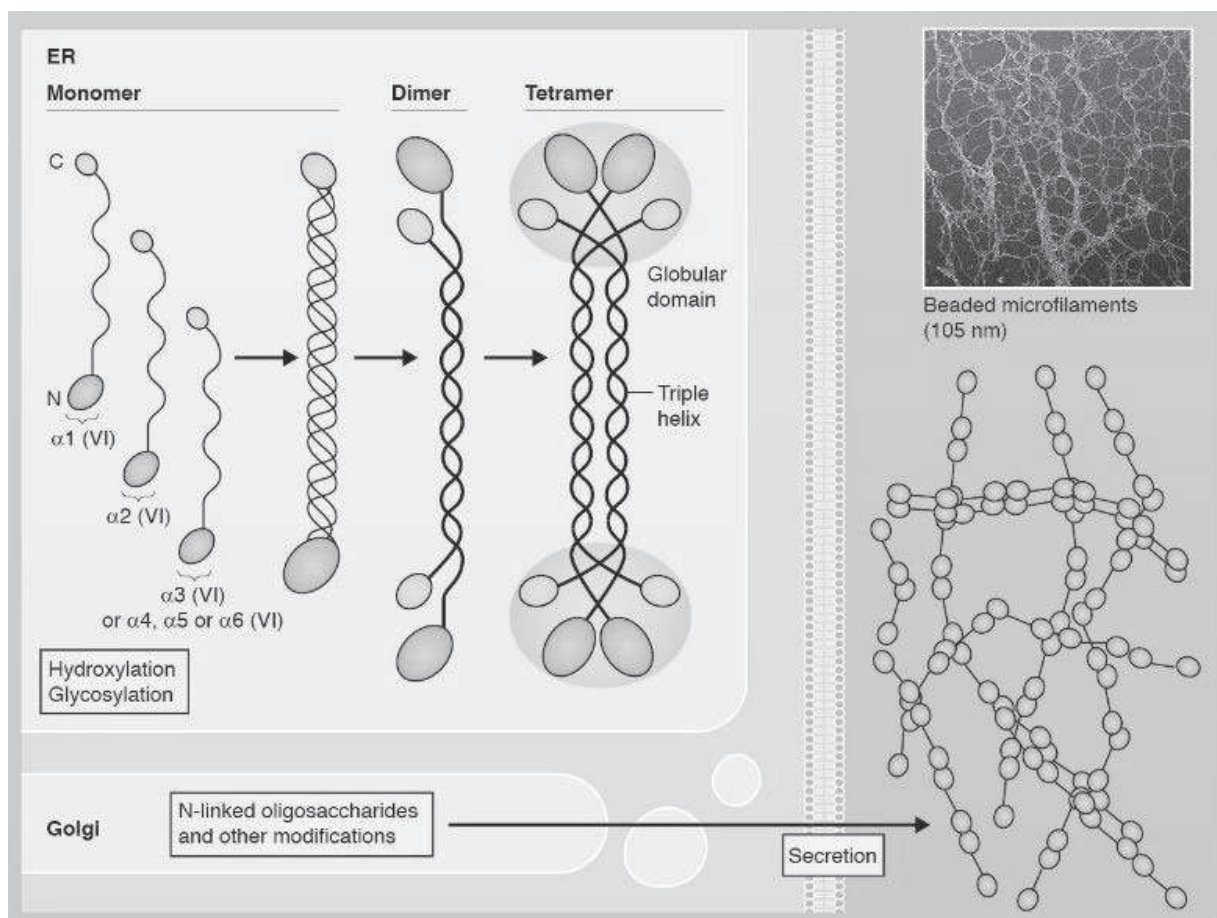

Figure 17. Assembly and secretion of ColVI molecule. Image modified from (Cescon et al., 2015).

At the molecular level, ColVI interacts with different components of the ECM, such as fibronectin (Tillet et al., 1994), type I and type IV collagens (Kuo, Maslen, Keene, & Glanville, 1997; Specks, Nerlich, Colby, Wiest, & Timpl, 1995) and heparin sulfate (Specks et al., 1992),

influencing therefore the activity of the ECM (Fig. 18). However, although these binding partners have been identified, the recognition of its functional receptors is missing. So far, few studies were able to identify putative receptors, such as integrin (Pfaff et al., 1993), Chondroitin sulfate proteoglycan 4 (also known as NG2) (Stallcup, Dahlin, & Healy, 1990; Tillet, Gential, Garrone, & Stallcup, 2002) and capillary morphogenesis gene 2 (CMG2/ANTXR2) (Burgi et al., 2017) receptors. ColVI has been particularly studied in the skeletal muscles; however, the interest on its functions has been recently expanded to other organs and tissues.
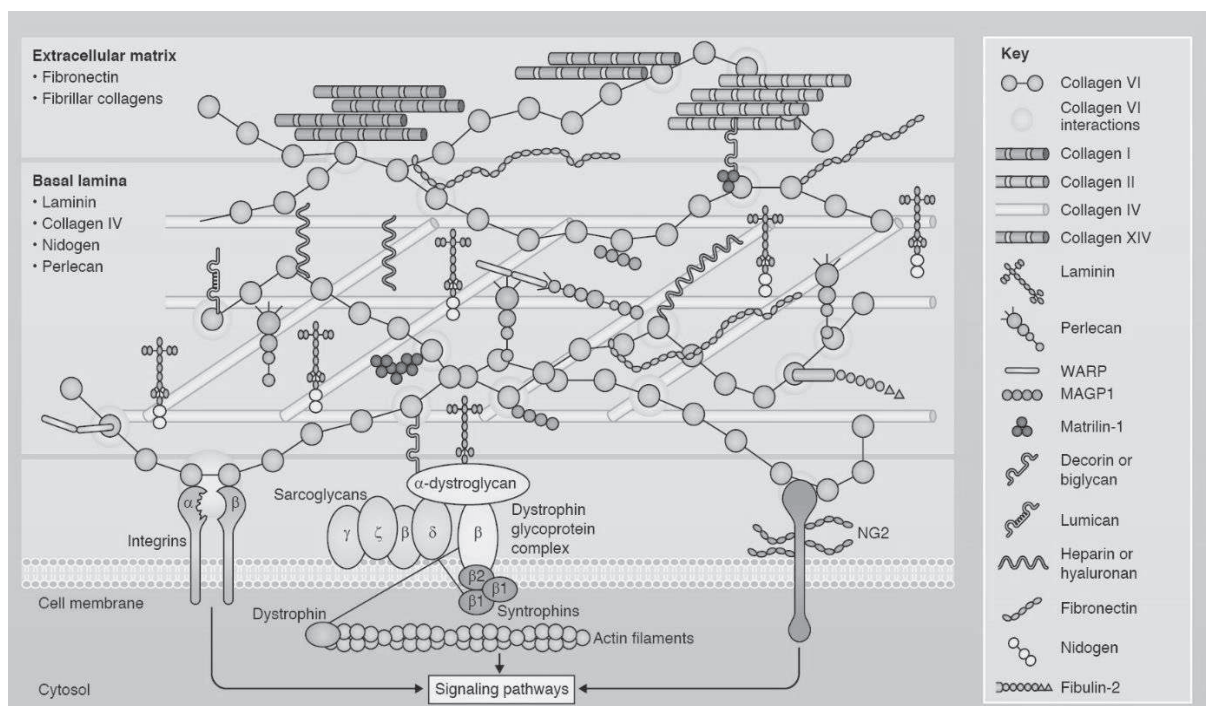
Figure 18. ColVI interactions in the ECM. Image modified from (Cescon, Gattazzo, Chen, & Bonaldo, 2015).

In the muscles, dominant or recessive mutations in the genes encoding for ColVI isoforms influence the way how this protein assembles and provoke genetic muscle disorders, such as Bethlem myopathy and the severe Ullrich congenital muscular dystrophy (Bernardi & Bonaldo, 2013; Bonnemann, 2011; Lamande, 2021). Loss of contractile strength of isolated *Col6a1*$^{-/-}$ muscles from mice has demonstrated to be associated with alteration of mitochondrial functions, possible due to an abnormal arrangement of the integrins (Irwin et al.,

2003; Werner & Werb, 2002) and induction of apoptosis (Irwin et al., 2003). In addition, the observation of abnormality in mitochondria has been associated with an impaired autophagy flux in $Col6a1^{-/-}$ muscles of mice, which can be rescued using the mTOR inhibitor rapamycin. Therefore, autophagy inhibition in $Col6a1^{-/-}$ muscles is probably responsible for the persistence of dysfunctional mitochondria and triggers apoptosis (Alexeev et al., 2014; Fitzwalter et al., 2018; Grumati et al., 2010). In line with that, proper autophagy is required for preserving muscle mass (Masiero et al., 2009). Looking at the signaling pathways, the impairment of autophagy flux followed by ColVI deficiency has been linked in mice to lower protein levels on beclin-1 and bnip3 and to a persistent activation of mTOR, indicated by the persistent phosphorylation of the downstream target eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1) and the upstream hyperphosphorylation of Akt (Grumati et al., 2010).

Interestingly, ColVI deficiency is additionally associated to changes in the distribution of adipose tissue in ColVI-related myopathy patients (Rodriguez et al., 2017). It has been suggested that the transplantation of human-adipose-derived stem cells into $Col6a1^{-/-}$ mice has a benefic effect against ColVI-related congenital muscular dystrophy (Alexeev et al., 2014; Cescon et al., 2015). Moreover, ColVI has special implications in obesity and insulin-resistance. $Col6a1^{-/-}$ ob/ob mice displayed an augmented number of caveolae structures which are important for insulin receptor stabilization (Gustavsson et al., 1999; Khan et al., 2009). This is translated into a major sensitivity for insulin and thus enhanced Akt activation upon insulin stimulation (Khan et al., 2009). Moreover, ColVI is highly expressed in adipose tissue in different metabolically challenged mouse models, contributing to the so-called "adipose tissue fibrosis" (Cescon et al., 2015; Khan et al., 2009). The metabolic deregulation due to ColVI in adipose tissue is not clear yet, but it is probably a consequence of the pro-fibrotic and pro-inflammatory effects of the COL6A3-derived signaling peptide called endotrophin. This is a soluble cleaved C5 fragment which is secreted by different cell types, especially adipocytes. Endotrophin triggers inflammation and fibrosis in adipose tissue, and its neutralization enhances insulin sensitivity (K. Sun et al., 2014). Moreover, level of endotrophin is upregulated

in obese mouse model. Coherently, also obese individuals show higher level of this fragment (K. Sun et al., 2014). Recently, a correlation was observed between serum level of endotrophin and risk of development of other disease, such as heart failure (Eruzun et al., 2019) and Evaluation of COPD Longitudinally to Identify Predictive Surrogate Endpoints (ECLIPSE) - chronic obstructive pulmonary disease progression (Ronnow et al., 2020), suggesting that this fragment may serve as valid serum biomarker. In addition to that, different ColVI fragments raised attention for their application as possible biomarkers for different fibrosis conditions and types of cancers (Lindholm et al., 2021; L. Williams, Layton, Yang, Feldmann, & Nanchahal, 2021; Willumsen, Bager, & Karsdal, 2019).

Given the implication of ColVI in different tissue by the regulation of several signaling pathways, it is worth to introduce its role in cancer progression. Indeed, it has been shown that different chains of ColVI may be differently expressed in tumors and metastasis (P. Chen, Cescon, & Bonaldo, 2013) and endotrophin has been associated with progression of mammary tumor (Park & Scherer, 2012) and increased angiogenesis (Park & Scherer, 2012). ColVI is expressed in different types of tumor, such as glioblastoma, melanoma, ovarian and breast cancer (Burchardt, Hein, & Bosserhoff, 2003; Ho, Chang, Yen, Hong, & Huang, 2021; Karousou et al., 2014; Turtoi et al., 2014) and it has recently been identified as inducer of breast cancer in cell invasion in metastasis and obesity (Wishart et al., 2020). In this scenario, inflammation covers a significant role and therefore macrophages. In this line, it has been shown that macrophages secrete ColVI (Gualandi et al., 2011; Schnoor et al., 2008). Moreover, ColVI is potentially involved in the recruitments of monocytes (Schnoor et al., 2008). At the signaling pathway level, the cancer progression is probably mediated by the interaction of ColVI with some receptors which are known to be highly expressed in tumor tissue. Especially, ColVI is believed to trigger Akt/glycogen synthase kinase-3 beta (GSK-3β) and β-catenin signaling, inducing the upregulation of chemotherapy resistance and tumor growth genes (P. Chen et al., 2013). Moreover, ColVI may contribute to cell invasion in via activation of NG2/EGFR and MAPK signaling (Wishart et al., 2020).

COLLAGEN VI AND HEPATIC FIBROSIS

Elevated levels of ColVI and derived peptides have been observed in serum of patients with hepatic fibrosis from different etiologies (Shahin et al., 1992; Stickel et al., 2001). Therefore, more and more studies have been conducted to evaluate the role of ColVI and its fragments as possible biomarkers for liver fibrosis (Karsdal et al., 2020; Veidal et al., 2011). Recently, ColVI has been identified as central regulator of fibrosis using a model of isolated cell tissue from patients with Dupuytren's disease, a localized fibrotic disorder of the palm (L. M. Williams et al., 2020). Moreover, ColVI has been found to act as key driver for fibroblast activation by inhibition of apoptosis (Ruhl et al., 1999). In addition to that, several animal studies of liver fibrosis show that ColVI is upregulated following injury (Fickert et al., 2007; Takahara, Sollberg, Muona, & Uitto, 1995). Newly, endotrophin may be a valid candidate biomarker of survival in cirrhotic patients with HCC (Leeming et al., 2020). According to the current evidence, ColVI is therefore an important driver of fibrosis development, functioning as structural component. Moreover, as described in the previous section, its role as signaling molecule in the context of disease development is gaining more and more attention.

RISK ASSESSMENT IN PATIENTS WITH CHRONIC HEPATITIS B INFECTION

The identification of the grade of fibrosis in HBV-infected patients strongly influences antiviral treatment choice and the follow up and it is particularly important considering that fibrosis can be reversed after elimination of liver injury (Asselah, Marcellin, & Bedossa, 2014). Although liver biopsy remains the gold standard for the assessment of liver fibrosis/cirrhosis grade, new non-invasive techniques and precision tools have been developed (Asselah et al., 2014; Y. Sun et al., 2018). Non-invasive assessment of liver fibrosis in patients with CHB may carried out using indirect and direct biomarkers (Nallagangula, Nagaraj, Venkataswamy, & Chandrappa, 2018; Nielsen, Leeming, Karsdal, & Krag, 2017). More details about biomarkers development for chronic viral hepatitis are described in a review which I first-authored and which is provided in the ANNEX (<u>Annex V: Profibrotic Signaling and HCC Risk during Chronic Viral Hepatitis: Biomarker Development</u>) (Virzi et al., 2021).

Indirect and direct biomarkers for liver fibrosis

Indirect and direct biomarkers reflect changes in liver functions and alterations of ECM composition, respectively (Nallagangula et al., 2018; Nielsen et al., 2017). Indirect markers are based on combinations of routine laboratory parameters, such as transaminases, albumin, bilirubin, prothrombin time, platelet levels and red cell distribution width (Parikh, Ryan, & Tsochatzis, 2017). From the combination of these measures, it is possible to calculate non-invasive scores of fibrosis. FIB-4 index, which was initially established for CHC/HIV co-infection (Sterling et al., 2006), results from the combination of aspartate aminotransferase (AST), ALT, and platelet count (age (yr) X AST (U/L)/[(PLT (109/L)]X [ALT (U/L)]1/2) (Vallet-Pichard et al., 2007). APRI marker consists in the AST/platelet ratio, and it was initially proposed for prediction of fibrosis and cirrhosis in CHC patients (Wai et al., 2003). FIB-4 and APRI scores have been defined good predictors for HBV-associated fibrosis (Teshale et al., 2014).

The direct serum markers are used for the assessment of the ECM composition, in order to evaluate the progression or regression of fibrosis within the liver (T. Liu, Wang, Karsdal, Leeming, & Genovese, 2012). Hyaluronic acid (HA), procollagen III amino terminal propeptide, LN and TIMP-1 are among the most studied markers belonging to this category. Serum HA level has been well correlated with the different fibrotic stages in CHB patients (Geramizadeh, Janfeshan, & Saberfiroozi, 2008), showing high reliability for differentiating between mild and extensive fibrotic stages (Orasan, Ciulei, Cozma, Sava, & Dumitrascu, 2016). TIMP-1 serum level may be correlated with fibrosis progression in CHB patients (Zhu, Li, Li, & Gao, 2012). Moreover, elevated serum levels of HA, LN, type III precollagen and type IV collagen can be associated to a higher risk of liver cirrhosis development in CHB patients (Yongdi Chen et al., 2019). In addition, more and more studies have evaluated the role of mac-2-binding protein glycosylation isomer (M2BPGi) as candidate biomarker of fibrosis and HCC in CHB patients (Hsu et al., 2018; Jun et al., 2019; J. Liu et al., 2017; Mak et al., 2019).

HBV-specific biomarkers

It is of fundamental importance to seek surrogates of cccDNA levels in the blood. Two viral serum biomarkers have been recently discovered to be associated to cccDNA level. These are represented by hepatitis B core-related antigen (HBcrAg) and HBV RNA (G. P. Caviglia, Armandi, et al., 2021; G. P. Caviglia, Zorzi, et al., 2021; Testoni et al., 2019). Different research groups have already studied the role of HBcrAg as viral biomarker for liver disease prediction, and the results obtained so far are encouraging. HBcrAg consists of three proteins, HBeAg, HBcAg, and a 22-kDa precore protein (Gian P Caviglia, Noviello, Pellicano, & Olivero, 2018). These can be measured together as HBcrAg via a serological test (Kimura et al., 2005; Kimura et al., 2002; Takahashi et al., 1983). Moreover, higher HBcrAg serum level has been found to be associated with higher risk of HCC development, independently from NAs therapy (Kumada et al., 2013). Remarkably, in a recent meta-analysis, HBcrAg immunoassay has emerged as a valid and low-cost alternative to HBV DNA PCR test for the identification of individuals with high viral load (Yoshida et al., 2021). This is particularly important considering

that nucleic acid testing is not an affordable tool in resource-limited countries, making harder the eradication of the virus and the related pathological consequences (Seck et al., 2018).

Beside HBcrAg, HBV RNA may also be used to quantify intrahepatic cccDNA and its transcriptional activity (Y. Liu, Jiang, Xue, Yan, & Liang, 2019). Evidence demonstrated that HBV RNA detected in patient's serum is contained in virus-like particles like HBV pgRNA virions (L. Jansen et al., 2016; Rokuhara et al., 2006; J. Wang et al., 2016). HBV pgRNA virions may accumulate after blocking the reverse transcription activity by the HBV DNA polymerase, e.g., during NA therapy. For this reason, pgRNA level may be associated with risk of viral rebound after suspension of NAs. Therefore, pgRNA virions quantification may be a potential biomarker for monitoring the effect of discontinuation of NA therapy in CHB patients (J. Wang et al., 2016). In this context, new highthroughput HBV RNA techniques have been recently developed but better standardization methods are still needed (Otsuka & Koike, 2020).

OBJECTIVE OF THE THESIS

Since the discovery of HBV, huge progress has been made in the field of CHB. The genome structure as well as the complete life cycle of HBV have been well characterized (Verrier, Colpitts, Schuster, et al., 2016). However, the molecular mechanisms and signaling pathways driving HBV-associated liver disease progression remain only partially understood and clinical studies on the effects of molecular therapeutics targeting the HBV-hijacked signaling pathways are relatively infrequent (Y. Yan et al., 2022). The gaps in our understanding hamper the development of new therapeutic options able to prevent HBV-associated liver disease and viral persistence. The persistence of liver disease risk after the current antiviral treatment indicates that unidentified factors and drivers are implicated in the pathological development.

Considering the enormous progress of -omics technologies and the urgent needs to elucidate the host molecular mechanisms triggered by HBV infection, my project aimed to identify the landscape of signaling pathways deregulated during a time-course HBV infection. Therefore, we established a large-scale infection of hepatocytes-like cells and by choosing a mass spectrometry method able to confer excellent sensitivity to the analysis (Mertins et al., 2018). Our atlas offers some important insights on previously uncovered aspects related to HBV pathogenesis and viral life cycle. In addition to that, this study makes a major contribution to research on HBV by providing a comprehensive phosphoproteomic and proteomic atlas for the creation and validation of working hypothesis.

The results of this PhD thesis are included in the manuscript prepared for submission as short article to *Cell Host and Microbe,* which I wrote as first author (see next section: SCIENTIFIC MANUSCRIPT: Phosphoproteomic atlas of HBV infection reveals a novel role of collagen VI and linker histone for liver pathogenesis and viral life cycle).

SCIENTIFIC MANUSCRIPT: Phosphoproteomic atlas of HBV infection reveals a novel role of collagen VI and linker histone for liver pathogenesis and viral life cycle

Research article

Virzì A, Boulahtouf Z, Heydmann L, Durand C. S, Ramberger E, Popp O, Moehlin J, Juhling F, Felli E, Mertins P, Pessaux P, Verrier E. R, Schuster C, Baumert T, Lupberger J.

This **short article prepared for submission in *Cell Host and Microbe*** includes the main results of my PhD thesis.

Phosphoproteomic atlas of HBV infection reveals a novel role of collagen VI and linker histone for liver pathogenesis and viral life cycle

Alessia Virzì[1], Zakaria Boulahtouf[1], Laura Heydmann[1], Sarah C. Durand[1], Evelyn Ramberger[2,3], Oliver Popp[3,4], Julien Moehlin[1], Frank Juhling[1], Emanuele Felli[1,4], Philipp Mertins[3,4], Patrick Pessaux[1,4,5], Eloi R. Verrier[1], Catherine Schuster[1], Thomas Baumert[1,4,6], Joachim Lupberger[1*]

[1]Université de Strasbourg, Inserm, Institut de Recherche sur les Maladies Virales et Hepatiques UMR_S1110, Strasbourg, France; [2]Proteomics Platform, Max Delbrück Center for Molecular Medicine in the Helmholtz Society, Berlin, Germany; [3]Berlin Institute of Health, Berlin, Germany; [4]Service d'hépato-gastroentérologie, Hôpitaux Universitaires de Strasbourg, Strasbourg, France; [5]Institut hospitalo-universitaire (IHU), Institute for Minimally Invasive Hybrid Image-Guided Surgery, Université de Strasbourg, Strasbourg, France; [6]Institut universitaire de France (IUF), Paris, France.

*Correspondence to: Dr. Joachim Lupberger, Inserm, U1110, Institut de Recherche sur les Maladies Virales et Hépatiques, 3 rue Koeberlé, Strasbourg 67000, France; phone +33368853715, joachim.lupberger@unistra.fr.

Key words: Hepatitis B virus, signal transduction, liver fibrosis, cccDNA, stellate cells

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Number of figures: 4

Summary

Despite a safe and effective vaccine, approximately 1.5 million individuals are chronically infected by hepatitis B virus. Chronic infection can only be controlled but rarely cured due to a persistent epigenetically-regulated viral minichromosome (cccDNA). Moreover, current antiviral therapies cannot fully prevent fibrotic liver disease progression due to unknown mechanisms. Therefore, the identification of pathways responsible for HBV-associated liver disease development and viral persistence is urgently needed.

Here, we provide the first proteomic and phosphoproteomic atlas of an HBV infection time course in permissive cells. The validity of our comprehensive dataset is confirmed by its ability to validate published findings. Beyond, the atlas provides novel insights in HBV-cell cycle interaction, cccDNA persistence and profibrotic signaling. We revealed a pro-viral effect of HBV-induced collagen VI in hepatocytes which promotes a pro-fibrotic phenotype switch in hepatic stellate cells. In addition, we revealed an HBV-induced linker histone phosphorylation in the G1 phase of the cell cycle, which impacts specifically HBV transcription with link to cccDNA. These findings provide insights on HBV-related liver disease progression and may help in the identification of druggable targets.

Highlights

➢ HBV induces profound changes in the cell cycle control and cell-matrix communication

➢ HBV stimulates the expression of collagen VI in hepatocytes, which promotes myofibro-blasts activation and liver fibrosis

➢ Collagen VI expression in hepatocytes has a pro-viral impact on HBV replication

➢ HBV induces linker histone H1 phosphorylation in G1 phase of the cell cycle stimulating specifically HBV transcription in the early phase of infection

Introduction

Chronic hepatitis B (CHB) represents the major risk factor for cirrhosis and hepatocellular carcinoma (HCC) worldwide (El-Serag, 2012). It is believed that during CHB a combination of direct and indirect factors contributes to a perturbed liver homeostasis, the buildup of excessive extracellular matrix (ECM), and pro-oncogenic pressure. The detection of fibrosis and necroinflammation within the liver is a key factor disease management of patients with CHB and it is crucial for cancer risk prognosis (Berumen et al., 2021; Parikh et al., 2017), considering that one third of the population with CHB will develops cirrhosis, liver failure and HCC, if untreated (EASL, 2012; Tao et al., 2020). Although fibrosis regression may occur after long term viral suppression, a sustained low level of HBV DNA can often promote fibrosis progression (Sun et al., 2020). However, despite an effective viral control, the risk of HCC development persists in both cirrhotic and non-cirrhotic patients (Grossi et al., 2017). At the cellular level, an important hallmark of fibrosis is the activation of hepatic stellate cells (HSCs), which is a transdifferentiation process converting HSCs from vitamin A-storing cells to ECM-deposing myofibroblasts (Puche et al., 2013). HSC activation is driven by parenchymal and non-parenchymal liver cells, i.e., hepatocytes, macrophages, Kupffer cells and biliary epithelial cells (Kikuchi et al., 2017; Tsuchida and Friedman, 2017; Ying et al., 2017). In contrast to HCV, the role of HBV in the activation of HSCs remains still poorly understood, whereas its impact on HCC has been more largely investigated (Lara-Pezzi et al., 2002; Lara-Pezzi et al., 2001a; Lara-Pezzi et al., 2001b). As urgently needed therapies targeting fibrosis are absent (Roehlen et al., 2020), the understanding of the hepatic intracellular communication and signaling promoting fibrosis is of fundamental importance to delineate new therapeutic strategies against HBV-associated disease.

Although an efficient HBV vaccine is available, an effective therapy for CHB is hampered as chronic HBV can only be controlled by the current antiviral options. Nucleos(t)ide analogues (NAs) are safe and more tolerated compared to pegylated-interferons (peg-IFNs) and can assure

partial cure in most of the patients with increased survival rate. However, a sustainable HBsAg loss (functional cure) is hardly realized (Tout et al., 2021). The failure of a resolutive treatment is largely associated with the persistence of the viral covalently closed circular DNA (cccDNA), retained in the nucleus of infected cells in the form of a minichromosome-like structure which is epigenetically regulated by post-translational modifications (PTMs) of histone and non-histone proteins (Bock et al., 2001). cccDNA is insensitive to the current antiviral therapy and therefore representing the main obstacle for HBV cure. Histones package the DNA and regulate gene expression via different epigenetic modifications, associated with active or repressive genomic transcription as well as cccDNA minichromosome regulation (Wang et al., 2020). cccDNA switches from a closed to an opened conformation subsequently to different epigenetic modifications, therefore sustaining a continuous production of HBV antigens (Wang *et al.*, 2020). Linker histone (H1) provides an additional higher level of structure organization, connecting two adjacent nucleosomes by binding short stretches of linker DNA (Davey et al., 2002; Hergeth and Schneider, 2015; Luger et al., 1997). As core histones, H1 are regulated by PTMs, i.e., phosphorylation, acetylation, and methylation (Andres et al., 2020). H1 phosphorylation predominantly causes a reduction of its binding affinity to chromatin with consequent detachment of H1 from active promoter regions (Belikov et al., 2007; Bhattacharjee et al., 2001; Koop et al., 2003; Vicent et al., 2011) with potential relevance for HBV minichromosome regulation.

As advances in -omics technologies including phosphoproteomics accelerated the profiling of signaling pathways in precision medicine (Nussinov et al., 2021), we provide an atlas of the HBV life cycle in NTCP-expressing HepG2 cells, using isobaric tagging mass spectrometry (Mertins et al., 2018). This proteomic and phosphoproteomic landscape reveals previously unknown drivers and circuits modulating different aspects of cccDNA regulation, viral life cycle and liver disease progression.

Experimental Procedures

Reagents, antibodies, RNAi. DMSO, polyethylene glycol 8000 (PEG-8000) and 2-(4-Amidino-phenyl)-6-indolecarbamidine dihydrochloride (DAPI) were obtained from Sigma. Paraformaldehyde (PFA) was purchased by Euromedex. Presto Blue cell viability assay was purchased by Invitrogen. For HSCs experiments, recombinant human TGF-β1 (Peprotech) and native human collagen VI protein (ab7538) were used at a concentration of 10 ng/mL and 0.5 μg/mL for four hours, respectively. The treatment was performed after 3 h serum starvation. HBsAg monoclonal HBsAg (Bio-Techne, clone 1044/329), collagen type VI, alpha 1 polyclonal antibody (Proteintech, 17023-1-AP), Alexa Fluor 647 AffiniPure Goat Anti-Rat IgG (H+L) and Alexa Fluor 488 AffiniPure Goat Anti-Rat IgG (H+L) were purchased from Jackson ImmunoResearch. Transfection was performed by reverse silencing in 96-well plate (Costar, Sigma-Aldrich) using Lipofectamine RNAiMAX (Invitrogen) in G418-free media for HepG2-NTCP. ON-TARGETplus Human COL6A1 siRNA, siRNA targeting Human HIST1H1E (H1.4), ON-TARGETplus non-targeting control pool were purchased from Dharmacon. Hepatitis B e-antigen (HBeAg) was detected by chemiluminescence essay (CLIA, Autobio). Isolated HSCs were marked by immunofluorescence staining for anti-alpha smooth muscle actin (α-SMA) protein (ab7817).

RNA, DNA extraction and gene expression analyses. Total RNA was extracted using ReliaPrep RNA Miniprep Systems (Promega) and quantified using NanoDrop 2000/2000c Spectrophotometer (Thermo Scientific). DNA was extracted using QiaAMP DNA MiniKit protocol (Qiagen). Reverse transcription was performed using Maxima First Strand cDNA Synthesis Kit (Thermo Scientific). Gene expression was measured by RT-qPCR using iTaq Universal SYBR Green Supermix (Bio-Rad) on the QuantStudio 3 instrument (Applied Biosystems). All the qPCR primers were synthetized by Sigma. GAPDH fw: 5'-TGC ACC ACC AAC TGC TTA; GAPDH bw: 5'-GGA TGC AGG GAT GAT GTT C; RPLP0 fw: 5'-AGC CCA GAA CAC TGG TCT C; RPLP0 bw: 5'-ACT CAG GAT TTC AAT GGT GCC; HPRT1 fw: 5'-CTG GAA AGA ATG TCT TGA TTG

TGG; HPRT1 bw: 5'-TTT GGA TTA TAC TGC CTG ACC AAG; COL1A1 fw: 5'-GTG CTA AGG GTG ATG CTG GT; COL1A1 bw: 5'-TTT GCC AGG AGA GCC ATC AG; HBx (for pregenomic and pre-core RNA) fw: 5'-CTG GAT CCT GCG CGG GAC GTC CTT T, bw: 5'-GTG AAA AAG TTG CAT GGT (Stockl et al., 2003); total HBV RNA fw: 5'-AAC ATG GAC ATC GAC CCT TA, bw: 5'-ACA TTG AGA TTC CCG AGA TTG A; H1.4 fw: 5'-CCG AGA AGA CTC CCG TGA AG; H1.4 bw: 5'-TGA GAG CGG CCA AAG ATA CG; COL6A1 fw: 5'-GAA GAA TGT CAC CGC CCA GA; COL6A1 bw: 5'-GGT GGT GTC AAA GTT GTG GC. ACTA2 fw: 5'-TGA AGA GCA TCC CAC CCT; ACTA2 bw: 5'-ACG AAG GAA TAG CCA CGC.

Cell lines, cell culture and HBV infection. HepG2-NTCP cells (Eller et al., 2020) were cultured in Dulbecco's Modified Eagle Medium 1x (DMEM, Gibco), supplemented with 10% heat-inacti-vated fetal bovine serum (FBS, Dominique Dutscher, France), 1% Minimum Essential Media Non-Essential Amino Acids Solution 100x (MEM NEAA, Gibco) and 0.5% Gentamicin (10 mg/mL) (Gibco). Opti-MEM (Gibco) was used as reduced-serum media. For HepG2-NTCP the medium was additionally supplemented with 0.25% Geneticin selection antibiotic (G418, Invitrogen). Ad-herent HepG2-NTCP were passaged 1:5 twice per week by trypsinization (0.25% Trypsin, 1 mM EDTA, Gibco) and passed 3 times through an 18-gauge needle prior plating and cultivated at 37 °C and 5% CO_2. Purification procedure of infectious recombinant HBV particles from human hepatoblastoma HepAD38 cell line has been described (Ladner et al., 1997). HepG2-NTCP cells were infected as described (Verrier et al., 2016a). Primary HSCs were isolated from non-paren-chymal cells (NPCs) as previously described (Kegel et al., 2016). Freshly isolated HSCs were cultured for up to two weeks to reach confluence. Stellate Cell medium SteCM (ScienCell, #5301) was reconstituted and replaced every four days for up to two weeks. HSCs were gently passaged using trypsin/DPBS (1:1) and were used for experiments between passages 3 to 5 and seeded in collagen I-coated plates.

Human subjects and patient cohorts. Primary NPCs for HSCs isolation were obtained from patients who had undergone liver resections at the Department of Gastroenterology at the University Hospital of Strasbourg, France. All patients provided a written informed consent, the protocol followed the ethical principles of the declaration of Helsinki and was approved by the ethics committee of the University Hospital of Strasbourg and the local independent ethics committee (*comités de protection des personnes*). Datasets were retrieved from the NCBI Gene Expression Omnibus database. HBV-infected primary human hepatocytes (PHHs) (GEO dataset GSE69590) (Yoneda et al., 2016), HBV-associated fibrosis (GEO dataset GSE84044) (Wang et al., 2017) and HBV-infected patients (GEO dataset GSE83148) (Zhou et al., 2017) were used for bioinformatic analysis and Gene Set Enrichment Analysis (GSEA) (Mootha et al., 2003; Subramanian et al., 2005).

Flow cytometry. HepG2-NTCP cells were infected in a 6-well plate 24 h after seeding. At day 10 post-infection (pi), cells were trypsinized, resuspended in media and washed with DPBS by centrifugation (1000xg, 5 min). Cells were resuspended and fixed in 2% PFA in DPBS for 10 min at room temperature (RT) prior permeabilization at RT for 30 min with permeabilization buffer (DPBS, 0.05% saponin, 1% FBS). Proteins were labeled using primary antibodies diluted in permeabilization buffer for 30 min at 4 °C and dye-coupled secondary antibodies for 30 min at 4 °C after washing with DPBS. Subsequently, cells were washed and resuspended in DPBS. 1.5×10^5 cells were then transferred in each well of a 96 well-plate (V-bottom) for detection using Cytoflex B2R2U0 cytometer (Beckman Coulter, BA47394) and CytExpert 2.3 software (Beckman Coulter) and then analyzed using FlowJo V10.5.3 (Beckman Coulter).

Proteomic and phosphoproteomic analyses. HepG2-NTCP cells were plated in F175 flasks and infected after 24 h with HBV genotype D (MOI of 500 as measured in genomic equivalents by qPCR) as described (Evripioti et al., 2019; Schulze et al., 2007; Verrier *et al.*, 2016a; Verrier et al., 2016b). Cells were lysed in 8.0 M urea, 75 mM NaCl, 50 mM Tris pH 8, 1 mM EDTA, aprotinin 2 µg/mL (Sigma-Aldrich), leupeptin 10 µg/mL (Roche), PMSF 1 mM (Sigma-Aldrich),

phosphatase inhibitor cocktail 2 and phosphatase inhibitor cocktail 3 (Sigma-Aldrich) (Mertins *et al.*, 2018) for 20 min at 4 °C. Subsequently, cells were scraped on ice and protein lysates were then cleared by centrifugation using a bench top centrifuge at maximum speed at 4 °C for 5 min. Protein quantification was performed using BCA bicinchoninic acid Protein Assay Kit (Pierce) and samples were stored at -80 °C until analysis. Samples were prepared for proteomic and phosphoproteomic analyses at the Max Delbrück center (MDC) for Molecular Medicine/Berlin Institute of Health (Berlin, Germany) using tandem mass tag (TMT) labeling and immobilized affinity chromatography (IMAC) as described (Mertins *et al.*, 2018). All the replicates were analyzed in a single acquisition improving the reproducibility of the analysis (Mertins et al., 2018).

Bioinformatic, statistical analysis and navigation instructions. Proteomic and phosphoproteomic analyses were performed from two biological replicates. The protein expression and the variation in phosphorylation were represented by the log2 fold change (logFC) between two conditions (Mock and HBV). From the proteomic and phosphoproteomic database, proteins enriched in one condition can be chosen by filtering with a logFC above or below zero. Values were scaled using a median-MAD normalization by calculating (x-median(x, na.rm=T))/mad(x, na.rm=T). The significance value is represented as adjusted p-value (adj.P.Val.), calculated by Benjamini-Hochberg (BH) post-test. An adj.P.Val. of 0.05 represents a 5% false discovery rate (FDR). The cured proteomic dataset was used for GSEA using Signal2Noise ratio, gene_set permutation and the default parameters (Mootha *et al.*, 2003; Subramanian *et al.*, 2005). Gene sets from the Molecular Signature Database (MSigDB, ver.7.0) (Liberzon et al., 2015; Liberzon et al., 2011) were included for the analysis: c2.all.v7.1.symbols.gmt; c6.all.v7.1.symbols.gmt; c7.all.v7.1.symbols.gmt; h.all.v7.1.symbols.gmt). REACOME and HALLMARK gene sets were used to categorize respectively the phosphoproteome (cutoff logFC= 0.5 day 2; adj.P.Val.< 0.05 day 2 and day 10) and the proteome (adj.P.Val.< 0.01) (Liberzon *et al.*, 2015; Liberzon *et al.*, 2011; Mootha *et al.*, 2003; Subramanian *et al.*, 2005). The dysregulated signaling pathways retrieved by GSEA were described using the normalized enrichment score (NES) and FDR q value <0.003. Biorender

was used for image creation (biorender.com). Morpheus was used for generation of heatmaps (https://software.broadinstitute.org/morpheus). Validation and functional experiments were per-formed at least in 3 independent replicates, unless otherwise stated. Statistical analysis was performed using GraphPad.

Result

Identification of a proteomic and phosphoproteomic atlas of HBV infection in HepG2-NTCP cells.

Signal transduction is a central regulator of cellular homeostasis in the liver and dysregulated during chronic injury (Pant et al., 2021; Virzi et al., 2021). To assess the landscape of HBV-in-duced signaling, we performed a global proteomic and phosphoproteomic analysis using HepG2-NTCP as highly efficient and reproducible infection model (Yan et al., 2012). To provide depth and increase sensitivity for mass spectrometry analysis, we upscaled HBV infection of HepG2-NTCP cells to gain 1.5 mg of protein per condition. To obtain a temporal resolution of the whole HBV life cycle we analyzed protein expression and phosphorylation at day 2 and day 10 pi. This protocol leads to a ~80% infection of cells as quantified by HBsAg and HBeAg (Figure S1A-B).

Following proteomic and phosphoproteomic analysis (Figure S1C), we identified in total 7611 proteins and 11197 unique phospho-sites, demonstrating an excellent sensitivity as well as high quality of the protocol comparable to previously published phosphoproteomic studies (Arshad et al., 2019; Friedrich et al., 2021; Lupberger et al., 2019; Mertins et al., 2016). In a first set of analysis of the data we applied a stringent cutoff (adj.P.Val.< 0.01 for proteomics, adj.P.Val. < 0.05 for phosphoproteomics) to identify top scoring candidates relevant throughout the infection period (Figure S2A-B). Subsequently, we retrieved 38 proteins (Figure 1A; Table S1) and 120 phospho-sites significantly dysregulated by HBV throughout the time-course infection (common day 2 + day 10) (Figure 1A; Table S2). Strikingly, HBV early infection induced a massive phos-phorylation, which tends to attenuate at the final stage (day 10), while the overall number of

proteins whose expression levels are dysregulated by HBV remained constant throughout infection (Figure 1A). This may reflect the early innate immune responses to infection although classical pathways of innate immunity were not identified in this extent (Figure S3). Thus, this emphasized the key role of HBV-induced signaling for the establishment of infection and cccDNA formation (Cooper et al., 2003; Diogo Dias et al., 2021). Applying the stringent cutoff, the most significantly functional categories dysregulated by HBV in the proteome and phosphoproteome appeared to be related with ECM remodeling and cell cycle deregulation, respectively (Figure 1B-C). We used this stringent set of candidates to validate the phosphoproteomic atlas by comparing the hits with recent published findings. We observed an HBV-induced increase of Apolipoprotein E (APOE) expression (Table S1), which is a known HBV host factor (Qiao and Luo, 2019). Moreover, Fibronectin 1 (FN1) (Ding et al., 2012) and plasminogen (PLG) (Table S1) were strongly upregulated by HBV and have been previously associated with HBV-related liver disease. PLG has been recently identified as a potential prognostic biomarker for HBV-related acute on chronic liver failure (Wu et al., 2020). Moreover, we identified previously unknown HBV-responsive phosphorylation sites of integrin beta-1 (ITGB1) and serine/threonine-protein kinase PLK1 (PLK1) (extended data available online). Both proteins are implicated in HBV life cycle signaling pathways (Diab et al., 2017; Foca et al., 2020; Wang *et al.*, 2017; Ye et al., 2021). Furthermore, ITGB1 has been lately recognized as a driver of fibrosis progression in HBV-patients (Wang *et al.*, 2017). PLK1 has recently been identified as pro-viral factor for HBV (Diab *et al.*, 2017; Foca *et al.*, 2020). Here, we revealed that the amino acids site Thr214 (adj.P.Val.= 0.009) and Thr210 (adj.P.Val.= 0.17) of PLK1(extended data available online) resulted to be hypo-phosphorylated during HBV infection indicating a possible preventive mechanism of mitotic entry (Tsvetkov and Stern, 2005). A more in-depth analysis of HBV-induced signals revealed a pronounced reorganization of apical junctions (Figure 1B; Table S3), such as tight junction protein ZO-1 (referred as well as TJP1) (Table S2), which are physically linked to the actin cytoskeleton (Miyoshi and Takai, 2008). Apical junction remodeling often reflets actin cytoskeleton modification, underlying

endocytosis mechanisms (Miyoshi and Takai, 2008). This suggests that HBV entry mainly depends on endocytosis pathways (Mooren et al., 2012). Interestingly, our data support the hypothesis that HBV enters the cell via clathrin-mediated endocytosis which we supposed to be strongly mediated by phosphorylation of adaptor-associated protein kinase 1 (AAK1) at Thr620, Thr674 and Ser678 (Table S2). Moreover, this is supported by perturbation of phosphorylation events at level of clathrin interactor 1 (CLINT1), clathrin light chain A (CLTA) respectively at day 2 and day 10 pi (extended data available online) and on intersectin-2 (ITSN2) (Table S2).This finding emphasizes the relevance of clathrin-mediated endocytosis for HBV entry and strongly corroborates recent finding on the HBV uptake mechanism (Herrscher et al., 2020). Therefore, targeting AAK1, CLINT1, CLTA and ITSN2 phosphorylation may represent a potential therapeutic strategy for the inhibition of HBV-associated entry.

HBV infection impacts extracellular matrix rearrangement and integrin signaling.

To get insight to virus-induced dysregulation of pathways and biological function, we performed differential expression analysis of the entire proteome using GSEA (Liberzon *et al.*, 2015; Liberzon *et al.*, 2011; Mootha *et al.*, 2003; Subramanian *et al.*, 2005). Consistent to the functional categories of the proteome analysis (Figure 1C), we revealed that HBV strongly perturbs components of ECM remodeling and integrin signaling (NABA_CORE_MATRISOME) (NABA_ECM_GLYCOPROTEINS) (REACTOME_DEGRADATION_OF_THE_EXTRACELLULAR_MATRIX) (REACTOME_INTEGRIN_CELL_SURFACE_INTERACTION) among the top 30 upregulated pathways in HBV-infected cells (FDR q-value < 0.003) (Figure 1D; Table S4). This result is further supported by transcriptomic data from HBV-infected primary human hepatocytes (PHHs) (GEO dataset GSE69590) (Figure 1D; Table S4). Therefore, the proteomic analysis suggests that HBV-infected HepG2-NTCP and PHHs actively influence ECM remodeling. To identify the main drivers of this observation we looked into the leading-edge genes of the enriched gene

sets studied. Among the 16 common leading-edge genes (Figure 1E), genes coding for FN1, ColVI and collagen XVIII are significantly (adj.P.Val.< 0.01) upregulated in both HepG2-NTCP and PHHs (Figure 1F).

Collagen VI is upregulated in HBV-infected hepatocytes acting as inducer of HBV replication.

GSEA analysis identified the alpha-1 chain of ColVI (COL6A1) as top-ranking leading-edge gene of the ECM and integrin gene sets pathways (Figure 1F). Moreover, also the alpha-2 chain (COL6A2) was significantly (at day 10, adj.P. Val= 0.0015) upregulated in HBV-infected HepG2-NTCP throughout the course of the infection (Table S1). This suggests a potential role of ColVI in HBV pathogenesis. ColVI is a known inducer of signaling and fibrosis in different organs and tissues (Castagnaro et al., 2018; Lamande and Bateman, 2018; Williams et al., 2020). To study its role in HBV-associated liver disease, we validated the induced ColVI-expression in HBV-infected HepG2-NTCP using an independent method. 10 days pi, we assessed the cytoplasmatic expression of ColVI in HBV-infected cells compared to the non-infected cells using flow cytometry (Figure 2A). Gating for HBsAg, HBV-infected cells displayed a significantly (p= 0.0286, Mann-Whitney U test) increased ColVI expression compared to uninfected cells. This was also validated in primary cells, as 40 h HBV infection of PHHs with a multiplicity of infection (MOI) of 50 (GEO dataset GSE69590) (Yoneda *et al.*, 2016) increased *COL6A1* mRNA levels (Figure 2B).

To investigate the clinical relevance of ColVI, we studied its expression in 122 liver tissues of HBV-infected (serum HBsAg or HBV DNA) patients compared with 6 uninfected patients (GEO dataset GSE83148) (Zhou *et al.*, 2017). Indeed, we observed an augmentation of *COL6A1* expression in these HBV-infected liver tissues (Figure 2C). This observation was validated in a second cohort of 124 liver biopsies of CHB-infected patients (GEO dataset GSE84044) (Wang *et al.*, 2017). CHB-patients' biopsies have been classified into five classes (0 to 4) for grade (G) and stage (S) according to the Scheuer system (Scheuer, 1991; Theise, 2007). Here, HBV-induced

COL6A1 expression directly correlated with higher S and G Scheuer score compared to S0 and G0 respectively (Figure 2D- E). Taken together, these results clearly demonstrate an association of HBV-induced hepatic ColVI expression with liver disease progression in patients. To reveal the role of ColVI for the HBV, we studied the impact of ColVI on infection by RNA interference (RNAi). Thereto, *COL6A1* was silenced in HepG2-NTCP prior HBV infection (Figure 2F). We confirmed *COL6A1* silencing 3 days pi and observed that silenced cells displayed a significant ($p < 0.0001$, Unpaired t-test) reduction in HBV transcripts levels (Figure 2G). This suggests that ColVI exerts a pro-viral role potentially through ColVI-associated signaling. This may be possibly mediated by subversion of the mammalian target of rapamycin (mTOR) signaling and autophagic machinery (Bernardi and Bonaldo, 2013; Castagnaro *et al.*, 2018; Grumati et al., 2010; Li et al., 2011; Lin et al., 2020).

Collagen VI contributes to fibrosis via activation of human liver myofibroblast.

The crosstalk between hepatocytes and NPCs is a major determinant of liver homeostasis and pathogenesis (Barbero-Becerra et al., 2015; Urushima et al., 2021; Zhang et al., 2021). HSCs are characterized by an elongated morphology which allows the establishment of cell-cell contacts with adjacent hepatocytes through the Disse space (Kitto and Henderson, 2021). During liver injury, HSCs are activated and differentiate into myofibroblasts which are largely accountable for ECM deposition during wound healing and fibrosis (Tsuchida and Friedman, 2017). It is thus conceivable that HBV-induced ColVI expressed in hepatocytes may influence the activation of HSCs. To answer this, primary HSCs were isolated as described (Figure 3A) (Kegel et al., 2016). HSCs were isolated from liver tissue of four patients that underwent liver resection for colorectal liver metastasis, adenocarcinoma or alcoholic liver without cirrhosis or inflammatory infiltrates. HSCs isolation was then confirmed by specific α-SMA staining characterizing cytoplasmic myofilaments along the cell axis (Figure 3B) (Carpino et al., 2005). Like TGF-β1, a known inducer of HSCs

differentiation (Desmouliere et al., 1993), ColVI treatment caused an increased gene expression of the myofibroblast markers smooth muscle actin alpha 2 (*ACTA2*) and collagen I (*COL1A1*) (Figure 3C-D). These results suggest a profibrotic effect of ColVI by reinforcing HSCs activation and promoting ECM deposition during fibrosis.

HBV-induced signaling perturbs cell cycle control.

To gain insight into the signaling landscape of HBV infection, we categorized differentially phosphorylated proteins in HBV-infected cells in corresponding gene sets retrieved for the MolSigDB (Liberzon *et al.*, 2015; Liberzon *et al.*, 2011). Strikingly, we found that most pathways phosphorylated by HBV belong to the G2/M checkpoint control of the cell cycle (Figure 1B; Table S3). This finding is in line with previous studies suggesting that HBV preferentially replicates in G1/G2 (Eller *et al.*, 2020; Wang et al., 2011; Xia et al., 2018) and thus further validated our screening approach. Beyond, our data allowed a more in-depth analysis, which identified HBV-induced hypo-phosphorylation of retinoblastoma-associated protein (pRb), at Thr356 and Ser788 at the early stage of HBV infection (extended data available online). Thr356 and Ser788 on pRb are known to be phosphorylated by CDK4-cyclin D1 (Connell-Crowley et al., 1997; Futatsugi et al., 2012; Lees et al., 1991; Zarkowska and Mittnacht, 1997) leading to a cell cycle progression and transcription of S-phase genes through the dissociation from the transcription factor E2F1 (Macdonald and Dick, 2012). Therefore, the identified HBV-induced pRb hypo-phosphorylation presumably leads to a G1 cell cycle arrest, which in turn promotes HBV infection (Eller *et al.*, 2020). This is further consolidated by the observed persistently perturbed phosphorylation of E2F1 targets (Figure 1B, Table S3).

Linker histone H1.4 phosphorylation promotes HBV transcription.

Further analysis of the top phosphorylation sites modulated by HBV infection revealed a massive linker histone phosphorylation at Ser and Thr including H1.4 at the early stage of the infection (day 2) (Figure 4A). Given the important role of H1 in chromatin remodeling, it is potentially linked to the HBV cell cycle control. Hyper-phosphorylation of H1 results generally in detachment of the protein from DNA during the S and M phase and associates to decompaction of chromatin (Fig-ure 4B) (Alexandrow and Hamlin, 2005; Bannister and Kouzarides, 2011) regulating gene expres-sion (Dou et al., 1999). As H1.4 is hardly phosphorylated in G1 (Sarg et al., 2006), the observed HBV-induced H1.4 phosphorylation may act pro-viral. To validate this, we simulated H1.4 detach-ment from the chromatin by hyper-phosphorylation (Figure 4B) using RNAi as previously de-scribed (Alexandrow and Hamlin, 2005; Bannister and Kouzarides, 2011). Silencing of H1.4 using siRNA reduced H1.4 expression (Figure 4C-D) and promoted HBV transcription (Figure 4E) with-out changing non-viral gene expression, i.e., Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH), Ribosomal Protein Lateral Stalk Subunit P0 (RPLP0), or Hypoxanthine Phosphoribo-syltransferase 1 (HRPT1) (Figure 4F) or reducing cell viability (Figure 4G). As H1 hyper-phos-phorylation is predominantly observed at day 2 but not at day 10, we investigated its role during established HBV infection. Therefore, we first infected HepG2-NTCP with HBV and then silenced H1.4 two days pi using RNAi. Consistently, the pro-viral role of H1.4 detachment was lost when the infection was already established suggesting a potential role in the early steps of the viral life cycle, potentially during cccDNA formation (Figure S4).

Discussion

Despite an effective vaccine, CHB can only be controlled but rarely cured due to the persistent viral cccDNA. Moreover, viral control cannot fully prevent fibrotic liver disease progression in CHB patients. This emphasizes the need to dissect the factors involved in cccDNA formation and viral pathogenesis. Addressing these unmet needs, we assessed for the first time the temporal signal-ing pattern in a phosphoproteomic atlas of the complete HBV viral cycle. Our model recapitulated known aspects of the virus life cycle and highlighted novel previously unrecognized factors involved in HBV-associated cell cycle control, epigenetic regulation and fibrosis development. The robustness of our phosphoproteomic atlas is further emphasized by the identification of a valida-tion set of targets which have been recently identified as pro-viral factors or related to HBV-asso-ciated endocytosis mechanism, fibrosis and cell cycle modulation.

We revealed that HBV infection cause a strong ECM remodeling. During liver injury, gene expression and secretome profiles, as well as the cellular microenvironment are dynamic. Stressed or injured hepatocytes contribute to the establishment of a profibrotic and proinflamma-tory environment by the secretion of damage-associated molecular patterns (DAMPs), IL-33 and other molecules triggering the trans-differentiation process of HSCs (Lee et al., 2017; Roehlen et al., 2020; Tan et al., 2018). Moreover, although HSCs are the main effector cells of liver fibrosis and ECM deposition (Geerts, 2001), the upregulation of specific ECM components has been al-ready observed in rat hepatocytes cultured in collagen sandwiches (Farkas et al., 2005). Our data uncovered that HBV-infected hepatocytes contribute to ECM formation by inducing ColVI expres-sion and engaging in integrin signaling and ECM remodeling. We identified previously unknown HBV-responsive phosphorylation sites on ITGB1, which is a putative receptor for ColVI (Jongewaard et al., 2002; Tulla et al., 2001) and a known driver of fibrosis progression in HBV-patients (Wang et al., 2017). We demonstrated that CoVI not only acts pro-viral but also acceler-ates fibrosis progression by sustaining patient-derived hepatic myofibroblast activation, which is consistent with the effects

of ColVI on HSCs in mice (Freise et al., 2021). Since ColVI exhibits apro-viral effect, it may have a role in signaling pathway related with HBV transcription or replication.

As HBV predominantly replicates in G1 of the cell cycle (Eller *et al.*, 2020; Wang *et al.*, 2011) we consistently identified known phosphorylation events of pro-viral host factors and proteins involved in cell cycle checkpoints. Generally, HBV is known to infect and replicate preferentially non-dividing hepatocytes (Cohen et al., 2010; Leupin et al., 2005; Ozer et al., 1996) and evidence suggested that HBV may disrupt cell cycle checkpoint to render the cellular environment more favorable for the infection (Eller *et al.*, 2020; Gearhart and Bouchard, 2010; Xia *et al.*, 2018). In PHH-engrafted mouse models, hepatocytes proliferation decreases all infection markers, including cccDNA (Allweiss et al., 2018). pRb is one of the major G1/S cell cycle checkpoint protein and its phosphorylation is cell-cycle dependent (Zarkowska and Mittnacht, 1997). Moreover, it is involved also in DNA repair mechanisms (Cook et al., 2015). When hypo-phosphorylated, pRb binds to E2F1 with consequent block of cell cycle progression and S phase entry (Adams and Kaelin, 1995). In this study, the identification of pRb hypo-phorylated sites at Thr356 and Ser788 sites, as well as the global downregulation of signaling pathways associated with E2F1 in HBV-infected cells, clearly supports a predominant G1 arrest in HBV-infected cells. In this study, we identified a previously unrecognized role of H1 for HBV replication in the G1 phase. Histones and PTMs have essential roles in cell cycle and are strongly implicated in the regulation of chromatin compaction (Zhang et al., 2014). Generally, phosphorylation of H1 is considered to cause conformational changes by electrostatic charge with consequent H1 detachment and chromatin relaxation (Belikov *et al.*, 2007; Bhattacharjee *et al.*, 2001; Koop *et al.*, 2003). Importantly, H1 is hardly phosphorylated during G1 phase (Whitefield et al., 2018) but we revealed that HBV is circumflexing this by inducing a massive phosphorylation of linker histones at the early phase of infection. Especially, we identified a strong phosphorylation profile of H1.4 at Ser and Thr aminoacids, which promotes specifically the HBV transcription while the transcription of nonviral genes seemed unaffected. Moreover, as H1 silencing after an established HBV infection had no effect, we believe

that H1.4 detachment may be implicated in cccDNA formation. However, currently it cannot be excluded that H1.4 phosphorylation is also involved in other pro-viral mechanisms that require chromatin decondensation such as DNA repair and integration mechanisms (Whitefield *et al.*, 2018).

Overall, our temporal proteomic and phosphoproteomic atlas represents a valuable tool for the community consolidating known and highlighting new aspects associated with HBV life cycle and pathogenesis. Targeting HBV-associated signaling components identified in our atlas thus may help to develop novel cccDNA-sensitive antiviral concepts and chemo-preventive strategies to reduce the burden of the HBV epidemic in the coming decades.

Bibliography

Adams, P.D., and Kaelin, W.G., Jr. (1995). Transcriptional control by E2F. Semin Cancer Biol *6*, 99-108. 10.1006/scbi.1995.0013.

Alexandrow, M.G., and Hamlin, J.L. (2005). Chromatin decondensation in S-phase involves recruitment of Cdk2 by Cdc45 and histone H1 phosphorylation. J Cell Biol *168*, 875-886. 10.1083/jcb.200409055.

Allweiss, L., Volz, T., Giersch, K., Kah, J., Raffa, G., Petersen, J., Lohse, A.W., Beninati, C., Pollicino, T., Urban, S., et al. (2018). Proliferation of primary human hepatocytes and prevention of hepatitis B virus reinfection efficiently deplete nuclear cccDNA in vivo. Gut *67*, 542-552. 10.1136/gutjnl-2016-312162.

Andres, M., Garcia-Gomis, D., Ponte, I., Suau, P., and Roque, A. (2020). Histone H1 Post-Translational Modifications: Update and Future Perspectives. Int J Mol Sci *21*. 10.3390/ijms21165941.

Arshad, O.A., Danna, V., Petyuk, V.A., Piehowski, P.D., Liu, T., Rodland, K.D., and McDermott, J.E. (2019). An Integrative Analysis of Tumor Proteomic and Phosphoproteomic Profiles to Examine the Relationships Between Kinase Activity and Phosphorylation. Mol Cell Proteomics *18*, S26-S36. 10.1074/mcp.RA119.001540.

Bannister, A.J., and Kouzarides, T. (2011). Regulation of chromatin by histone modifications. Cell Res *21*, 381-395. 10.1038/cr.2011.22.

Barbero-Becerra, V.J., Giraudi, P.J., Chavez-Tapia, N.C., Uribe, M., Tiribelli, C., and Rosso, N. (2015). The interplay between hepatic stellate cells and hepatocytes in an in vitro model of NASH. Toxicol In Vitro *29*, 1753-1758. 10.1016/j.tiv.2015.07.010.

Belikov, S., Astrand, C., and Wrange, O. (2007). Mechanism of histone H1-stimulated glucocorticoid receptor DNA binding in vivo. Mol Cell Biol *27*, 2398-2410. 10.1128/MCB.01509-06.

Bernardi, P., and Bonaldo, P. (2013). Mitochondrial dysfunction and defective autophagy in the pathogenesis of collagen VI muscular dystrophies. Cold Spring Harb Perspect Biol *5*, a011387. 10.1101/cshperspect.a011387.

Berumen, J., Baglieri, J., Kisseleva, T., and Mekeel, K. (2021). Liver fibrosis: Pathophysiology and clinical implications. WIREs Mech Dis *13*, e1499. 10.1002/wsbm.1499.

Bhattacharjee, R.N., Banks, G.C., Trotter, K.W., Lee, H.L., and Archer, T.K. (2001). Histone H1 phosphorylation by Cdk2 selectively modulates mouse mammary tumor virus transcription through chromatin remodeling. Mol Cell Biol *21*, 5417-5425. 10.1128/MCB.21.16.5417-5425.2001.

Bock, C.T., Schwinn, S., Locarnini, S., Fyfe, J., Manns, M.P., Trautwein, C., and Zentgraf, H. (2001). Structural organization of the hepatitis B virus minichromosome. J Mol Biol *307*, 183-196. 10.1006/jmbi.2000.4481.

Carpino, G., Morini, S., Ginanni Corradini, S., Franchitto, A., Merli, M., Siciliano, M., Gentili, F., Onetti Muda, A., Berloco, P., Rossi, M., et al. (2005). Alpha-SMA expression in hepatic stellate cells and quantitative analysis of hepatic fibrosis in cirrhosis and in recurrent chronic hepatitis after liver transplantation. Dig Liver Dis *37*, 349-356. 10.1016/j.dld.2004.11.009.

Castagnaro, S., Chrisam, M., Cescon, M., Braghetta, P., Grumati, P., and Bonaldo, P. (2018). Extracellular Collagen VI Has Prosurvival and Autophagy Instructive Properties in Mouse Fibroblasts. Front Physiol *9*, 1129. 10.3389/fphys.2018.01129.

Cohen, D., Adamovich, Y., Reuven, N., and Shaul, Y. (2010). Hepatitis B virus activates deoxynucleotide synthesis in nondividing hepatocytes by targeting the R2 gene. Hepatology *51*, 1538-1546. 10.1002/hep.23519.

Connell-Crowley, L., Harper, J.W., and Goodrich, D.W. (1997). Cyclin D1/Cdk4 regulates retinoblastoma protein-mediated cell cycle arrest by site-specific phosphorylation. Mol Biol Cell *8*, 287-301. 10.1091/mbc.8.2.287.

Cook, R., Zoumpoulidou, G., Luczynski, M.T., Rieger, S., Moquet, J., Spanswick, V.J., Hartley, J.A., Rothkamm, K., Huang, P.H., and Mittnacht, S. (2015). Direct involvement of retinoblastoma family proteins in DNA repair by non-homologous end-joining. Cell Rep *10*, 2006-2018. 10.1016/j.celrep.2015.02.059.

Cooper, A., Paran, N., and Shaul, Y. (2003). The earliest steps in hepatitis B virus infection. Biochim Biophys Acta *1614*, 89-96. 10.1016/s0005-2736(03)00166-4.

Davey, C.A., Sargent, D.F., Luger, K., Maeder, A.W., and Richmond, T.J. (2002). Solvent mediated interactions in the structure of the nucleosome core particle at 1.9 a resolution. J Mol Biol *319*, 1097-1113. 10.1016/S0022-2836(02)00386-8.

Desmouliere, A., Geinoz, A., Gabbiani, F., and Gabbiani, G. (1993). Transforming growth factor-beta 1 induces alpha-smooth muscle actin expression in granulation tissue myofibroblasts and in quiescent and growing cultured fibroblasts. J Cell Biol *122*, 103-111. 10.1083/jcb.122.1.103.

Diab, A., Foca, A., Fusil, F., Lahlali, T., Jalaguier, P., Amirache, F., N'Guyen, L., Isorce, N., Cosset, F.L., Zoulim, F., et al. (2017). Polo-like-kinase 1 is a proviral host factor for hepatitis B virus replication. Hepatology *66*, 1750-1765. 10.1002/hep.29236.

Ding, D., Lou, X., Hua, D., Yu, W., Li, L., Wang, J., Gao, F., Zhao, N., Ren, G., Li, L., and Lin, B. (2012). Recurrent targeted genes of hepatitis B virus in the liver cancer genomes identified by a

next-generation sequencing-based approach. PLoS Genet *8*, e1003065.
10.1371/journal.pgen.1003065.

Diogo Dias, J., Sarica, N., and Neuveut, C. (2021). Early Steps of Hepatitis B Life Cycle: From
Capsid Nuclear Import to cccDNA Formation. Viruses *13*. 10.3390/v13050757.

Dou, Y., Mizzen, C.A., Abrams, M., Allis, C.D., and Gorovsky, M.A. (1999). Phosphorylation of
linker histone H1 regulates gene expression in vivo by mimicking H1 removal. Mol Cell *4*, 641-
647. 10.1016/s1097-2765(00)80215-4.

EASL (2012). EASL clinical practice guidelines: Management of chronic hepatitis B virus
infection. J Hepatol *57*, 167-185. 10.1016/j.jhep.2012.02.010.

El-Serag, H.B. (2012). Epidemiology of viral hepatitis and hepatocellular carcinoma.
Gastroenterology *142*, 1264-1273 e1261. 10.1053/j.gastro.2011.12.061.

Eller, C., Heydmann, L., Colpitts, C.C., El Saghire, H., Piccioni, F., Juhling, F., Majzoub, K.,
Pons, C., Bach, C., Lucifora, J., et al. (2020). A genome-wide gain-of-function screen identifies
CDKN2C as a HBV host factor. Nat Commun *11*, 2707. 10.1038/s41467-020-16517-w.

Evripioti, A.A., Ortega-Prieto, A.M., Skelton, J.K., Bazot, Q., and Dorner, M. (2019).
Phosphodiesterase-induced cAMP degradation restricts hepatitis B virus infection. Philos Trans
R Soc Lond B Biol Sci *374*, 20180292. 10.1098/rstb.2018.0292.

Farkas, D., Bhat, V.B., Mandapati, S., Wishnok, J.S., and Tannenbaum, S.R. (2005).
Characterization of the secreted proteome of rat hepatocytes cultured in collagen sandwiches.
Chem Res Toxicol *18*, 1132-1139. 10.1021/tx0500225.

Foca, A., Dhillon, A., Lahlali, T., Lucifora, J., Salvetti, A., Rivoire, M., Lee, A., and Durantel, D. (2020). Antiviral activity of PLK1-targeting siRNA delivered by lipid nanoparticles in HBV-infected hepatocytes. Antivir Ther *25*, 151-162. 10.3851/IMP3361.

Freise, C., Lee, H., Chronowski, C., Chan, D., Cziomer, J., Ruhl, M., Dagdelen, T., Losekann, M., Erben, U., Catic, A., et al. (2021). Alpha-single chains of collagen type VI inhibit the fibrogenic effects of triple helical collagen VI in hepatic stellate cells. PLoS One *16*, e0254557. 10.1371/journal.pone.0254557.

Friedrich, C., Schallenberg, S., Kirchner, M., Ziehm, M., Niquet, S., Haji, M., Beier, C., Neudecker, J., Klauschen, F., and Mertins, P. (2021). Comprehensive micro-scaled proteome and phosphoproteome characterization of archived retrospective cancer repositories. Nat Commun *12*, 3576. 10.1038/s41467-021-23855-w.

Futatsugi, A., Utreras, E., Rudrabhatla, P., Jaffe, H., Pant, H.C., and Kulkarni, A.B. (2012). Cyclin-dependent kinase 5 regulates E2F transcription factor through phosphorylation of Rb protein in neurons. Cell Cycle *11*, 1603-1610. 10.4161/cc.20009.

Gearhart, T.L., and Bouchard, M.J. (2010). Replication of the hepatitis B virus requires a calcium-dependent HBx-induced G1 phase arrest of hepatocytes. Virology *407*, 14-25. 10.1016/j.virol.2010.07.042.

Geerts, A. (2001). History, heterogeneity, developmental biology, and functions of quiescent hepatic stellate cells. Semin Liver Dis *21*, 311-335. 10.1055/s-2001-17550.

Grossi, G., Vigano, M., Loglio, A., and Lampertico, P. (2017). Hepatitis B virus long-term impact of antiviral therapy nucleot(s)ide analogues (NUCs). Liver Int *37 Suppl 1*, 45-51. 10.1111/liv.13291.

Grumati, P., Coletto, L., Sabatelli, P., Cescon, M., Angelin, A., Bertaggia, E., Blaauw, B., Urciuolo, A., Tiepolo, T., Merlini, L., et al. (2010). Autophagy is defective in collagen VI muscular dystrophies, and its reactivation rescues myofiber degeneration. Nat Med *16*, 1313-1320. 10.1038/nm.2247.

Hergeth, S.P., and Schneider, R. (2015). The H1 linker histones: multifunctional proteins beyond the nucleosomal core particle. EMBO Rep *16*, 1439-1453. 10.15252/embr.201540749.

Herrscher, C., Pastor, F., Burlaud-Gaillard, J., Dumans, A., Seigneuret, F., Moreau, A., Patient, R., Eymieux, S., de Rocquigny, H., Hourioux, C., et al. (2020). Hepatitis B virus entry into HepG2-NTCP cells requires clathrin-mediated endocytosis. Cell Microbiol *22*, e13205. 10.1111/cmi.13205.

Jongewaard, I.N., Lauer, R.M., Behrendt, D.A., Patil, S., and Klewer, S.E. (2002). Beta 1 integrin activation mediates adhesive differences between trisomy 21 and non-trisomic fibroblasts on type VI collagen. Am J Med Genet *109*, 298-305. 10.1002/ajmg.10413.

Kegel, V., Deharde, D., Pfeiffer, E., Zeilinger, K., Seehofer, D., and Damm, G. (2016). Protocol for Isolation of Primary Human Hepatocytes and Corresponding Major Populations of Non-parenchymal Liver Cells. J Vis Exp, e53069. 10.3791/53069.

Kikuchi, A., Pradhan-Sundd, T., Singh, S., Nagarajan, S., Loizos, N., and Monga, S.P. (2017). Platelet-Derived Growth Factor Receptor alpha Contributes to Human Hepatic Stellate Cell Proliferation and Migration. Am J Pathol *187*, 2273-2287. 10.1016/j.ajpath.2017.06.009.

Kitto, L.J., and Henderson, N.C. (2021). Hepatic Stellate Cell Regulation of Liver Regeneration and Repair. Hepatol Commun *5*, 358-370. 10.1002/hep4.1628.

Koop, R., Di Croce, L., and Beato, M. (2003). Histone H1 enhances synergistic activation of the MMTV promoter in chromatin. EMBO J *22*, 588-599. 10.1093/emboj/cdg052.

Ladner, S.K., Otto, M.J., Barker, C.S., Zaifert, K., Wang, G.H., Guo, J.T., Seeger, C., and King, R.W. (1997). Inducible expression of human hepatitis B virus (HBV) in stably transfected hepatoblastoma cells: a novel system for screening potential inhibitors of HBV replication. Antimicrob Agents Chemother *41*, 1715-1720. 10.1128/AAC.41.8.1715.

Lamande, S.R., and Bateman, J.F. (2018). Collagen VI disorders: Insights on form and function in the extracellular matrix and beyond. Matrix Biol *71-72*, 348-367. 10.1016/j.matbio.2017.12.008.

Lara-Pezzi, E., Gomez-Gaviro, M.V., Galvez, B.G., Mira, E., Iniguez, M.A., Fresno, M., Martinez, A.C., Arroyo, A.G., and Lopez-Cabrera, M. (2002). The hepatitis B virus X protein promotes tumor cell invasion by inducing membrane-type matrix metalloproteinase-1 and cyclooxygenase-2 expression. J Clin Invest *110*, 1831-1838. 10.1172/JCI15887.

Lara-Pezzi, E., Majano, P.L., Yanez-Mo, M., Gomez-Gonzalo, M., Carretero, M., Moreno-Otero, R., Sanchez-Madrid, F., and Lopez-Cabrera, M. (2001a). Effect of the hepatitis B virus HBx protein on integrin-mediated adhesion to and migration on extracellular matrix. J Hepatol *34*, 409-415. 10.1016/s0168-8278(00)00090-8.

Lara-Pezzi, E., Serrador, J.M., Montoya, M.C., Zamora, D., Yanez-Mo, M., Carretero, M., Furthmayr, H., Sanchez-Madrid, F., and Lopez-Cabrera, M. (2001b). The hepatitis B virus X protein (HBx) induces a migratory phenotype in a CD44-dependent manner: possible role of HBx in invasion and metastasis. Hepatology *33*, 1270-1281. 10.1053/jhep.2001.1270.

Lee, Y.S., Kim, S.Y., Ko, E., Lee, J.H., Yi, H.S., Yoo, Y.J., Je, J., Suh, S.J., Jung, Y.K., Kim, J.H., et al. (2017). Exosomes derived from palmitic acid-treated hepatocytes induce fibrotic activation of hepatic stellate cells. Sci Rep *7*, 3710. 10.1038/s41598-017-03389-2.

Lees, J.A., Buchkovich, K.J., Marshak, D.R., Anderson, C.W., and Harlow, E. (1991). The retinoblastoma protein is phosphorylated on multiple sites by human cdc2. EMBO J *10*, 4279-4290.

Leupin, O., Bontron, S., Schaeffer, C., and Strubin, M. (2005). Hepatitis B virus X protein stimulates viral genome replication via a DDB1-dependent pathway distinct from that leading to cell death. J Virol *79*, 4238-4245. 10.1128/JVI.79.7.4238-4245.2005.

Li, J., Liu, Y., Wang, Z., Liu, K., Wang, Y., Liu, J., Ding, H., and Yuan, Z. (2011). Subversion of cellular autophagy machinery by hepatitis B virus for viral envelopment. J Virol *85*, 6319-6333. 10.1128/JVI.02627-10.

Liberzon, A., Birger, C., Thorvaldsdottir, H., Ghandi, M., Mesirov, J.P., and Tamayo, P. (2015). The Molecular Signatures Database (MSigDB) hallmark gene set collection. Cell Syst *1*, 417-425. 10.1016/j.cels.2015.12.004.

Liberzon, A., Subramanian, A., Pinchback, R., Thorvaldsdottir, H., Tamayo, P., and Mesirov, J.P. (2011). Molecular signatures database (MSigDB) 3.0. Bioinformatics *27*, 1739-1740. 10.1093/bioinformatics/btr260.

Lin, Y., Zhao, Z., Huang, A., and Lu, M. (2020). Interplay between Cellular Autophagy and Hepatitis B Virus Replication: A Systematic Review. Cells *9*. 10.3390/cells9092101.

Luger, K., Mader, A.W., Richmond, R.K., Sargent, D.F., and Richmond, T.J. (1997). Crystal structure of the nucleosome core particle at 2.8 A resolution. Nature *389*, 251-260. 10.1038/38444.

Lupberger, J., Croonenborghs, T., Roca Suarez, A.A., Van Renne, N., Juhling, F., Oudot, M.A., Virzi, A., Bandiera, S., Jamey, C., Meszaros, G., et al. (2019). Combined Analysis of Metabolomes, Proteomes, and Transcriptomes of Hepatitis C Virus-Infected Cells and Liver to

Identify Pathways Associated With Disease Development. Gastroenterology *157*, 537-551 e539. 10.1053/j.gastro.2019.04.003.

Macdonald, J.I., and Dick, F.A. (2012). Posttranslational modifications of the retinoblastoma tumor suppressor protein as determinants of function. Genes Cancer *3*, 619-633. 10.1177/1947601912473305.

Mertins, P., Mani, D.R., Ruggles, K.V., Gillette, M.A., Clauser, K.R., Wang, P., Wang, X., Qiao, J.W., Cao, S., Petralia, F., et al. (2016). Proteogenomics connects somatic mutations to signalling in breast cancer. Nature *534*, 55-62. 10.1038/nature18003.

Mertins, P., Tang, L.C., Krug, K., Clark, D.J., Gritsenko, M.A., Chen, L., Clauser, K.R., Clauss, T.R., Shah, P., Gillette, M.A., et al. (2018). Reproducible workflow for multiplexed deep-scale proteome and phosphoproteome analysis of tumor tissues by liquid chromatography-mass spectrometry. Nat Protoc *13*, 1632-1661. 10.1038/s41596-018-0006-9.

Miyoshi, J., and Takai, Y. (2008). Structural and functional associations of apical junctions with cytoskeleton. Biochim Biophys Acta *1778*, 670-691. 10.1016/j.bbamem.2007.12.014.

Mooren, O.L., Galletta, B.J., and Cooper, J.A. (2012). Roles for actin assembly in endocytosis. Annu Rev Biochem *81*, 661-686. 10.1146/annurev-biochem-060910-094416.

Mootha, V.K., Lindgren, C.M., Eriksson, K.F., Subramanian, A., Sihag, S., Lehar, J., Puigserver, P., Carlsson, E., Ridderstrale, M., Laurila, E., et al. (2003). PGC-1alpha-responsive genes involved in oxidative phosphorylation are coordinately downregulated in human diabetes. Nat Genet *34*, 267-273. 10.1038/ng1180.

Nussinov, R., Jang, H., Nir, G., Tsai, C.J., and Cheng, F. (2021). A new precision medicine initiative at the dawn of exascale computing. Signal Transduct Target Ther *6*, 3. 10.1038/s41392-020-00420-3.

Ozer, A., Khaoustov, V.I., Mearns, M., Lewis, D.E., Genta, R.M., Darlington, G.J., and Yoffe, B. (1996). Effect of hepatocyte proliferation and cellular DNA synthesis on hepatitis B virus replication. Gastroenterology *110*, 1519-1528. 10.1053/gast.1996.v110.pm8613059.

Pant, A., Dsouza, L., and Yang, Z. (2021). Alteration in Cellular Signaling and Metabolic Reprogramming during Viral Infection. mBio *12*, e0063521. 10.1128/mBio.00635-21.

Parikh, P., Ryan, J.D., and Tsochatzis, E.A. (2017). Fibrosis assessment in patients with chronic hepatitis B virus (HBV) infection. Ann Transl Med *5*, 40. 10.21037/atm.2017.01.28.

Puche, J.E., Saiman, Y., and Friedman, S.L. (2013). Hepatic stellate cells and liver fibrosis. Compr Physiol *3*, 1473-1492. 10.1002/cphy.c120035.

Qiao, L., and Luo, G.G. (2019). Human apolipoprotein E promotes hepatitis B virus infection and production. PLoS Pathog *15*, e1007874. 10.1371/journal.ppat.1007874.

Roehlen, N., Crouchet, E., and Baumert, T.F. (2020). Liver Fibrosis: Mechanistic Concepts and Therapeutic Perspectives. Cells *9*. 10.3390/cells9040875.

Sarg, B., Helliger, W., Talasz, H., Forg, B., and Lindner, H.H. (2006). Histone H1 phosphorylation occurs site-specifically during interphase and mitosis: identification of a novel phosphorylation site on histone H1. J Biol Chem *281*, 6573-6580. 10.1074/jbc.M508957200.

Scheuer, P.J. (1991). Classification of chronic viral hepatitis: a need for reassessment. J Hepatol *13*, 372-374. 10.1016/0168-8278(91)90084-o.

Schulze, A., Gripon, P., and Urban, S. (2007). Hepatitis B virus infection initiates with a large surface protein-dependent binding to heparan sulfate proteoglycans. Hepatology *46*, 1759-1768. 10.1002/hep.21896.

Stockl, L., Berting, A., Malkowski, B., Foerste, R., Hofschneider, P.H., and Hildt, E. (2003). Integrity of c-Raf-1/MEK signal transduction cascade is essential for hepatitis B virus gene expression. Oncogene *22*, 2604-2610. 10.1038/sj.onc.1206320.

Subramanian, A., Tamayo, P., Mootha, V.K., Mukherjee, S., Ebert, B.L., Gillette, M.A., Paulovich, A., Pomeroy, S.L., Golub, T.R., Lander, E.S., and Mesirov, J.P. (2005). Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. Proc Natl Acad Sci U S A *102*, 15545-15550. 10.1073/pnas.0506580102.

Sun, Y., Wu, X., Zhou, J., Meng, T., Wang, B., Chen, S., Liu, H., Wang, T., Zhao, X., Wu, S., et al. (2020). Persistent Low Level of Hepatitis B Virus Promotes Fibrosis Progression During Therapy. Clin Gastroenterol Hepatol *18*, 2582-2591 e2586. 10.1016/j.cgh.2020.03.001.

Tan, Z., Liu, Q., Jiang, R., Lv, L., Shoto, S.S., Maillet, I., Quesniaux, V., Tang, J., Zhang, W., Sun, B., and Ryffel, B. (2018). Interleukin-33 drives hepatic fibrosis through activation of hepatic stellate cells. Cell Mol Immunol *15*, 388-398. 10.1038/cmi.2016.63.

Tao, Y., Wu, D., Zhou, L., Chen, E., Liu, C., Tang, X., Jiang, W., Han, N., Li, H., and Tang, H. (2020). Present and Future Therapies for Chronic Hepatitis B. Adv Exp Med Biol *1179*, 137-186. 10.1007/978-981-13-9151-4_6.

Theise, N.D. (2007). Liver biopsy assessment in chronic viral hepatitis: a personal, practical approach. Mod Pathol *20 Suppl 1*, S3-14. 10.1038/modpathol.3800693.

Tout, I., Lampertico, P., Berg, T., and Asselah, T. (2021). Perspectives on stopping nucleos(t)ide analogues therapy in patients with chronic hepatitis B. Antiviral Res *185*, 104992. 10.1016/j.antiviral.2020.104992.

Tsuchida, T., and Friedman, S.L. (2017). Mechanisms of hepatic stellate cell activation. Nat Rev Gastroenterol Hepatol *14*, 397-411. 10.1038/nrgastro.2017.38.

Tsvetkov, L., and Stern, D.F. (2005). Phosphorylation of Plk1 at S137 and T210 is inhibited in response to DNA damage. Cell Cycle *4*, 166-171. 10.4161/cc.4.1.1348.

Tulla, M., Pentikainen, O.T., Viitasalo, T., Kapyla, J., Impola, U., Nykvist, P., Nissinen, L., Johnson, M.S., and Heino, J. (2001). Selective binding of collagen subtypes by integrin alpha 1I, alpha 2I, and alpha 10I domains. J Biol Chem *276*, 48206-48212. 10.1074/jbc.M104058200.

Urushima, H., Yuasa, H., Matsubara, T., Kuroda, N., Hara, Y., Inoue, K., Wake, K., Sato, T., Friedman, S.L., and Ikeda, K. (2021). Activation of Hepatic Stellate Cells Requires Dissociation of E-Cadherin-Containing Adherens Junctions with Hepatocytes. Am J Pathol *191*, 438-453. 10.1016/j.ajpath.2020.12.007.

Verrier, E.R., Colpitts, C.C., Bach, C., Heydmann, L., Weiss, A., Renaud, M., Durand, S.C., Habersetzer, F., Durantel, D., Abou-Jaoude, G., et al. (2016a). A targeted functional RNA interference screen uncovers glypican 5 as an entry factor for hepatitis B and D viruses. Hepatology *63*, 35-48. 10.1002/hep.28013.

Verrier, E.R., Colpitts, C.C., Schuster, C., Zeisel, M.B., and Baumert, T.F. (2016b). Cell Culture Models for the Investigation of Hepatitis B and D Virus Infection. Viruses *8*. 10.3390/v8090261.

Vicent, G.P., Nacht, A.S., Font-Mateu, J., Castellano, G., Gaveglia, L., Ballare, C., and Beato, M. (2011). Four enzymes cooperate to displace histone H1 during the first minute of hormonal gene activation. Genes Dev *25*, 845-862. 10.1101/gad.621811.

Virzi, A., Gonzalez-Motos, V., Tripon, S., Baumert, T.F., and Lupberger, J. (2021). Profibrotic Signaling and HCC Risk during Chronic Viral Hepatitis: Biomarker Development. J Clin Med *10*. 10.3390/jcm10050977.

Wang, M., Gong, Q., Zhang, J., Chen, L., Zhang, Z., Lu, L., Yu, D., Han, Y., Zhang, D., Chen, P., et al. (2017). Characterization of gene expression profiles in HBV-related liver fibrosis

patients and identification of ITGBL1 as a key regulator of fibrogenesis. Sci Rep *7*, 43446. 10.1038/srep43446.

Wang, T., Zhao, R., Wu, Y., Kong, D., Zhang, L., Wu, D., Li, C., Zhang, C., Yu, Z., and Jin, X. (2011). Hepatitis B virus induces G1 phase arrest by regulating cell cycle genes in HepG2.2.15 cells. Virol J *8*, 231. 10.1186/1743-422X-8-231.

Wang, Z., Wang, W., and Wang, L. (2020). Epigenetic regulation of covalently closed circular DNA minichromosome in hepatitis B virus infection. Biophysics Reports *6*, 115-126. 10.1007/s41048-020-00112-z.

Whitefield, D.B., Spagnol, S.T., Armiger, T.J., Lan, L., and Dahl, K.N. (2018). Quantifying site-specific chromatin mechanics and DNA damage response. Sci Rep *8*, 18084. 10.1038/s41598-018-36343-x.

Williams, L.M., McCann, F.E., Cabrita, M.A., Layton, T., Cribbs, A., Knezevic, B., Fang, H., Knight, J., Zhang, M., Fischer, R., et al. (2020). Identifying collagen VI as a target of fibrotic diseases regulated by CREBBP/EP300. Proc Natl Acad Sci U S A *117*, 20753-20763. 10.1073/pnas.2004281117.

Wu, D., Zhang, S., Xie, Z., Chen, E., Rao, Q., Liu, X., Huang, K., Yang, J., Xiao, L., Ji, F., et al. (2020). Plasminogen as a prognostic biomarker for HBV-related acute-on-chronic liver failure. J Clin Invest *130*, 2069-2080. 10.1172/JCI130197.

Xia, Y., Cheng, X., Li, Y., Valdez, K., Chen, W., and Liang, T.J. (2018). Hepatitis B Virus Deregulates the Cell Cycle To Promote Viral Replication and a Premalignant Phenotype. J Virol *92*. 10.1128/JVI.00722-18.

Yan, H., Zhong, G., Xu, G., He, W., Jing, Z., Gao, Z., Huang, Y., Qi, Y., Peng, B., Wang, H., et al. (2012). Sodium taurocholate cotransporting polypeptide is a functional receptor for human hepatitis B and D virus. Elife *1*, e00049. 10.7554/eLife.00049.

Ye, F., Huang, W., Xue, Y., Tang, E., Wang, M., Shi, F., Wei, D., Han, Y., Chen, P., Zhang, X., and Yu, D. (2021). Serum Levels of ITGBL1 as an Early Diagnostic Biomarker for Hepatocellular Carcinoma with Hepatitis B Virus Infection. J Hepatocell Carcinoma *8*, 285-300. 10.2147/JHC.S306966.

Ying, H.Z., Chen, Q., Zhang, W.Y., Zhang, H.H., Ma, Y., Zhang, S.Z., Fang, J., and Yu, C.H. (2017). PDGF signaling pathway in hepatic fibrosis pathogenesis and therapeutics (Review). Mol Med Rep *16*, 7879-7889. 10.3892/mmr.2017.7641.

Yoneda, M., Hyun, J., Jakubski, S., Saito, S., Nakajima, A., Schiff, E.R., and Thomas, E. (2016). Hepatitis B Virus and DNA Stimulation Trigger a Rapid Innate Immune Response through NF-kappaB. J Immunol *197*, 630-643. 10.4049/jimmunol.1502677.

Zarkowska, T., and Mittnacht, S. (1997). Differential phosphorylation of the retinoblastoma protein by G1/S cyclin-dependent kinases. J Biol Chem *272*, 12738-12746. 10.1074/jbc.272.19.12738.

Zhang, B., Dong, Q., Su, H., Birchler, J.A., and Han, F. (2014). Histone phosphorylation: its role during cell cycle and centromere identity in plants. Cytogenet Genome Res *143*, 144-149. 10.1159/000360435.

Zhang, B., Lapenta, K., Wang, Q., Nam, J.H., Chung, D., Robert, M.E., Nathanson, M.H., and Yang, X. (2021). Trefoil factor 2 secreted from damaged hepatocytes activates hepatic stellate cells to induce fibrogenesis. J Biol Chem *297*, 100887. 10.1016/j.jbc.2021.100887.

Zhou, W., Ma, Y., Zhang, J., Hu, J., Zhang, M., Wang, Y., Li, Y., Wu, L., Pan, Y., Zhang, Y., et al. (2017). Predictive model for inflammation grades of chronic hepatitis B: Large-scale analysis of clinical parameters and gene expressions. Liver Int *37*, 1632-1641. 10.1111/liv.13427.

FIGURE LEGENDS

Figure 1. **Proteomic and phosphoproteomic changes induced by HBV in hepatocytes-like cells during an infection time course**. **A.** Phospho-sites (in green) and protein expression (in blues) overlap between day 2 and day 10 pi (see Table S1, S2). **B-C.** Chord analysis of major biological pathways dysregulated by HBV on the phosphoproteomics using HALLMARK database from MSigDB (120 common hits; adj.P.Val.< 0.05, cutoff logFC= 0.5; see Table S3) and on the proteomics using REACTOME database from MsigDB (38 common hits; adj.P.Val.< 0.01). **D.** HBV perturbs pathways involved in ECM remodeling and integrin interaction at the proteomic and transcriptomic level in both HepG2-NTCP and PHHs, respectively. In red: significantly (FDR q-value < 0.003) upregulated, in grey: not significantly dysregulated (FDR q-value > 0.003). Refer to Table S4 for additional information. **E**. Leading-edge genes of gene sets enriched at proteomic and transcriptomic level partially overlap. **F**. *COL6A1* is among the leading-edge genes, and it is significantly (in red: adj.P.Val.< 0.01) upregulated in HepG2-NTCP and PHHs.

Figure 2. HBV-induced ColVI expression is required for viral infection. A. ColVI is upregulated in HBsAg-positive HepG2-NTCP cells (red pick) versus HBsAg negative (light-blue pick) measured by flow cytometry analysis (n= 4; p= 0.0286; Mann-Whitney U test). **B.** Augmented *COL6A1 mRNA* expression level in HBV-infected PHHs (GEO dataset GSE69590) (n= 3; p= 0.0005; unpaired t-test) **C.** *COL6A1* mRNA expression is upregulated in HBV-infected patients (n=122) compared with healthy patients (n= 6) (GEO dataset GSE83148) (p= 0.1234 (ns); 0.0332 (*); 0.0021 (**); 0.0002 (***); < 0.0001 (****); One-way ANOVA). **D-E.** *COL6A1* mRNA expression correlates with fibrosis progression in HBV-associated fibrosis patients (GEO dataset GSE84044); S0= 43; S1= 20, S2= 33, S4= 10; G1= 37; G2= 33; G3= 34; G4= 15 (p= 0.1234 (ns); 0.0332 (*); 0.0021 (**); 0.0002 (***); <0.0001 (****); One-way ANOVA). **F-G.** Silencing of *COL6A1* reduces the expression of HBV transcripts. *pgRNA/pcRNA*: p < 0.0001, Unpaired t-test; total HBV mRNA: p= 0.0083, Unpaired t-test.

Figure 3. ColVI promotes stellate cell activation. A. Experimental outline of patients-derived HSCs isolation for ColVI treatment (parts figure was created with *BioRender.com*). **B.** Isolated myofibroblasts cultivated for two weeks prior experiment to reach confluency and good experimental condition were stained for α-SMA. **C-D.** Myofibroblasts derived from four different donors treated with ColVI (0.5 µg/mL) and TGF-β1 (10 ng/mL) expressed higher mRNA level of activation markers (*COL1A1* and *ACTA2*) compared to the non-treated (mock). One-way ANOVA test*;* p= 0.1234 (ns); 0.0332 (*); 0.0021 (**); 0.0002 (***); < 0.0001 (****).

Figure 4. **H1.4 phosphorylation by gene silencing enhances HBV transcription**. **A**. HBV infection significantly induces linker histones phosphorylation at day 2 pi. In red: upregulated adj.P.Val.< 0.05; in grey: not significantly dysregulated). **B**. H1 phosphorylation is associated with H1 detachment and chromatin decompaction (Belikov *et al.*, 2007; Bhattacharjee *et al.*, 2001; Koop *et al.*, 2003; Vicent *et al.*, 2011). Created with *BioRender.com*. **C-D**. siRNA silencing of H1.4 measured by WB and qPCR. **E.** H1.4 phosphorylation mimicked H1.4 silencing increased the transcription of HBV-associated transcripts (*pgRNA/pcRNA*: p< 0.0001, Unpaired t-test; total HBV mRNA transcript: p= 0.0002, Unpaired t-test). **F.** Silencing of H1.4 does not impact housekeeping gene expression level. **G**. Silencing of H1.4 does not affect cell viability (presto blue viability assay).

Figure S1. HBV infection for proteomic and phosphoproteomic analysis. A. HBsAg staining at day 10 pi **B**. HBeAg measurement at day 2 and day 10 pi on the supernatant of HBV-infected cells. Scale bar: 100 µm. **C.** Experimental design for global proteomic and phosphoproteomic analysis.

Figure S2. **Volcano plot of the proteomic and phosphoproteomic analysis**. **A**. Volcano plot showing the logFC plotted against the -log 10 P-value, illustrate proteins expression changes in HBV-infected cell over Mock (non-infected) for day 2 and day 10 pi with a cutoff for adj.P.Val.= 0.01 (green dots: significantly downregulated; red dots: significantly upregulated; gray

dots: non-significantly dysregulated). **B.** Volcano plot showing the logFC plotted against the -log 10 P-value, depicts phospho-peptide changes in HBV-infected cells over Mock (non-infected) for day 2 and day 10 pi, with a cutoff of adj.P.Val.= 0.05 (green dots: significantly downregulated; red dots: significantly upregulated; gray dots: non-significantly dysregulated).

Figure S3. Phosphoproteomic changes at day 2 and day 10 post infection. Chord analysis of major biological pathways dysregulated by HBV on the phosphoproteomics using HALLMARK database from MSigDB (created using phosphoproteome hits with adj.P.Val. < 0.05, cutoff logFC= 0.5 for day 2; adj.P.Val.< 0.05 for day 10).

Figure S4. Silencing of H1.4 on established HBV infection is not associated with augmentation of HBV transcripts level. HepG2-NTCP cells were infected for 3 days following 3 days silencing (*pgRNA/pcRNA*: p= 0.0017, Unpaired t-test; HBV total mRNA p= 0.1615, Mann-Whitney U test).

Fig. 1

Phosphoproteomic

A

HBV phospho-proteomics

1431

120
day 10
day 2
202

281   **38**   314
day 2   day 10

HBV proteomics

B

MYC TARGET

MITOTIC SPINDLE

TGFB SIGNALING

APICAL JUNCTION

E2F TARGET

G2/M CHECKPOINT

C

Proteomic

PLASMA LIPOPROTEIN
REMODELING

REGULATION OF IGF TRANSPORT AND
UPTAKE BY IGFBPS

INTEGRIN SIGNALING

PLASMA LIPOPROTEIN ASSEMBLY
REMODELING CLEARANCE

**INTEGRIN CELL SURFACE
INTERACTIONS**

FIBRIN CLOT
FORMATION

**EXTRACELLULAR
MATRIX ORGANIZATION**

REPONSE TO ELEVATED
PLATELET CYTOSOLIC
Ca++

GRB2 SOS PROVIDES LINKAGE TO
MAPK SIGNALING FOR INTEGRINS

P130CAS LINKAGE TO MAPK
SIGNALING FOR INTEGRIN

D

NES

-3.00   0.00   3.00

	Protein day2	Protein day10	RNA (PHH)	id
				NABA_CORE_MATRISOME
				NABA_ECM_GLYCOPROTEINS
				REACTOME_INTEGRIN_CELL_SURFACE_INTERACTIONS
				REACTOME_DEGRADATION_OF_THE_EXTRACELLULAR_MATRIX

F

logFC

-1.00   0.00   1.00

	PHH	HepG2-NTCP (day2)	id
			CDH1
			FGG
			PLG
			COL18A1
			FN1
			COL6A1
			FGL1
			ICAM1
			SMOC1
			VWA1

E

PHH

HepG2-NTCP

120

16

14

day 2

Fig. 2

A

B

C

D

E

F

G

Fig. 3

A

Liver resection

↓

NPCs extraction

Density gradient centrifugation

HSCs isolation and culture

ColVI treatment

Activation fibrotic markers

B

DAPI   α-SMA

C

D

Fig. 4

Fig. S1

A

DAPI HBsAg DAPI HBsAg

B

C

Fig. S2

A   Proteomic

B   Phosphoproteomic

Fig. S3

Phosphoproteomic (day 2)

Phosphoproteomic (day 10)

Fig. S4

Supplementary Table S1. Significant (adj.P.Val.<0.01) proteomic hits common day 2 + day 10 pi. Related to Fig. 1A.

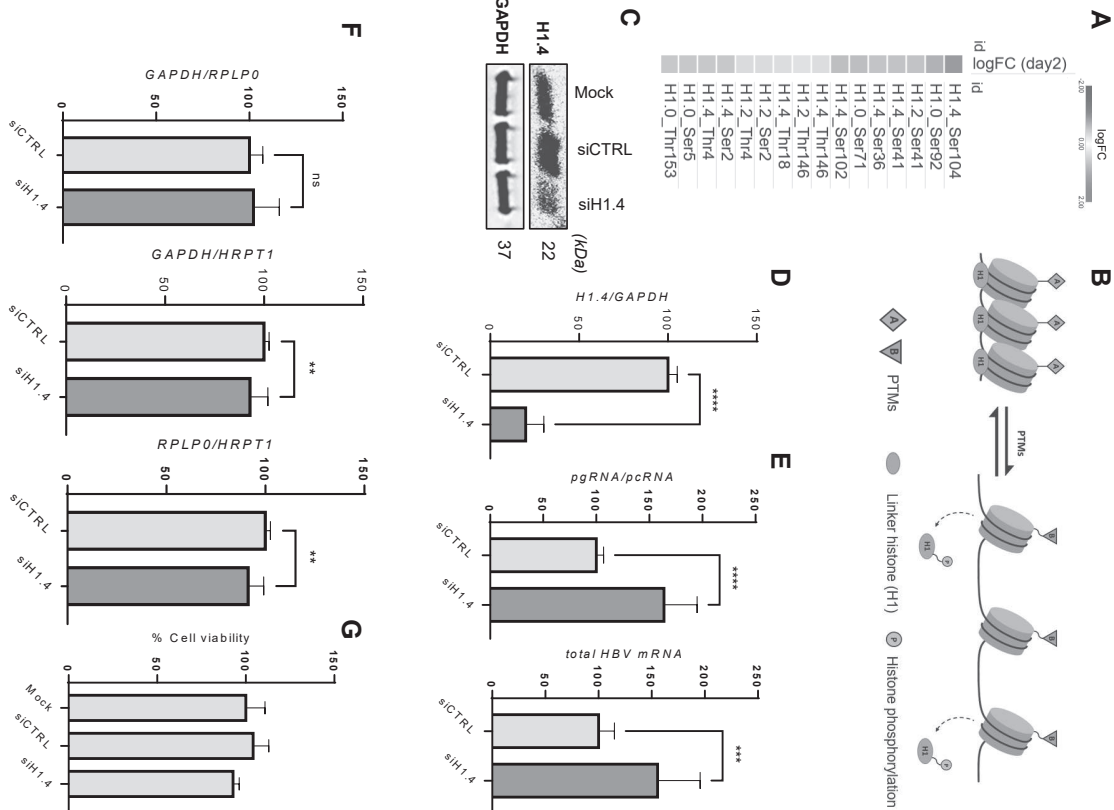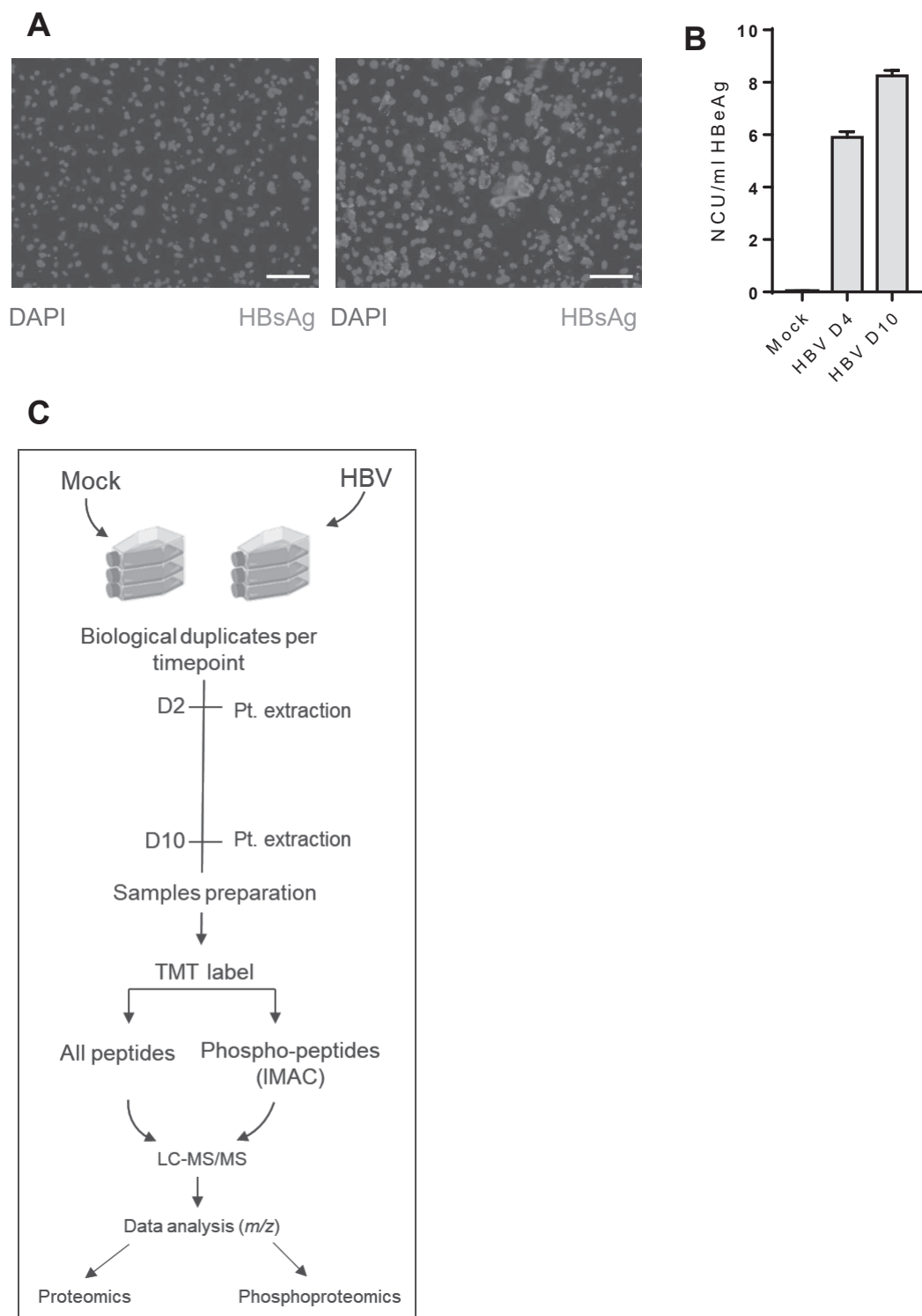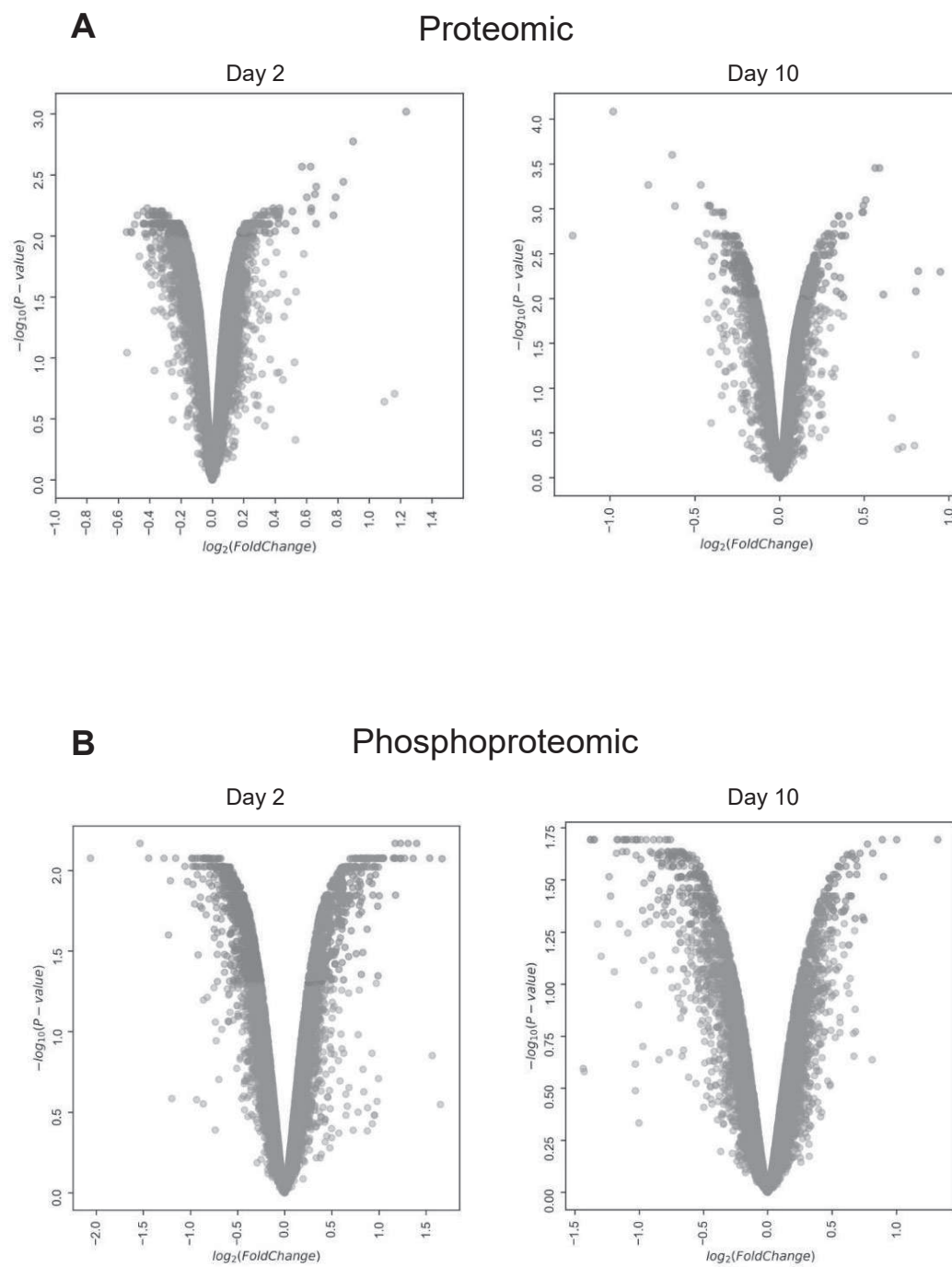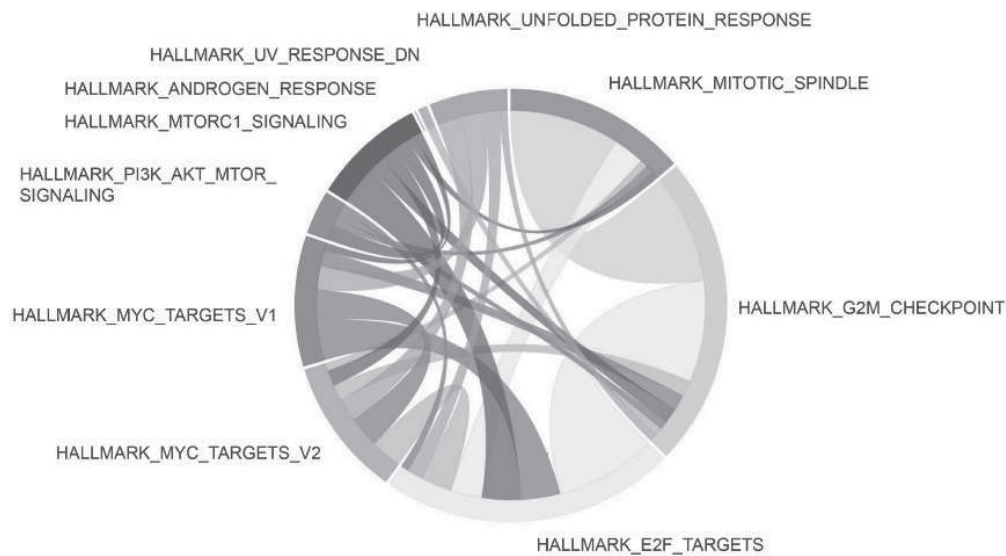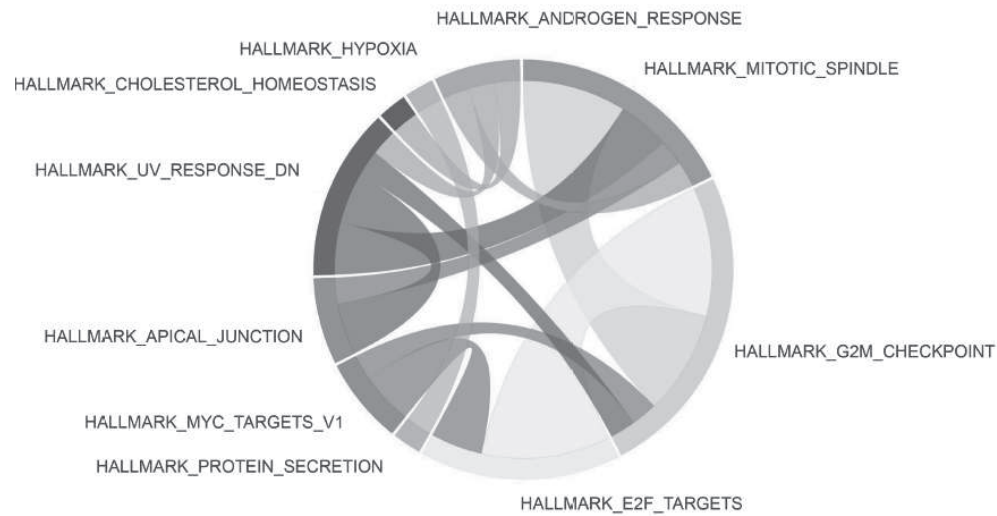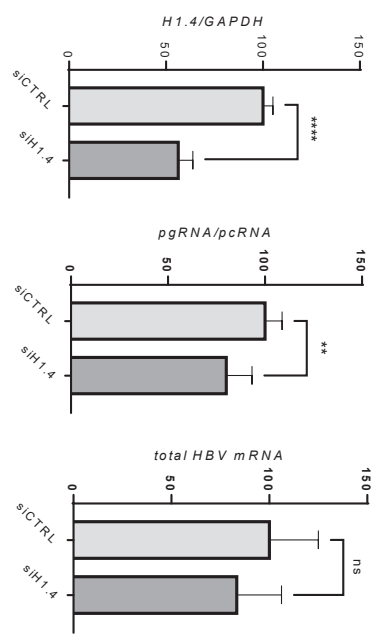| Id	Uniprot	DAY 2 (adj.P.Val.)	DAY 10 (adj.P.Val.)	DAY 2 (logFC)	DAY 10 (logFC)
ABHD17A	Q96GS6-3	0.00483397	0.004448207	0.602625909	0.205077875
AGR2	O95994	0.008057835	0.002001741	-0.299791647	0.311385044
ALKBH3	Q96Q83	0.009273633	0.008407496	0.199937492	0.152405556
ANPEP	P15144	0.007964844	0.005151501	0.288739892	0.210112454
APOA4	P06727	0.002712889	0.001479781	0.62715063	0.371151251
APOA5	Q6Q788	0.007964844	0.008360001	0.467392116	0.337689217
APOE	P02649	0.00456172	0.001207425	0.654510174	0.348837406
C19orf80	Q6UXH0	0.006794477	0.00035198	0.77287222	0.591326245
C5	P01031	0.0063032	0.001207425	0.629604471	0.412193051
CCDC126	C9J0J0	0.007964844	0.003872377	-0.438732477	-0.207071564
CCL20	P78556-2	0.0063032	0.006598688	0.431367205	-0.171529766
COL18A1	P39060-2	0.005938776	0.005895273	0.428453191	0.360168633
COL6A1	P12109	0.008032586	0.008532623	0.245954868	0.197384657
COL6A2	P12110-3	0.002712889	0.001479781	0.571886817	0.322413696
CXorf57	Q6NSI4-4	0.003610387	0.000801793	0.835540181	0.509232151
DAB2	P98082	0.008057835	0.004483557	0.244955927	0.18340316
EVA1A	B8ZZF5	0.007964844	0.001207425	0.298986334	0.35184491
F5	A0A0A0MRJ7	0.008571506	0.003270569	0.228366712	0.240108816
FASN	P49327	0.007964844	0.005374255	-0.236032308	0.177462062
FGA	P02671-2	0.0063032	0.002558296	0.511410895	0.307120433
FGB	P02675	0.001685115	0.00035198	0.897925102	0.565722386
FGG	P02679	0.00856003	0.002558296	0.228583927	0.237060101
FN1	P02751-17	0.009204628	0.003082441	0.399568874	0.319382095
GLG1	Q92896	0.006776465	0.005879383	-0.37084474	-0.168173629
GPR112	Q8IZF6-2	0.00483397	0.001901838	0.786761686	0.326283362
H6PD	R4GMU1	0.009457705	0.004976394	-0.177618443	-0.202326212
KDELC2	Q7Z4H8	0.007964844	0.008333652	-0.333388994	-0.152407876
LDLR	J3KMZ9	0.007964844	0.0033061	0.366262566	0.211072443
MASP2	O00187-2	0.009577771	0.002001741	0.366130966	0.369903701
NDUFA13	Q9P0J0	0.008057835	0.004641704	-0.282970198	-0.176454316
NDUFA9	Q16795	0.007964844	0.008532623	-0.334006575	-0.139493962
PLG	P00747	0.000960079	0.004976394	1.236096946	0.81957317
PTPRF	P10586-2	0.009416593	0.003319366	0.337977931	0.260372475
RAB27A	H3BU81	0.009329198	0.004641704	-0.546339738	-0.180393474
RGS10	O43665-2	0.009577771	0.004960415	0.183833494	-0.203827925
SMC1B	Q8NDV3-2	0.007964844	0.002493188	0.325197128	-0.277201253
SPAG5	Q96R06	0.007964844	0.0033061	-0.35817838	0.26996392
TM9SF3	Q9HD45	0.007964844	0.004976394	-0.432155164	-0.27426752

Supplementary Table S2. Significant (adj.P.Val.< 0.05) phosphoproteomic hits common day 2 + day 10. Related to Fig. 1A.

Id	Uniprot	DAY2 (adj.P.Val.)	DAY 10 (adj.P.Val.)	DAY2 (logFC)	DAY10 (logFC)
_51	A0A0U1RQE4	0.021232	0.03783	-0.441524959	-0.45515
AAK1_620	Q2M2I8-2	0.011386	0.040908	0.5210325	0.506259
AAK1_674	A0A096LNZ0	0.046519	0.027193	0.324401804	0.695247
AAK1_678	A0A096LNZ0	0.020086	0.023576	0.572607987	0.816801
ABCF1_167	Q5STZ8	0.038625	0.042945	0.391191158	0.412762
ACO1_138	P21399	0.018644	0.041064	0.56526734	0.465661
AGAP3_324	Q96P47	0.042047	0.023924	-0.43559744	-0.8525
AMPD3_85	E9PPG2	0.022424	0.041787	0.37052999	-0.51018
AP1AR_195	Q63HQ0-2	0.008409	0.041064	-0.986375561	-0.46064
ARHGEF28_311	Q8N1W1-5	0.01719	0.023144	0.547716236	-0.77084
ATP1A1_55	P05023-4	0.01719	0.023576	-0.637286628	0.717904
BAIAP2_366	Q9UQB8-4	0.032763	0.038394	-0.419307029	-0.80774
C19orf43_114	Q9BQ61	0.017262	0.0241	0.400366155	-0.74495
CAMSAP1_217	Q5T5Y3	0.018644	0.020281	0.401231899	0.890816
CARHSP1_30	H3BTK3	0.01245	0.030643	0.481669677	0.546523
CAST_34	P20810-9	0.017177	0.030528	-0.415130725	-0.52935
CBLB_479	B5MC15	0.047034	0.039707	0.494223651	0.475191
CCT2_254	P78371	0.012683	0.0241	0.514905188	-0.64489
CDC20_59	Q12834	0.014254	0.02523	-0.697865331	-0.9854
CDK12_893	Q9NYV4	0.014254	0.045752	0.459909717	-0.42252
CENPF_3119	P49454	0.008437	0.045272	-0.751082922	-0.80802
CTNND1_927	O60716-5	0.008437	0.046547	-0.704628227	-0.53324
DDX3X_579	F6S8Q4	0.00973	0.028038	0.638744019	-0.56149
DDX50_678	A0A087WVC1	0.008409	0.020281	-2.060357673	-1.08881
DNAJC8_81	O75937	0.010643	0.020281	0.553151988	-1.10477
DTX2_269	E7ET89	0.00971	0.03783	-0.629247089	-0.54625
EIF4B_354	P23588	0.008437	0.024921	0.810267606	-0.61513
EPB41L2_598	H0Y5B0	0.00955	0.041787	-0.667408465	-0.44674
ETV5_248	P41161	0.040425	0.039446	0.288720352	0.44933
FAM129B_683	Q96TA1-2	0.010312	0.029804	0.651339686	0.692591
GIGYF2_30	Q6Y7W6	0.008409	0.029804	0.981181185	0.634946
GSPT2_191	Q8IYD1	0.008409	0.020281	1.539891633	1.322285
GTF2F1_385	P35269	0.036705	0.034537	-0.33634317	0.469043
H1F0_92	P07305	0.009408	0.02523	0.950852892	0.686189
HN1L_85	A6NGP5	0.014491	0.040699	-0.495573017	-0.4635
IGF2BP1_436	Q9NZI8	0.019235	0.020281	0.416434773	-1.3766
IGF2BP1_438	Q9NZI8	0.019235	0.020281	0.416434773	-1.3766

IQSEC1_90	A0A2R8Y7T6	0.019759	0.023144	0.452383423	-0.91177
IRS1_794	P35568	0.008409	0.030643	-1.280235022	-0.484
ITGAD_333	Q13349	0.008437	0.020281	1.004798442	1.002919
ITSN2_120	A0A087WVF7	0.018092	0.029804	-0.467189418	-0.56187
KCNQ1_109	A0A2R8YEQ9	0.033467	0.039898	0.429745847	0.60588
KIAA1462_1319	Q9P266	0.014254	0.027193	0.588292676	-0.73698
KLC1_199	H0YG16	0.031999	0.023144	0.5057867	-0.67552
LAD1_161	E9PDI4	0.015993	0.03293	0.590444491	0.520776
LBR_99	C9JXK0	0.021567	0.041787	-0.356091611	-0.45482
LGALS2_126	P05162	0.008437	0.027193	0.852977547	-0.69205
LGALS2_127	P05162	0.008409	0.02523	0.887642301	-0.74772
LMO7_460	E9PMS6	0.008899	0.023144	-0.788502486	-0.72757
LRRFIP2_3	C9JSU1	0.025192	0.020281	-1.23156605	-1.34823
LUZP1_423	Q86V48-2	0.021791	0.045073	-0.430250056	-0.42323
MAP1B_744	P46821	0.026211	0.042945	-0.365013241	-0.41736
MAP2_434	P11137-2	0.011956	0.023924	0.622143324	0.617396
MAP3K1_250	Q13233	0.01815	0.036087	-0.655765005	-0.6838
MARK2_367	E7ETY4	0.00955	0.042945	-0.67551459	-0.42473
MINK1_535	Q8N4C8	0.011911	0.034725	-0.539819307	-0.60637
MLLT4_1287	A8MQ02	0.019268	0.03783	-0.572391935	-0.55144
NHSL1_1085	Q5SYE7-2	0.047378	0.044865	0.526707466	0.452044
NUP98_1043	P52948-6	0.009445	0.029335	-0.977207355	-0.54007
OTUD4_350	Q01804-5	0.027984	0.030528	0.451710471	-0.53381
P57789-3_7	P57789-3	0.029577	0.040136	-0.406723181	-0.78038
PAK2_143	Q13177	0.009445	0.027495	-0.731804364	-0.74291
PARD3_174	Q8TEW0-8	0.020567	0.020281	-0.43611551	-1.17065
PARG_178	Q86W56-3	0.035072	0.039898	0.296900309	-0.42159
PKP2_251	Q99959-2	0.008437	0.042311	-0.97933244	-0.43352
PKP4_447	Q99569-2	0.018291	0.029968	0.435460316	-0.54604
PLEC_2719	Q15149-3	0.044255	0.029804	0.814323858	-0.50012
PLIN3_241	O60664-3	0.014254	0.029804	1.176989894	-0.57181
PPFIA1_733	A0A2R8Y7R9	0.00971	0.02523	0.592037966	0.585699
PPP1R12A_366	O14974-5	0.024857	0.031077	-0.380851229	-0.55438
PPP1R12A_385	O14974-5	0.048605	0.0241	-0.502508007	-0.74427
PPP1R12A_608	O14974-5	0.034959	0.020281	0.363412353	-1.09844
PPP1R3D_77	O95685	0.009445	0.024526	0.698796837	0.663817
RAB11FIP1_8	E7EX40	0.016476	0.023144	-0.493109589	-0.82621
RBMX_352	P38159	0.011233	0.041787	-0.664002504	-0.44812
RHPN2_504	Q8IUC4-2	0.044052	0.030643	-0.401024152	-0.67282
ROCK1_1341	Q13464	0.020843	0.020281	0.570915791	-1.01666
RPL10_53	F8W7C6	0.033231	0.024191	0.508207835	-0.73837
RPL27A_11	E9PJD9	0.021647	0.02523	0.464635806	-0.7009

RPL28_92	P46779	0.015652	0.027193	0.808953032	-0.59141
RPL37A_21	G5E9R3	0.016476	0.0241	0.793441833	-1.02264
RPS11_67	P62280	0.012841	0.020281	0.723322688	-1.0291
RPS17_519	H3BNC9	0.012511	0.035749	0.506384296	-0.84521
RPS2_92	H0YEN5	0.016476	0.039241	0.426192931	-0.42556
RPS25_82	P62851	0.012461	0.031077	0.507412962	-0.49562
RPS27_78	P42677	0.011974	0.030643	0.550314996	-0.50237
RRBP1_191	Q9P2E9	0.008437	0.046837	-0.79741232	-0.44816
SAFB_604	Q15424-2	0.008409	0.030528	-1.441835036	-0.61453
SCAPER_759	H3BS25	0.00955	0.023576	0.963231958	-0.77202
SH2D4A_216	Q9H788-2	0.038225	0.042945	-0.342544025	-0.47496
SH3D19_345	A0A0U1RQE4	0.011956	0.047818	0.518035916	0.472649
SH3PXD2A_333	H0Y507	0.033215	0.034325	-0.429522185	-0.54599
SLTM_912	Q9NWH9	0.008409	0.040123	-0.848022808	-0.41348
SMTN_360	A0A087WVP4	0.016703	0.029804	-0.408792464	-0.56107
SNIP1_128	Q8TAD8	0.016181	0.03783	-0.954451726	-0.45467
SRRM1_388	A9Z1X7	0.044871	0.034725	0.315302662	0.470286
SRRM1_674	A9Z1X7	0.036183	0.041787	-0.346516421	-0.50561
SRRM1_676	A9Z1X7	0.026433	0.040768	-0.383672175	-0.61822
SRRM2_1880	Q9UQ35	0.022829	0.031786	0.41358453	0.488166
SRRM2_1893	Q9UQ35	0.011386	0.03783	0.582781884	0.435648
SRRM2_1982	Q9UQ35	0.014874	0.043496	0.445217983	0.387844
SRRM2_1984	Q9UQ35	0.044964	0.048264	0.301631782	0.441574
SRRM2_2692	Q9UQ35	0.012289	0.023576	-0.638827849	-0.84506
SSFA2_1118	E9PHV5	0.008437	0.023144	-0.675074834	-0.68155
STIM1_402	E9PNJ4	0.037184	0.023576	-0.399197644	-0.65783
STIM2_680	A0A1X7SBY3	0.014684	0.034248	-0.893465389	-0.49249
STK10_417	O94804	0.012683	0.024946	-0.46902985	-0.63415
TBC1D10B_661	Q4KMP7	0.009445	0.03783	-1.058543349	-0.661
TJP1_354	G3V1L9	0.043024	0.034248	-0.389189086	-0.51792
TMPO_433	P42166	0.016703	0.049475	-0.895411597	-0.68174
TNKS1BP1_1652	Q9C0C2	0.028366	0.03201	-0.356574134	-0.49801
TNS3_392	Q68CZ2-2	0.04499	0.031002	-0.468064029	-0.69675
TRIP10_318	M0R0F9	0.019714	0.030528	-0.439889032	-0.54107
TSC22D4_165	Q9Y3Q8	0.023935	0.032759	-0.665341236	-0.96081
TSC22D4_177	Q9Y3Q8	0.022076	0.0241	-0.362285738	-0.83196
TWF1_224	Q12792-4	0.043061	0.039898	0.416097049	-0.4627
VASP_85	K7EM16	0.011733	0.023576	-1.029903159	-1.1754
ZC3HAV1_378	C9J6P4	0.033793	0.048563	-0.394770336	-0.37732
ZKSCAN1_357	P17029	0.016534	0.038394	0.462710854	-0.77674
ZKSCAN1_361	P17029	0.019235	0.020281	0.427105383	-0.98702

Supplementary table S3. Key signaling pathways of the phosphoproteome significantly dysregulated (logFC cutoff: 0.5 Adj.P. Val.< 0.05) at day 2 +day 10 pi. Related to Fig. 1B.

Gene Set Name	# Genes in Gene Set (K)	Description	# Genes in Overlap (k)	k/K	FDR q-value
HALLMARK_MITOTIC_SPINDLE	199	Genes important for mitotic spindle assembly.	9	0.0452	7.74E-8
HALLMARK_E2F_TARGETS	200	Genes encoding cell cycle related targets of E2F transcription factors.	7	0.0350	1.04E-5
HALLMARK_G2M_CHECKPOINT	200	Genes involved in the G2/M checkpoint. as in progression through the cell division cycle.	7	0.0350	1.04E-5
HALLMARK_APICAL_JUNCTION	200	Genes encoding components of apical junction complex.	6	0.0300	1.24E-4
HALLMARK_TGF_BETA_SIGNALING	54	Genes up-regulated in response to TGFB1 [GeneID=7040].	3	0.0556	3.14E-3
HALLMARK_MYC_TARGETS_V1	200	A subgroup of genes regulated by MYC - version 1 (v1).	4	0.0200	1.22E-2

Supplementary Table S4. Top 30 signaling pathways by GSEA analysis upregulated by HBV at day 2 pi. Related to Fig. 1D.

NAME	NES	FDR q-value
REACTOME_RESPONSE_OF_EIF2AK4_GCN2_TO_AMINO_ACID_DEFICIENCY	2.6535008	0.0
REACTOME_EUKARYOTIC_TRANSLATION_ELONGATION	2.632291	0.0
REACTOME_SELENOAMINO_ACID_METABOLISM	2.5926435	0.0
KEGG_RIBOSOME	2.58636	0.0
REACTOME_NONSENSE_MEDIATED_DECAY_NMD	2.5411894	0.0
REACTOME_INTEGRIN_CELL_SURFACE_INTERACTIONS	2.5236378	0.0
REACTOME_EUKARYOTIC_TRANSLATION_INITIATION	2.4752605	0.0
REACTOME_REGULATION_OF_EXPRESSION_OF_SLITS_AND_ROBOS	2.4303813	0.0
NABA_CORE_MATRISOME	2.3784692	1.3539488E-4
PID_UPA_UPAR_PATHWAY	2.3727489	1.2185538E-4
REACTOME_SIGNALING_BY_ROBO_RECEPTORS	2.33694	1.1077762E-4
RODRIGUES_NTN1_AND_DCC_TARGETS	2.3163311	1.01546146E-4
GSE22229_RENAL_TRANSPLANT_VS_HEALTHY_PBMC_DN	2.2892718	9.373491E-5
BILANGES_SERUM_AND_RAPAMYCIN_SENSITIVE_GENES	2.2846448	1.7398423E-4
TIEN_INTESTINE_PROBIOTICS_6HR_UP	2.2679105	4.860247E-4
REACTOME_DEGRADATION_OF_THE_EXTRACELLULAR_MATRIX	2.259016	5.3141505E-4
POMEROY_MEDULLOBLASTOMA_DESMOPLASIC_VS_CLASSIC_DN	2.2516253	5.71835E-4
PECE_MAMMARY_STEM_CELL_UP	2.2351112	7.429559E-4
REACTOME_ACTIVATION_OF_THE_MRNA_UPON_BINDING_OF_THE_CAP_BINDING_ COMPLEX_ AND_EIFS_AND_SUBSEQUENT_BINDING_TO_43S	2.2028759	0.0011506862
CHNG_MULTIPLE_MYELOMA_HYPERPLOID_UP	2.1698813	0.00194305
REACTOME_ENDOSOMAL_SORTING_COMPLEX_REQUIRED_FOR_TRANSPORT_ ESCRT	2.1619465	0.0020815588
NABA_ECM_GLYCOPROTEINS	2.1570737	0.0024286867
PID_EPHRINB_REV_PATHWAY	2.1539178	0.0023230915
REACTOME_IMMUNOREGULATORY_INTERACTIONS_BETWEEN_A_LYMPHOID_ AND_A_NON_LYMPHOID_CELL	2.1422915	0.0029315893
TGFB_UP.V1_UP	2.141325	0.0028629485
BOQUEST_STEM_CELL_UP	2.1314576	0.0030827192
BHATI_G2M_ARREST_BY_2METHOXYESTRADIOL_DN	2.130345	0.003014073
HINATA_NFKB_TARGETS_KERATINOCYTE_UP	2.1276588	0.0030366813
GSE42088_UNINF_VS_LEISHMANIA_INF_DC_2H_DN	2.120211	0.0032649385
BEIER_GLIOMA_STEM_CELL_DN	2.1149318	0.003317672

GENERAL DISCUSSION

Beyond the main manuscript, I contributed to additional studies linked to my PhD project, which deepened my expertise on signaling in chronic hepatitis and associated pathogenesis (see ANNEX).

HBV and HCV are two very different viruses which represent the leading causes of liver disease progression and hepatocarcinogenesis. Nevertheless, they differ in structure and viral life cycle. HCV belongs to the *Flaviviridae* family and it is an RNA virus which contains a single ORF and its replication occurs in the cytosol by the formation of a membrane-associated replication complex (Moradpour, Penin, & Rice, 2007). On the other hand, HBV has a more complex life cycle characterized by integration events and cccDNA persistence (Tsukuda & Watashi, 2020). The comparison of HBV/HCV-induced signaling pathways (Annex IV: Signaling Induced by Chronic Viral Hepatitis: Dependence and Consequences) underlined that these two viruses perturb common cellular circuits even though employing different molecular strategies (Boulahtouf et al., 2022). Importantly, several studies identified epigenetic imprinting imposed by these viruses, which persists even after antiviral cure promoting silently liver-disease progression and cancer (Andrisani, 2021; Hamdane et al., 2019; Juhling et al., 2021). For example, modification on H3K27ac by HCV infection (but also induced by HBV and NASH) increased the risk of HCC development after DAA therapy (Hamdane et al., 2019; Juhling et al., 2021; Moran-Salvador & Mann, 2017). In addition to that, epigenetic changes modulated aspects of fibrosis development (Moran-Salvador & Mann, 2017).

Understanding the virus-induced perturbations of signaling pathways is required to delineate the landscape of kinases and phosphatases responsible for the development of the disease. In this context, my team recognized an impaired expression of protein tyrosine phosphatase receptor type D (PTPRD) during CHC, which in turn renders STAT3 less dephosphorylated therefore more active, associated with a reduction of patients' survival (Annex I: Impaired hepatic protein tyrosine phosphatase delta is a driver of metabolic syndrome) (Van Renne et al., 2018). In addition, the integrated analysis of metabolomes, proteomes, and transcriptomes

performed by my team allowed the identification of key pathways related to HCV-associated disease (Annex III: Combined analysis of metabolome, proteomes, and transcriptomes of HCV- infected cells and liver to identify pathways associated with disease development) (Lupberger et al., 2019). HCV infection, but not HBV, alters peroxisome function by STAT3 persistent activation, also observed at clinical level. This alteration, as proof of principle, can be potentially rescued using STAT3 inhibitors (Lupberger et al., 2019). However, despite the remarkable breakthrough in HCV antiviral therapy using the DAAs (Mohamed et al., 2020), therapeutic strategies to guarantee functional and complete cure for chronically HBV infected patients are still missing (Dandri & Petersen, 2020).

To address this yet unmet medical need, several challenges in research remain to be overcome. Since the discovery of HBV, the scientific research was restricted by the absence of a robust model capable to guarantee reproducibility and perform large scale screening. Consequently, few comprehensive *-omic* analysis have been conducted in the field of HBV, and the published studies are restricted on patients' samples or specialized models for immune response studies (Ding, Wei, Sun, Zhang, & Tian, 2009; Q. Gao et al., 2019; Kan et al., 2017; Katrinli et al., 2016; Ren et al., 2018). Moreover, lack of robust model of infection led to biased or partial results since research on the subject has been mostly restricted to the use of individual viral proteins, such as HBx (Kongkavitoon, Tangkijvanich, Hirankarn, & Palaga, 2016; Rawat & Bouchard, 2015). In the last decades, scientific progress led to the development of robust and reliable models for HBV infection such as HepG2-NTCP, one of the most used cell lines for the study of the molecular mechanisms associated with HBV infection (van de Klundert et al., 2016). At the same time, technologies and bioinformatic approaches tremendously evolved encouraging proteomic research. Combined with advanced mass spectrometry technologies, proteomic analysis ensures high sensitivity and accuracy of the results (Graves & Haystead, 2002; Pino, Rose, O'Broin, Shah, & Schilling, 2020). With the latest tandem mass tag (TMT) MS method, samples can be labeled and analyzed all in one single acquisition, therefore further decreasing the variability (Mertins et al., 2018).

To help addressing the current gaps in the understanding of signaling pathways responsible for persistence and progression of CHB infection, we created a proteomic and phospho-proteomic atlas of the complete HBV viral cycle. Our atlas confirmed previously identified pro-viral factors and thus represents a valid database for hypothesis development and validation available for the scientific community. Beyond, it identified host-dependent factors potentially responsible for cccDNA formation/modulation and highlighted potentially druggable circuits dysregulated during HBV-associated fibrosis development.

In detail, this study revealed previously unrecognized factors related to ECM remodeling, cell cycle regulation and epigenetic modification. First, the analysis of the proteomics database via GSEA revealed that HBV-infected hepatocytes contribute to ECM remodeling by the expression of ColVI already at early-stage post infection. In vitro studies shown that injured hepatocytes may undergo through epithelial mesenchymal transition (EMT) and acquire myofibroblast phenotypes. This process is caracterized by the downregulation of epithelial markers, such as E-cadherin and albumin, and by the upregulation of mesenchymal markers, such as Snail1 a Collagen I (X. Liu, Huang, Remmers, & Hollingsworth, 2014; G. Xie & Diehl, 2013). In our atlas, I do not observe any indicator of EMT. Interestingly, E-cadherin resulted to be significantly upregulated in HBV infected HepG2-NTCP cells, with possible influences on the distribution of NTCP receptors (Q. Hu et al., 2020).

We moreover identified previously unknown HBV-responsive phosphorylation sites of ITGβ1, which represents a putative receptor for ColVI (Jongewaard, Lauer, Behrendt, Patil, & Klewer, 2002; Tulla et al., 2001) and a known driver of fibrosis progression in CHB-patients (M. Wang et al., 2017). This is also important considering integrin subunits are differentially expressed in diverse tissue and disease, representing therefore interesting therapeutic targets (Yokosaki & Nishimichi, 2021). By the analysis of different cohorts of patients, I demonstrated that the overexpression of ColVI by HBV chronic infection is relevant, and it can be proportionally related to the grade of fibrosis severity in CHB-fibrotic patients. It is likely that, in patients, *COL6A1* originates from both HBV-infected hepatocytes and myofibroblasts.

Interestingly, I found that ColVI expressed by HBV-infected hepatocytes is not only implicated in the development of a pro-fibrotic environment but may also act as proviral factor. During liver injury, hepatocytes release a wide list of mediators of inflammation and fibrosis which affect profoundly the behavior of the surrounding cells (Y. S. Lee et al., 2017; Roehlen et al., 2020). My study demonstrates that ColVI, which is potentially secreted by HBV-infected hepatocytes, causes an upregulation of fibrotic hallmarks such as *COL1A1* and *ACTA2* in patients-derived myofibroblasts, consistently with the effects of ColVI on mice HSCs (Freise et al., 2021). The underlying mechanisms remain to be clarified the ability of the hepatocytes to secrete ColVI should be consolidated. Preliminary observations in our proteomics suggest that HBV induces upregulation of proteins involved in the secretion of collagens, components of the coat protein complex II (COPII) vesicles, such as protein transport protein Sec23, Sec24 and Cullin (Malhotra & Erlmann, 2015). Moreover, we found an upregulation of annexin A2 (ANXA2) in HBV-infected cells. ANXA2 is known to modulate HBsAg secretion (Y. Liu & Gao, 2014) and potentially promotes ColVI secretion in hepatocytes as ANXA2 interacts with ColVI mediating its secretion in experimental model of bronchial epithelial cells (Dassah et al., 2014). Possible effects of ColVI on downstream integrin signaling as FAK remain to be investigated, given the potential role of FAK molecular inhibitors in anti-fibrotic therapy for liver and lungs (Kinoshita et al., 2013; X. K. Zhao et al., 2017). The identification of more specific receptors for ColVI is lacking and it represents an important aspect for the development of anti-fibrotic therapeutic strategies. Despite the development of non-invasive biomarker for detection of liver fibrosis, liver biopsy remains the gold standard to detect with accuracy the histological grade and stage of liver fibrosis/cirrhosis (Asselah et al., 2014). In future, the analysis of ColVI fragments in the plasma of HBV-infected patients may be useful to understand the potential of ColVI as biomarker for HBV-associated liver disease progression and/or for other liver disease etiologies as previously suggested (Leeming et al., 2020; L. Williams et al., 2021).

In addition, my findings highlighted a previously unrecognized pro-viral role of ColVI during HBV infection as *COL6A1* silencing hampered HBV infection. This may be linked to an impairment of autophagy caused by diminished level of ColVI as similarly observed in other

tissue and pathological conditions (Bernardi & Bonaldo, 2013; Castagnaro et al., 2018; Grumati et al., 2010; J. Li et al., 2011; Lin, Zhao, Huang, & Lu, 2020). HBV requires induction of autophagy. For example, low glucose concentrations induce autophagy in infected cells by activating AMPK/mTOR-ULK1 and thereby promote HBV replication (X. Wang et al., 2020). Moreover, another study demonstrated that inhibition of autophagy may reduce HBV replication (N. Xie et al., 2016). ColVI silencing/rescue experiments using inhibitors of mTOR like rapamycin (X. Wang, Wei, Jiang, Meng, & Lu, 2021), may help to understand the mechanism of action of ColVI during HBV infection. The proteomic data presented in this work emphasize a role of HBV-infected hepatocytes in ColVI expression and ECM remodeling leading to a model in which HBV-induced ColVI acts pro-viral and exacerbates liver fibrosis by promoting HSCs activation.

The assessment of the phosphorylation events in HBV-infected cells revealed a remarkably profound impact on cell cycle checkpoint regulators. This is consistent with previous results which prove a cell cycle arrest in G1 phase induced by HBV (Eller et al., 2020). Moreover, this further accords with our observation of a strong hypophosphorylated state of pRb during HBV infection, which clearly emphasizing a predominant G1 arrest (Eller et al., 2020). The understanding of virus-associated dysregulation of cell cycle control is relevant considering that facilitating hepatocyte regeneration and cell proliferation may affect cccDNA formation/maintenance and thus contribute to viral eradication (Allweiss et al., 2018; C. L. Chong et al., 2011). In this line, I demonstrated that HBV infection resulted in a strong phosphorylation of linker histone H1, which is an important regulator of chromatin structure (Hergeth & Schneider, 2015; Luger et al., 1997). Generally, phosphorylation of linker histones is associated with chromatin detachment (Belikov et al., 2007; Bhattacharjee et al., 2001; Koop et al., 2003; Vicent et al., 2011). Interestingly, H1 does not result generally phosphorylated during G1 phase (Whitefield et al., 2018). This suggests that HBV may promote linker histones phosphorylation to sustain cccDNA formation. However, it is also possible that the observed massive H1 phosphorylation reflects a cellular response counteracting the HBV-imposed G1 arrest. In this study, I simulated hyperphosphorylation-associated H1.4 detachment from the

chromatin by using RNA interference technique as previously proven (Alexandrow & Hamlin, 2005; Bannister & Kouzarides, 2011; Dou et al., 1999). Using this strategy, we observed an increased HBV transcription level at the early phase of infection while non-viral genes levels seem unaffected. This is in line with previous studies underling that phosphorylation of H1.4 associates with transcription of specific genes rather than globally activating gene transcription (Belikov et al., 2007; Dou et al., 1999; Saha et al., 2020). Importantly, I did not observe any fluctuation on HBV transcription levels by silencing the variant H1.2, arguing for a virus-specific effect of H1.4 function on HBV transcription. This is in line with the fact that H1.2 variant has a short C-terminal tail and weekly compact the chromatin (Clausell et al., 2009; Prendergast & Reinberg, 2021). Silencing of H1.4 prior infection resulted in increased HBV mRNA transcripts, while the silencing on the established infection did not affect the level of HBV mRNA transcripts. A possible explanation might be that H1.4 is required for cccDNA formation and establishment of the infection. To consolidate these findings, a trans-dominant expression of a H1.4 phospho-mutant could be applied. Thereto, HBV-mediated phosphorylated serines and threonines will be substituted by alanines to abrogate the negative charge of the phosphorylation. A substitution glutamate can mimic a constitutively phosphorylated negative charge state in H1.4. Overexpression of these mutants followed by HBV infection will transdominantly act over any effects of the residual wild type H1.4 on HBV infection. If H1.4 phosphorylation is required for the early steps of the infection, we should observe an increase of the transcription rate in the cells transfected with the phospho-mimicking H1.4 mutant, whereas a decrease of transcription should occur in the phospho-dead mutant H1.4. These mutants could be also used to study a physical interaction with cccDNA with H1.4. Linker histones have been slightly detected in complex with the cccDNA in comparison with other histones such as H3 (Bock et al., 2001). This is most likely due to the observed H1.4 hyperphosphorylation which associated with detachment from the chromatin. The transdominant phospho-negative mutant however could stick to cccDNA during HBV infection, which may be studied by a ChIP-PCR approach. The challenge of such a mutation strategy will be the number of phosphorylation sites that as targeted by HBV as identified by the

phosphoproteomics. Such a large number of substitutions may impact folding of the protein and cause artefacts. However, proof of concept has been provided by *Dou et al.,* mutating 5 different phosphorylation site of linker histone sites (Dou et al., 1999). Optimization of the procedure in our model is ongoing.

Despite the absent effects on housekeeping gene expression shown in the paper provided in the Annex I section, the indirect effect of H1.4 phosphorylation on host genome transcription cannot be excluded. Phosphorylation of H1.4 may influence transcription of ligases and host factors required for cccDNA formation. Moreover, H1.4 phosphorylation may be also involved in other mechanisms such as DNA repair (Whitefield et al., 2018), which preferentially occur in open chromatin regions (Furuta et al., 2018; Peneau et al., 2022). Globally, this research has thrown up many new imminent questions in need of further investigation. The present study provides additional evidence with respect to HBV-induced cell cycle modulation and highlights for the first time the potential role of H1.4 in cccDNA formation. What is now needed is the identification of the specific kinases and phosphatase responsible for H1.4 regulation, which may open the way to new cccDNA-targeting therapeutic strategies. The predicted kinases involved in H1.4 hyperphosphorylations are CDK1, CDK2 and protein kinase A (PKA), which should be validated in further work. The role of H1.4 phosphorylation in cell cycle progression during HBV infection and the interaction between cccDNA and linker histones will potentially shed new light on the mechanism of HBV persistence.

Overall, our temporal proteomic and phosphoproteomic atlas recapitulates not only known aspects of the virus life cycle but also it highlights previously unrecognized factors involved in HBV-associated cell cycle control, epigenetic regulation and disease progression. The clinical relevance of my findings is that targeting of ColVI regulators during HBV-associated liver disease may attenuate fibrosis progression and being part a of novel anti-fibrotic therapeutic concept. Beyond, the removal of the underlying etiology is essential for a halt of disease progression. As signaling pathways are established targets in cancer therapy (Yip & Papa, 2021) targeting HBV-associated signaling events found in our atlas may

contribute to develop novel and effective antiviral strategies. Especially targeting cccDNA formation and maintenance, potentially via epigenetic drugs of signaling inhibitors hold the potential to eliminate cccDNA and by such contribute of an effective HBV cure in the coming decades.

BIBLIOGRAPHY

Abdel-Misih, S. R., & Bloomston, M. (2010). Liver anatomy. *Surg Clin North Am, 90*(4), 643-653. doi:10.1016/j.suc.2010.04.017

Adams, P. D., & Kaelin, W. G., Jr. (1995). Transcriptional control by E2F. *Semin Cancer Biol, 6*(2), 99-108. doi:10.1006/scbi.1995.0013

Agarwal, K., Afdhal, N., Coffin, C., Fung, S., Dusheiko, G., Foster, G., . . . Iyer, R. (2020). Liver toxicity in the Phase 2 Catalyst 206 trial of Inarigivir 400 mg daily added to a nucleoside in HBV EAg negative patients. *Journal of Hepatology, 73*, S125.

Agarwal, K., Ahn, S. H., Elkhashab, M., Lau, A. H., Gaggar, A., Bulusu, A., . . . Nguyen, M. H. (2018). Safety and efficacy of vesatolimod (GS-9620) in patients with chronic hepatitis B who are not currently on antiviral treatment. *J Viral Hepat, 25*(11), 1331-1340. doi:10.1111/jvh.12942

Aird, W. C. (2011). Discovery of the cardiovascular system: from Galen to William Harvey. *J Thromb Haemost, 9 Suppl 1*, 118-129. doi:10.1111/j.1538-7836.2011.04312.x

Aizarani, N., Saviano, A., Sagar, Mailly, L., Durand, S., Herman, J. S., . . . Grun, D. (2019). A human liver cell atlas reveals heterogeneity and epithelial progenitors. *Nature, 572*(7768), 199-204. doi:10.1038/s41586-019-1373-2

Al-Mahtab, M., Bazinet, M., & Vaillant, A. (2016). Safety and Efficacy of Nucleic Acid Polymers in Monotherapy and Combined with Immunotherapy in Treatment-Naive Bangladeshi Patients with HBeAg+ Chronic Hepatitis B Infection. *PLoS One, 11*(6), e0156667. doi:10.1371/journal.pone.0156667

Alam, N., Goel, H. L., Zarif, M. J., Butterfield, J. E., Perkins, H. M., Sansoucy, B. G., . . . Languino, L. R. (2007). The integrin-growth factor receptor duet. *J Cell Physiol, 213*(3), 649-653. doi:10.1002/jcp.21278

Alexandrow, M. G., & Hamlin, J. L. (2005). Chromatin decondensation in S-phase involves recruitment of Cdk2 by Cdc45 and histone H1 phosphorylation. *J Cell Biol, 168*(6), 875-886. doi:10.1083/jcb.200409055

Alexeev, V., Arita, M., Donahue, A., Bonaldo, P., Chu, M. L., & Igoucheva, O. (2014). Human adipose-derived stem cell transplantation as a potential therapy for collagen VI-related congenital muscular dystrophy. *Stem Cell Res Ther, 5*(1), 21. doi:10.1186/scrt411

Alexopoulou, A., Vasilieva, L., & Karayiannis, P. (2020). New Approaches to the Treatment of Chronic Hepatitis B. *J Clin Med, 9*(10). doi:10.3390/jcm9103187

Allweiss, L., & Dandri, M. (2017). The Role of cccDNA in HBV Maintenance. *Viruses, 9*(6). doi:10.3390/v9060156

Allweiss, L., Giersch, K., Pirosu, A., Volz, T., Muench, R. C., Beran, R. K., . . . Dandri, M. (2022). Therapeutic shutdown of HBV transcripts promotes reappearance of the SMC5/6 complex and silencing of the viral genome in vivo. *Gut, 71*(2), 372-381. doi:10.1136/gutjnl-2020-322571

Allweiss, L., Volz, T., Giersch, K., Kah, J., Raffa, G., Petersen, J., . . . Dandri, M. (2018). Proliferation of primary human hepatocytes and prevention of hepatitis B virus reinfection efficiently deplete nuclear cccDNA in vivo. *Gut, 67*(3), 542-552. doi:10.1136/gutjnl-2016-312162

Alter, H. J., Holland, P. V., & Schmidt, P. J. (1970). Hepatitis-associated antigen. To test or not to test? *Lancet, 2*(7664), 142-143. doi:10.1016/s0140-6736(70)92717-0

Alter, M. J. (2003). Epidemiology of hepatitis B in Europe and worldwide. *J Hepatol, 39 Suppl 1*, S64-69. doi:10.1016/s0168-8278(03)00141-7

Amin, O. E., Colbeck, E. J., Daffis, S., Khan, S., Ramakrishnan, D., Pattabiraman, D., . . . Pallett, L. J. (2021). Therapeutic Potential of TLR8 Agonist GS-9688 (Selgantolimod) in Chronic Hepatitis B: Remodeling of Antiviral and Regulatory Mediators. *Hepatology, 74*(1), 55-71. doi:10.1002/hep.31695

Anderson, K. R., Haeussler, M., Watanabe, C., Janakiraman, V., Lund, J., Modrusan, Z., . . . Warming, S. (2018). CRISPR off-target analysis in genetically engineered rats and mice. *Nat Methods, 15*(7), 512-514. doi:10.1038/s41592-018-0011-5

Andres, M., Garcia-Gomis, D., Ponte, I., Suau, P., & Roque, A. (2020). Histone H1 Post-Translational Modifications: Update and Future Perspectives. *Int J Mol Sci, 21*(16). doi:10.3390/ijms21165941

Andrisani, O. (2021). Epigenetic mechanisms in hepatitis B virus-associated hepatocellular carcinoma. *Hepatoma Res, 7*. doi:10.20517/2394-5079.2020.83

Araujo, N. M., Teles, S. A., & Spitz, N. (2020). Comprehensive Analysis of Clinically Significant Hepatitis B Virus Mutations in Relation to Genotype, Subgenotype and Geographic Region. *Front Microbiol, 11*, 616023. doi:10.3389/fmicb.2020.616023

Ardito, F., Giuliani, M., Perrone, D., Troiano, G., & Lo Muzio, L. (2017). The crucial role of protein phosphorylation in cell signaling and its use as targeted therapy (Review). *Int J Mol Med, 40*(2), 271-280. doi:10.3892/ijmm.2017.3036

Argentou, N., Germanidis, G., Hytiroglou, P., Apostolou, E., Vassiliadis, T., Patsiaoura, K., . . . Speletas, M. (2016). TGF-beta signaling is activated in patients with chronic HBV infection and repressed by SMAD7 overexpression after successful antiviral treatment. *Inflamm Res, 65*(5), 355-365. doi:10.1007/s00011-016-0921-6

Arpino, V., Brock, M., & Gill, S. E. (2015). The role of TIMPs in regulation of extracellular matrix proteolysis. *Matrix Biol, 44-46*, 247-254. doi:10.1016/j.matbio.2015.03.005

Arzberger, S., Hosel, M., & Protzer, U. (2010). Apoptosis of hepatitis B virus-infected hepatocytes prevents release of infectious virus. *J Virol, 84*(22), 11994-12001. doi:10.1128/JVI.00653-10

Asrani, S. K., Devarbhavi, H., Eaton, J., & Kamath, P. S. (2019). Burden of liver diseases in the world. *J Hepatol, 70*(1), 151-171. doi:10.1016/j.jhep.2018.09.014

Asselah, T., Lada, O., Moucari, R., Martinot, M., Boyer, N., & Marcellin, P. (2007). Interferon therapy for chronic hepatitis B. *Clin Liver Dis, 11*(4), 839-849, viii. doi:10.1016/j.cld.2007.08.010

Asselah, T., Loureiro, D., Boyer, N., & Mansouri, A. (2019). Targets and future direct-acting antiviral approaches to achieve hepatitis B virus cure. *Lancet Gastroenterol Hepatol, 4*(11), 883-892. doi:10.1016/S2468-1253(19)30190-6

Asselah, T., Loureiro, D., Tout, I., Castelnau, C., Boyer, N., Marcellin, P., & Mansouri, A. (2020). Future treatments for hepatitis delta virus infection. *Liver Int, 40 Suppl 1*, 54-60. doi:10.1111/liv.14356

Asselah, T., Marcellin, P., & Bedossa, P. (2014). Improving performance of liver biopsy in fibrosis assessment. *J Hepatol, 61*(2), 193-195. doi:10.1016/j.jhep.2014.03.006

Baatout, S., & Derradji, H. (2006). About histone H1 phosphorylation during mitosis. *Cell Biochem Funct, 24*(2), 93-94. doi:10.1002/cbf.1293

Bagga, S., & Bouchard, M. J. (2014). Cell cycle regulation during viral infection. *Methods Mol Biol, 1170*, 165-227. doi:10.1007/978-1-4939-0888-2_10

Balhorn, R., Chalkley, R., & Granner, D. (1972). Lysine-rich histone phosphorylation. A positive correlation with cell replication. *Biochemistry, 11*(6), 1094-1098. doi:10.1021/bi00756a023

Banales, J. M., Huebert, R. C., Karlsen, T., Strazzabosco, M., LaRusso, N. F., & Gores, G. J. (2019). Cholangiocyte pathobiology. *Nat Rev Gastroenterol Hepatol, 16*(5), 269-281. doi:10.1038/s41575-019-0125-y

Bannister, A. J., & Kouzarides, T. (2011). Regulation of chromatin by histone modifications. *Cell Res, 21*(3), 381-395. doi:10.1038/cr.2011.22

Bardens, A., Doring, T., Stieler, J., & Prange, R. (2011). Alix regulates egress of hepatitis B virus naked capsid particles in an ESCRT-independent manner. *Cell Microbiol, 13*(4), 602-619. doi:10.1111/j.1462-5822.2010.01557.x

Bazinet, M., Pantea, V., Cebotarescu, V., Cojuhari, L., Jimbei, P., Albrecht, J., . . . Vaillant, A. (2017). Safety and efficacy of REP 2139 and pegylated interferon alfa-2a for treatment-naive patients with chronic hepatitis B virus and hepatitis D virus co-

infection (REP 301 and REP 301-LTF): a non-randomised, open-label, phase 2 trial. *Lancet Gastroenterol Hepatol, 2*(12), 877-889. doi:10.1016/S2468-1253(17)30288-1

Beck, J., & Nassal, M. (2007). Hepatitis B virus replication. *World J Gastroenterol, 13*(1), 48-64. doi:10.3748/wjg.v13.i1.48

Bednar, J., Garcia-Saez, I., Boopathi, R., Cutter, A. R., Papai, G., Reymer, A., . . . Dimitrov, S. (2017). Structure and Dynamics of a 197 bp Nucleosome in Complex with Linker Histone H1. *Mol Cell, 66*(3), 384-397 e388. doi:10.1016/j.molcel.2017.04.012

Belikov, S., Astrand, C., & Wrange, O. (2007). Mechanism of histone H1-stimulated glucocorticoid receptor DNA binding in vivo. *Mol Cell Biol, 27*(6), 2398-2410. doi:10.1128/MCB.01509-06

Belloni, L., Pollicino, T., De Nicola, F., Guerrieri, F., Raffa, G., Fanciulli, M., . . . Levrero, M. (2009). Nuclear HBx binds the HBV minichromosome and modifies the epigenetic regulation of cccDNA function. *Proc Natl Acad Sci U S A, 106*(47), 19975-19979. doi:10.1073/pnas.0908365106

Bernardi, P., & Bonaldo, P. (2013). Mitochondrial dysfunction and defective autophagy in the pathogenesis of collagen VI muscular dystrophies. *Cold Spring Harb Perspect Biol, 5*(5), a011387. doi:10.1101/cshperspect.a011387

Bertoli, C., Skotheim, J. M., & de Bruin, R. A. (2013). Control of cell cycle transcription during G1 and S phases. *Nat Rev Mol Cell Biol, 14*(8), 518-528. doi:10.1038/nrm3629

Berumen, J., Baglieri, J., Kisseleva, T., & Mekeel, K. (2021). Liver fibrosis: Pathophysiology and clinical implications. *WIREs Mech Dis, 13*(1), e1499. doi:10.1002/wsbm.1499

Bhattacharjee, R. N., Banks, G. C., Trotter, K. W., Lee, H. L., & Archer, T. K. (2001). Histone H1 phosphorylation by Cdk2 selectively modulates mouse mammary tumor virus transcription through chromatin remodeling. *Mol Cell Biol, 21*(16), 5417-5425. doi:10.1128/MCB.21.16.5417-5425.2001

Bialek, S. R., Bower, W. A., Novak, R., Helgenberger, L., Auerbach, S. B., Williams, I. T., & Bell, B. P. (2008). Persistence of protection against hepatitis B virus infection among adolescents vaccinated with recombinant hepatitis B vaccine beginning at birth: a 15-year follow-up study. *Pediatr Infect Dis J, 27*(10), 881-885. doi:10.1097/INF.0b013e31817702ba

Bismuth, H. (1982). Surgical anatomy and anatomical surgery of the liver. *World J Surg, 6*(1), 3-9. doi:10.1007/BF01656368

Block, T. M., Alter, H. J., London, W. T., & Bray, M. (2016). A historical perspective on the discovery and elucidation of the hepatitis B virus. *Antiviral Res, 131*, 109-123. doi:10.1016/j.antiviral.2016.04.012

Blumberg, B. S. (1977). Australia antigen and the biology of hepatitis B. *Science, 197*(4298), 17-25. doi:10.1126/science.325649

Blumberg, B. S. (1984). Hepatitis B virus and the control of hepatocellular carcinoma. *IARC Sci Publ*(63), 243-261. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/6100273

Blumberg, B. S., Sutnick, A. I., & London, W. T. (1968). Hepatitis and leukemia: their relation to Australia antigen. *Bull N Y Acad Med, 44*(12), 1566-1586. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/5247325

Blumberg, B. S., Sutnick, A. I., London, W. T., & Millman, I. (1970). Australia antigen and hepatitis. *N Engl J Med, 283*(7), 349-354. doi:10.1056/NEJM197008132830707

Bock, C. T., Schranz, P., Schroder, C. H., & Zentgraf, H. (1994). Hepatitis B virus genome is organized into nucleosomes in the nucleus of the infected cell. *Virus Genes, 8*(3), 215-229. doi:10.1007/BF01703079

Bock, C. T., Schwinn, S., Locarnini, S., Fyfe, J., Manns, M. P., Trautwein, C., & Zentgraf, H. (2001). Structural organization of the hepatitis B virus minichromosome. *J Mol Biol, 307*(1), 183-196. doi:10.1006/jmbi.2000.4481

Boni, C., Janssen, H. L. A., Rossi, M., Yoon, S. K., Vecchi, A., Barili, V., . . . Ferrari, C. (2019). Combined GS-4774 and Tenofovir Therapy Can Improve HBV-Specific T-Cell Responses in Patients With Chronic Hepatitis. *Gastroenterology, 157*(1), 227-241 e227. doi:10.1053/j.gastro.2019.03.044

Bonnemann, C. G. (2011). The collagen VI-related myopathies Ullrich congenital muscular dystrophy and Bethlem myopathy. *Handb Clin Neurol, 101*, 81-96. doi:10.1016/B978-0-08-045031-5.00005-0

Bonus, M., Haussinger, D., & Gohlke, H. (2021). Liver cell hydration and integrin signaling. *Biol Chem, 402*(9), 1033-1045. doi:10.1515/hsz-2021-0193

Boulahtouf, Z., Virzi, A., Baumert, T. F., Verrier, E. R., & Lupberger, J. (2022). Signaling Induced by Chronic Viral Hepatitis: Dependence and Consequences. *Int J Mol Sci, 23*(5). doi:10.3390/ijms23052787

Boyd, A., Lacombe, K., Lavocat, F., Maylin, S., Miailhes, P., Lascoux-Combe, C., . . . Zoulim, F. (2016). Decay of ccc-DNA marks persistence of intrahepatic viral DNA synthesis under tenofovir in HIV-HBV co-infected patients. *J Hepatol, 65*(4), 683-691. doi:10.1016/j.jhep.2016.05.014

Bradbury, E. M., Cary, P. D., Chapman, G. E., Crane-Robinson, C., Danby, S. E., Rattle, H. W., . . . Aviles, F. J. (1975). Studies on the role and mode of operation of the very-lysine-rich histone H1 (F1) in eukaryote chromatin. The conformation of histone H1. *Eur J Biochem, 52*(3), 605-613. doi:10.1111/j.1432-1033.1975.tb04032.x

Brandstadter, R., & Katz Sand, I. (2017). The use of natalizumab for multiple sclerosis. *Neuropsychiatr Dis Treat, 13*, 1691-1702. doi:10.2147/NDT.S114636

Bremer, C. M., Bung, C., Kott, N., Hardt, M., & Glebe, D. (2009). Hepatitis B virus infection is dependent on cholesterol in the viral envelope. *Cell Microbiol, 11*(2), 249-260. doi:10.1111/j.1462-5822.2008.01250.x

Burchardt, E. R., Hein, R., & Bosserhoff, A. K. (2003). Laminin, hyaluronan, tenascin-C and type VI collagen levels in sera from patients with malignant melanoma. *Clin Exp Dermatol, 28*(5), 515-520. doi:10.1046/j.1365-2230.2003.01326.x

Burchill, M. A., Goldberg, A. R., & Tamburini, B. A. J. (2019). Emerging Roles for Lymphatics in Chronic Liver Disease. *Front Physiol, 10*, 1579. doi:10.3389/fphys.2019.01579

Burgi, J., Kunz, B., Abrami, L., Deuquet, J., Piersigilli, A., Scholl-Burgi, S., . . . van der Goot, F. G. (2017). CMG2/ANTXR2 regulates extracellular collagen VI which accumulates in hyaline fibromatosis syndrome. *Nat Commun, 8*, 15861. doi:10.1038/ncomms15861

Butterworth, J., Gregoire, D., Peter, M., Roca Suarez, A. A., Desandre, G., Simonin, Y., . . . Hibner, U. (2021). GOLT1B Activation in Hepatitis C Virus-Infected Hepatocytes Links ER Trafficking and Viral Replication. *Pathogens, 11*(1). doi:10.3390/pathogens11010046

Buynak, E. B., Roehm, R. R., Tytell, A. A., Bertland, A. U., 2nd, Lampson, G. P., & Hilleman, M. R. (1976). Development and chimpanzee testing of a vaccine against human hepatitis B. *Proc Soc Exp Biol Med, 151*(4), 694-700. doi:10.3181/00379727-151-39288

Caballeria, L., Pera, G., Arteaga, I., Rodriguez, L., Aluma, A., Morillas, R. M., . . . Gines, P. (2018). High Prevalence of Liver Fibrosis Among European Adults With Unknown Liver Disease: A Population-Based Study. *Clin Gastroenterol Hepatol, 16*(7), 1138-1145 e1135. doi:10.1016/j.cgh.2017.12.048

Cabral-Pacheco, G. A., Garza-Veloz, I., Castruita-De la Rosa, C., Ramirez-Acuna, J. M., Perez-Romero, B. A., Guerrero-Rodriguez, J. F., . . . Martinez-Fierro, M. L. (2020). The Roles of Matrix Metalloproteinases and Their Inhibitors in Human Diseases. *Int J Mol Sci, 21*(24). doi:10.3390/ijms21249739

CADTH. (2018). *Clinical Review Report: Tenofovir Alafenamide (Vemlidy): (Gilead Sciences Canada, Inc.): Indication: Treatment of chronic hepatitis B in adults with compensated liver disease.Appendix 7Summary of Natural History, Diagnosis, Management, and Prognosis of HBV.*

Caligiuri, P., Cerruti, R., Icardi, G., & Bruzzone, B. (2016). Overview of hepatitis B virus mutations and their implications in the management of infection. *World J Gastroenterol, 22*(1), 145-154. doi:10.3748/wjg.v22.i1.145

Cargill, T., & Barnes, E. (2021). Therapeutic vaccination for treatment of chronic hepatitis B. *Clin Exp Immunol, 205*(2), 106-118. doi:10.1111/cei.13614

Castagnaro, S., Chrisam, M., Cescon, M., Braghetta, P., Grumati, P., & Bonaldo, P. (2018). Extracellular Collagen VI Has Prosurvival and Autophagy Instructive Properties in Mouse Fibroblasts. *Front Physiol, 9*, 1129. doi:10.3389/fphys.2018.01129

Cavalcanti de, A. M. A., & Martins, C. (2013). History of liver anatomy: Mesopotamian liver clay models. *HPB (Oxford), 15*(4), 322-323. doi:10.1111/j.1477-2574.2012.00555.x

Caviglia, G. P., Armandi, A., Rosso, C., Ribaldone, D. G., Pellicano, R., & Fagoonee, S. (2021). Hepatitis B Core-Related Antigen as Surrogate Biomarker of Intrahepatic Hepatitis B Virus Covalently-Closed-Circular DNA in Patients with Chronic Hepatitis B: A Meta-Analysis. *Diagnostics (Basel), 11*(2). doi:10.3390/diagnostics11020187

Caviglia, G. P., Noviello, D., Pellicano, R., & Olivero, A. (2018). Role of serum hepatitis B core-related antigen in chronic hepatitis B infection. *Minerva Biotecnol, 30*, 29-35.

Caviglia, G. P., Zorzi, A., Rizzetto, M., Mirandola, M., Olivero, A., & Carolo, G. (2021). Hepatitis B Virus Reactivation upon Immunosuppression: Is There a Role for Hepatitis B Core-Related Antigen in Patients with Immune-Escape Mutants? A Case Report. *Diagnostics (Basel), 11*(12). doi:10.3390/diagnostics11122185

Cescon, M., Gattazzo, F., Chen, P., & Bonaldo, P. (2015). Collagen VI at a glance. *J Cell Sci, 128*(19), 3525-3531. doi:10.1242/jcs.169748

Chakraborty, A., Ko, C., Henning, C., Lucko, A., Harris, J. M., Chen, F., . . . McKeating, J. A. (2020). Synchronised infection identifies early rate-limiting steps in the hepatitis B virus life cycle. *Cell Microbiol, 22*(12), e13250. doi:10.1111/cmi.13250

Chang, J., Lucas, M. C., Leonte, L. E., Garcia-Montolio, M., Singh, L. B., Findlay, A. D., . . . Cox, T. R. (2017). Pre-clinical evaluation of small molecule LOXL2 inhibitors in breast cancer. *Oncotarget, 8*(16), 26066-26078. doi:10.18632/oncotarget.15257

Chathuranga, K., Weerawardhana, A., Dodantenna, N., & Lee, J. S. (2021). Regulation of antiviral innate immune signaling and viral evasion following viral genome sensing. *Exp Mol Med, 53*(11), 1647-1668. doi:10.1038/s12276-021-00691-y

Cheemerla, S., & Balakrishnan, M. (2021). Global Epidemiology of Chronic Liver Disease. *Clin Liver Dis (Hoboken), 17*(5), 365-370. doi:10.1002/cld.1061

Chen, A., & Brown, C. (2012). Distinct families of cis-acting RNA replication elements epsilon from hepatitis B viruses. *RNA Biol, 9*(2), 130-136. doi:10.4161/rna.18649

Chen, C. F., Lee, W. C., Yang, H. I., Chang, H. C., Jen, C. L., Iloeje, U. H., . . . Associated Liver Disease/Cancer in, H. B. V. S. G. (2011). Changes in serum levels of HBV DNA and alanine aminotransferase determine risk for hepatocellular carcinoma. *Gastroenterology, 141*(4), 1240-1248, 1248 e1241-1242. doi:10.1053/j.gastro.2011.06.036

Chen, J., Xu, W., Chen, Y., Xie, X., Zhang, Y., Ma, C., . . . Wu, J. (2017). Matrix Metalloproteinase 9 Facilitates Hepatitis B Virus Replication through Binding with Type I Interferon (IFN) Receptor 1 To Repress IFN/JAK/STAT Signaling. *J Virol, 91*(8). doi:10.1128/JVI.01824-16

Chen, P., Cescon, M., & Bonaldo, P. (2013). Collagen VI in cancer and its biological mechanisms. *Trends Mol Med, 19*(7), 410-417. doi:10.1016/j.molmed.2013.04.001

Chen, T. S., & Chen, P. S. (1988). William Harvey as hepatologist. *Am J Gastroenterol, 83*(11), 1274-1277. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/3055945

Chen, Y., Cai, G., Zhang, C., Yao, J., Wang, Z., Wang, Z., . . . Zhou, B. (2019). The association of serum markers of fibrosis and development of liver cirrhosis in chronic hepatitis B patients: A systematic review and meta-analysis. *Cogent Medicine, 6*(1), 1619896. doi:10.1080/2331205X.2019.1619896

Chen, Y., Maguire, T., Hileman, R. E., Fromm, J. R., Esko, J. D., Linhardt, R. J., & Marks, R. M. (1997). Dengue virus infectivity depends on envelope protein binding to target cell heparan sulfate. *Nat Med, 3*(8), 866-871. doi:10.1038/nm0897-866

Cheng, X., Xia, Y., Serti, E., Block, P. D., Chung, M., Chayama, K., . . . Liang, T. J. (2017). Hepatitis B virus evades innate immunity of hepatocytes but activates cytokine production by macrophages. *Hepatology, 66*(6), 1779-1793. doi:10.1002/hep.29348

Chong, C. K., Cheng, C. Y. S., Tsoi, S. Y. J., Huang, F. Y., Liu, F., Seto, W. K., . . . Wong, D. K. (2017). Role of hepatitis B core protein in HBV transcription and recruitment of

histone acetyltransferases to cccDNA minichromosome. *Antiviral Res, 144*, 1-7. doi:10.1016/j.antiviral.2017.05.003

Chong, C. L., Chen, M. L., Wu, Y. C., Tsai, K. N., Huang, C. C., Hu, C. P., . . . Chang, C. (2011). Dynamics of HBV cccDNA expression and transcription in different cell growth phase. *J Biomed Sci, 18*, 96. doi:10.1186/1423-0127-18-96

Chu, C. S., Hsu, P. H., Lo, P. W., Scheer, E., Tora, L., Tsai, H. J., . . . Juan, L. J. (2011). Protein kinase A-mediated serine 35 phosphorylation dissociates histone H1.4 from mitotic chromosome. *J Biol Chem, 286*(41), 35843-35851. doi:10.1074/jbc.M111.228064

Chubb, J. E., & Rea, S. (2010). Core and linker histone modifications involved in the DNA damage response. *Subcell Biochem, 50*, 17-42. doi:10.1007/978-90-481-3471-7_2

Clausell, J., Happel, N., Hale, T. K., Doenecke, D., & Beato, M. (2009). Histone H1 subtypes differentially modulate chromatin condensation without preventing ATP-dependent remodeling by SWI/SNF or NURF. *PLoS One, 4*(10), e0007243. doi:10.1371/journal.pone.0007243

Clayton, R. F., Rinaldi, A., Kandyba, E. E., Edward, M., Willberg, C., Klenerman, P., & Patel, A. H. (2005). Liver cell lines for the study of hepatocyte functions and immunological response. *Liver Int, 25*(2), 389-402. doi:10.1111/j.1478-3231.2005.01017.x

Contreras, A., Hale, T. K., Stenoien, D. L., Rosen, J. M., Mancini, M. A., & Herrera, R. E. (2003). The dynamic mobility of histone H1 is regulated by cyclin/CDK phosphorylation. *Mol Cell Biol, 23*(23), 8626-8636. doi:10.1128/MCB.23.23.8626-8636.2003

Cooper, A., & Shaul, Y. (2006). Clathrin-mediated endocytosis and lysosomal cleavage of hepatitis B virus capsid-like core particles. *J Biol Chem, 281*(24), 16563-16569. doi:10.1074/jbc.M601418200

Cooper, G. M. (2000). The Cell: A Molecular Approach. 2nd edition. Sinauer Associates.

Cornberg, M., Lok, A. S., Terrault, N. A., Zoulim, F., & Faculty, E.-A. H. T. E. C. (2019). Guidance for design and endpoints of clinical trials in chronic hepatitis B - Report from the 2019 EASL-AASLD HBV Treatment Endpoints Conference. *Hepatology*. doi:10.1002/hep.31030

Cox, A. L., El-Sayed, M. H., Kao, J. H., Lazarus, J. V., Lemoine, M., Lok, A. S., & Zoulim, F. (2020). Progress towards elimination goals for viral hepatitis. *Nat Rev Gastroenterol Hepatol, 17*(9), 533-542. doi:10.1038/s41575-020-0332-6

D'Amico, G., Morabito, A., D'Amico, M., Pasta, L., Malizia, G., Rebora, P., & Valsecchi, M. G. (2018). New concepts on the clinical course and stratification of compensated and decompensated cirrhosis. *Hepatol Int, 12*(Suppl 1), 34-43. doi:10.1007/s12072-017-9808-z

Dandri, M., & Petersen, J. (2017). Animal models of HBV infection. *Best Pract Res Clin Gastroenterol, 31*(3), 273-279. doi:10.1016/j.bpg.2017.04.014

Dandri, M., & Petersen, J. (2020). cccDNA Maintenance in Chronic Hepatitis B - Targeting the Matrix of Viral Replication. *Infect Drug Resist, 13*, 3873-3886. doi:10.2147/IDR.S240472

Dane, D. S., Cameron, C. H., & Briggs, M. (1970). Virus-like particles in serum of patients with Australia-antigen-associated hepatitis. *Lancet, 1*(7649), 695-698. doi:10.1016/s0140-6736(70)90926-8

Das, S., Ramakrishnan, K., Behera, S. K., Ganesapandian, M., Xavier, A. S., & Selvarajan, S. (2019). Hepatitis B Vaccine and Immunoglobulin: Key Concepts. *J Clin Transl Hepatol, 7*(2), 165-171. doi:10.14218/JCTH.2018.00037

Dassah, M., Almeida, D., Hahn, R., Bonaldo, P., Worgall, S., & Hajjar, K. A. (2014). Annexin A2 mediates secretion of collagen VI, pulmonary elasticity and apoptosis of bronchial epithelial cells. *J Cell Sci, 127*(Pt 4), 828-844. doi:10.1242/jcs.137802

Daujat, S., Zeissler, U., Waldmann, T., Happel, N., & Schneider, R. (2005). HP1 binds specifically to Lys26-methylated histone H1.4, whereas simultaneous Ser27 phosphorylation blocks HP1 binding. *J Biol Chem, 280*(45), 38090-38095. doi:10.1074/jbc.C500229200

Davey, C. A., Sargent, D. F., Luger, K., Maeder, A. W., & Richmond, T. J. (2002). Solvent mediated interactions in the structure of the nucleosome core particle at 1.9 a resolution. *J Mol Biol, 319*(5), 1097-1113. doi:10.1016/S0022-2836(02)00386-8

de Villiers, M. J., Nayagam, S., & Hallett, T. B. (2021). The impact of the timely birth dose vaccine on the global elimination of hepatitis B. *Nat Commun, 12*(1), 6223. doi:10.1038/s41467-021-26475-6

Decaris, M. L., Gatmaitan, M., FlorCruz, S., Luo, F., Li, K., Holmes, W. E., . . . Emson, C. L. (2014). Proteomic analysis of altered extracellular matrix turnover in bleomycin-induced pulmonary fibrosis. *Mol Cell Proteomics, 13*(7), 1741-1752. doi:10.1074/mcp.M113.037267

Decorsiere, A., Mueller, H., van Breugel, P. C., Abdul, F., Gerossier, L., Beran, R. K., . . . Strubin, M. (2016). Hepatitis B virus X protein identifies the Smc5/6 complex as a host restriction factor. *Nature, 531*(7594), 386-389. doi:10.1038/nature17170

Deroubaix, A., & Kramvis, A. (2021). In vitro expression of precore proteins of hepatitis B virus subgenotype A1 is affected by HBcAg, and can affect HBsAg secretion. *Sci Rep, 11*(1), 8167. doi:10.1038/s41598-021-87529-9

Dewidar, B., Meyer, C., Dooley, S., & Meindl-Beinker, A. N. (2019). TGF-beta in Hepatic Stellate Cell Activation and Liver Fibrogenesis-Updated 2019. *Cells, 8*(11). doi:10.3390/cells8111419

Dimri, M., & Satyanarayana, A. (2020). Molecular Signaling Pathways and Therapeutic Targets in Hepatocellular Carcinoma. *Cancers (Basel), 12*(2). doi:10.3390/cancers12020491

Ding, C., Wei, H., Sun, R., Zhang, J., & Tian, Z. (2009). Hepatocytes proteomic alteration and seroproteome analysis of HBV-transgenic mice. *Proteomics, 9*(1), 87-105. doi:10.1002/pmic.200701053

Diogo Dias, J., Sarica, N., & Neuveut, C. (2021). Early Steps of Hepatitis B Life Cycle: From Capsid Nuclear Import to cccDNA Formation. *Viruses, 13*(5). doi:10.3390/v13050757

Doane, K. J., Howell, S. J., & Birk, D. E. (1998). Identification and functional characterization of two type VI collagen receptors, alpha 3 beta 1 integrin and NG2, during avian corneal stromal development. *Invest Ophthalmol Vis Sci, 39*(2), 263-275. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/9477982

Dogan, U. B., Ozturk, A. B., Akin, M. S., Yalaki, S., & Sayan, M. (2014). A case of entecavir resistance which is developed after complete viral suppression during entecavir treatment for nucleoside-naive chronic hepatitis B. *Turk J Gastroenterol, 25 Suppl 1*, 206-209. doi:10.5152/tjg.2014.3605

Dou, Y., & Gorovsky, M. A. (2000). Phosphorylation of linker histone H1 regulates gene expression in vivo by creating a charge patch. *Mol Cell, 6*(2), 225-231. doi:10.1016/s1097-2765(00)00024-1

Dou, Y., Mizzen, C. A., Abrams, M., Allis, C. D., & Gorovsky, M. A. (1999). Phosphorylation of linker histone H1 regulates gene expression in vivo by mimicking H1 removal. *Mol Cell, 4*(4), 641-647. doi:10.1016/s1097-2765(00)80215-4

Dunn, C., Peppa, D., Khanna, P., Nebbia, G., Jones, M., Brendish, N., . . . Maini, M. K. (2009). Temporal analysis of early immune responses in patients with acute hepatitis B virus infection. *Gastroenterology, 137*(4), 1289-1300. doi:10.1053/j.gastro.2009.06.054

Durantel, D., & Zoulim, F. (2009). Innate response to hepatitis B virus infection: observations challenging the concept of a stealth virus. *Hepatology, 50*(6), 1692-1695. doi:10.1002/hep.23361

EASL. (2012). EASL clinical practice guidelines: Management of chronic hepatitis B virus infection. *J Hepatol, 57*(1), 167-185. doi:10.1016/j.jhep.2012.02.010

EASL. (2017). EASL 2017 Clinical Practice Guidelines on the management of hepatitis B virus infection. *J Hepatol, 67*(2), 370-398. doi:10.1016/j.jhep.2017.03.021

El-Zayadi, A. R. (2009). Hepatitis C comorbidities affecting the course and response to therapy. *World J Gastroenterol, 15*(40), 4993-4999. doi:10.3748/wjg.15.4993

Eller, C., Heydmann, L., Colpitts, C. C., El Saghire, H., Piccioni, F., Juhling, F., . . . Baumert, T. F. (2020). A genome-wide gain-of-function screen identifies CDKN2C as a HBV host factor. *Nat Commun, 11*(1), 2707. doi:10.1038/s41467-020-16517-w

Eruzun, H., Toprak, I. D., Arman, Y., Yilmaz, U., Ozcan, M., Kutlu, Y., . . . Tukek, T. (2019). Serum endotrophin levels in patients with heart failure with reduced and mid-range ejection fraction. *Eur J Intern Med, 64*, 29-32. doi:10.1016/j.ejim.2019.04.016

Evans, H. M. (1908). "The liver in antiquity and the beginnings of anatomy," by Prof. Morris Jastrow. University of Pennsylvania Medical Bulletin, Vol. XX, No. 11, January, 1908."An omen school text;" Old testament and semitic studies in memory of William Rainey Harper, vol. II, Chicago, 1908. In: Wiley Subscription Services, Inc., A Wiley Company Hoboken.

Ezzikouri, S., Kayesh, M. E. H., Benjelloun, S., Kohara, M., & Tsukiyama-Kohara, K. (2020). Targeting Host Innate and Adaptive Immunity to Achieve the Functional Cure of Chronic Hepatitis B. *Vaccines (Basel), 8*(2). doi:10.3390/vaccines8020216

Fanning, G. C., Zoulim, F., Hou, J., & Bertoletti, A. (2019). Therapeutic strategies for hepatitis B virus infection: towards a cure. *Nat Rev Drug Discov, 18*(11), 827-844. doi:10.1038/s41573-019-0037-0

Fattovich, G., Bortolotti, F., & Donato, F. (2008). Natural history of chronic hepatitis B: special emphasis on disease progression and prognostic factors. *J Hepatol, 48*(2), 335-352. doi:10.1016/j.jhep.2007.11.011

Feray, C., & Lopez-Labrador, F. X. (2019). Is PD-1 blockade a potential therapy for HBV? *JHEP Rep, 1*(3), 142-144. doi:10.1016/j.jhepr.2019.07.007

Fickert, P., Stoger, U., Fuchsbichler, A., Moustafa, T., Marschall, H. U., Weiglein, A. H., . . . Trauner, M. (2007). A new xenobiotic-induced mouse model of sclerosing cholangitis and biliary fibrosis. *Am J Pathol, 171*(2), 525-536. doi:10.2353/ajpath.2007.061133

Fitzwalter, B. E., Towers, C. G., Sullivan, K. D., Andrysik, Z., Hoh, M., Ludwig, M., . . . Thorburn, A. (2018). Autophagy Inhibition Mediates Apoptosis Sensitization in Cancer Therapy by Relieving FOXO3a Turnover. *Dev Cell, 44*(5), 555-565 e553. doi:10.1016/j.devcel.2018.02.014

Floreani, A., Baldo, V., Cristofoletti, M., Renzulli, G., Valeri, A., Zanetti, C., & Trivello, R. (2004). Long-term persistence of anti-HBs after vaccination against HBV: an 18 year experience in health care workers. *Vaccine, 22*(5-6), 607-610. doi:10.1016/j.vaccine.2003.09.001

Francis, D. P., Hadler, S. C., Thompson, S. E., Maynard, J. E., Ostrow, D. G., Altman, N., . . . Redeker, A. G. (1982). The prevention of hepatitis B with vaccine. Report of the centers for disease control multi-center efficacy trial among homosexual men. *Ann Intern Med, 97*(3), 362-366. doi:10.7326/0003-4819-97-3-362

Freise, C., Lee, H., Chronowski, C., Chan, D., Cziomer, J., Ruhl, M., . . . Sahin, E. (2021). Alpha-single chains of collagen type VI inhibit the fibrogenic effects of triple helical collagen VI in hepatic stellate cells. *PLoS One, 16*(9), e0254557. doi:10.1371/journal.pone.0254557

Fricker, Z. P., & Reddy, K. R. (2019). When (and When Not) to Treat Patients With HBV Infection. *Clin Gastroenterol Hepatol, 17*(13), 2644-2647. doi:10.1016/j.cgh.2019.05.039

Furuta, M., Tanaka, H., Shiraishi, Y., Unida, T., Imamura, M., Fujimoto, A., . . . Nakagawa, H. (2018). Characterization of HBV integration patterns and timing in liver cancer and HBV-infected livers. *Oncotarget, 9*(38), 25075-25088. doi:10.18632/oncotarget.25308

Furutani, Y., Toguchi, M., Shiozaki-Sato, Y., Qin, X. Y., Ebisui, E., Higuchi, S., . . . Kojima, S. (2019). An interferon-like small chemical compound CDM-3008 suppresses hepatitis B virus through induction of interferon-stimulated genes. *PLoS One, 14*(6), e0216139. doi:10.1371/journal.pone.0216139

Fyodorov, D. V., Zhou, B. R., Skoultchi, A. I., & Bai, Y. (2018). Emerging roles of linker histones in regulating chromatin structure and function. *Nat Rev Mol Cell Biol, 19*(3), 192-206. doi:10.1038/nrm.2017.94

Gane, E., Verdon, D. J., Brooks, A. E., Gaggar, A., Nguyen, A. H., Subramanian, G. M., . . . Dunbar, P. R. (2019). Anti-PD-1 blockade with nivolumab with and without therapeutic vaccination for virally suppressed chronic hepatitis B: A pilot study. *J Hepatol, 71*(5), 900-907. doi:10.1016/j.jhep.2019.06.028

Ganem, D. (1991). Assembly of hepadnaviral virions and subviral particles. *Curr Top Microbiol Immunol, 168*, 61-83. doi:10.1007/978-3-642-76015-0_4

Gao, B., Jeong, W. I., & Tian, Z. (2008). Liver: An organ with predominant innate immunity. *Hepatology, 47*(2), 729-736. doi:10.1002/hep.22034

Gao, Q., Zhu, H., Dong, L., Shi, W., Chen, R., Song, Z., . . . Fan, J. (2019). Integrated Proteogenomic Characterization of HBV-Related Hepatocellular Carcinoma. *Cell, 179*(2), 561-577 e522. doi:10.1016/j.cell.2019.08.052

Geerts, A. (2001). History, heterogeneity, developmental biology, and functions of quiescent hepatic stellate cells. *Semin Liver Dis, 21*(3), 311-335. doi:10.1055/s-2001-17550

Geramizadeh, B., Janfeshan, K., & Saberfiroozi, M. (2008). Serum hyaluronic acid as a noninvasive marker of hepatic fibrosis in chronic hepatitis B. *Saudi J Gastroenterol, 14*(4), 174-177. doi:10.4103/1319-3767.43274

Giacinti, C., & Giordano, A. (2006). RB and cell cycle progression. *Oncogene, 25*(38), 5220-5227. doi:10.1038/sj.onc.1209615

Gilbert, R. J., Beales, L., Blond, D., Simon, M. N., Lin, B. Y., Chisari, F. V., . . . Rowlands, D. J. (2005). Hepatitis B small surface antigen particles are octahedral. *Proc Natl Acad Sci U S A, 102*(41), 14783-14788. doi:10.1073/pnas.0505062102

Giroglou, T., Florin, L., Schafer, F., Streeck, R. E., & Sapp, M. (2001). Human papillomavirus infection requires cell surface heparan sulfate. *J Virol, 75*(3), 1565-1570. doi:10.1128/JVI.75.3.1565-1570.2001

Global Burden of Disease Liver Cancer, C., Akinyemiju, T., Abera, S., Ahmed, M., Alam, N., Alemayohu, M. A., . . . Fitzmaurice, C. (2017). The Burden of Primary Liver Cancer and Underlying Etiologies From 1990 to 2015 at the Global, Regional, and National Level: Results From the Global Burden of Disease Study 2015. *JAMA Oncol, 3*(12), 1683-1691. doi:10.1001/jamaoncol.2017.3055

Goldstein, S. T., Alter, M. J., Williams, I. T., Moyer, L. A., Judson, F. N., Mottram, K., . . . Margolis, H. S. (2002). Incidence and risk factors for acute hepatitis B in the United States, 1982-1998: implications for vaccination programs. *J Infect Dis, 185*(6), 713-719. doi:10.1086/339192

Gong, Q., Chen, S., Guo, J., Sun, H., Zheng, G., Liu, Q., . . . He, S. (2011). Chromosome remodeling related to hepatitis B virus replication in HepG2 cells. *DNA Cell Biol, 30*(6), 347-354. doi:10.1089/dna.2010.1172

Graves, P. R., & Haystead, T. A. (2002). Molecular biologist's guide to proteomics. *Microbiol Mol Biol Rev, 66*(1), 39-63; table of contents. doi:10.1128/MMBR.66.1.39-63.2002

Gripon, P., Diot, C., & Guguen-Guillouzo, C. (1993). Reproducible high level infection of cultured adult human hepatocytes by hepatitis B virus: effect of polyethylene glycol on adsorption and penetration. *Virology, 192*(2), 534-540. doi:10.1006/viro.1993.1069

Gripon, P., Diot, C., Theze, N., Fourel, I., Loreal, O., Brechot, C., & Guguen-Guillouzo, C. (1988). Hepatitis B virus infection of adult human hepatocytes cultured in the presence of dimethyl sulfoxide. *J Virol, 62*(11), 4136-4143. doi:10.1128/JVI.62.11.4136-4143.1988

Grumati, P., Coletto, L., Sabatelli, P., Cescon, M., Angelin, A., Bertaggia, E., . . . Bonaldo, P. (2010). Autophagy is defective in collagen VI muscular dystrophies, and its reactivation rescues myofiber degeneration. *Nat Med, 16*(11), 1313-1320. doi:10.1038/nm.2247

Gualandi, F., Curci, R., Sabatelli, P., Martoni, E., Bovolenta, M., Maraldi, M. N., . . . Ferlini, A. A. (2011). Macrophages: a minimally invasive tool for monitoring collagen VI myopathies. *Muscle Nerve, 44*(1), 80-84. doi:10.1002/mus.21999

Guan, J. L. (2010). Integrin signaling through FAK in the regulation of mammary stem cells and breast cancer. *IUBMB Life, 62*(4), 268-276. doi:10.1002/iub.303

Guo, Y. H., Li, Y. N., Zhao, J. R., Zhang, J., & Yan, Z. (2011). HBc binds to the CpG islands of HBV cccDNA and promotes an epigenetic permissive state. *Epigenetics, 6*(6), 720-726. doi:10.4161/epi.6.6.15815

Gustavsson, J., Parpal, S., Karlsson, M., Ramsing, C., Thorn, H., Borg, M., . . . Stralfors, P. (1999). Localization of the insulin receptor in caveolae of adipocyte plasma membrane. *FASEB J, 13*(14), 1961-1971. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/10544179

Hagenbuch, B., & Dawson, P. (2004). The sodium bile salt cotransport family SLC10. *Pflugers Arch, 447*(5), 566-570. doi:10.1007/s00424-003-1130-z

Hamdane, N., Juhling, F., Crouchet, E., El Saghire, H., Thumann, C., Oudot, M. A., . . . Baumert, T. F. (2019). HCV-Induced Epigenetic Changes Associated With Liver Cancer Risk Persist After Sustained Virologic Response. *Gastroenterology, 156*(8), 2313-2329 e2317. doi:10.1053/j.gastro.2019.02.038

Han, J., Zhang, X., Lau, J. K., Fu, K., Lau, H. C., Xu, W., . . . Yu, J. (2019). Bone marrow-derived macrophage contributes to fibrosing steatohepatitis through activating hepatic stellate cells. *J Pathol, 248*(4), 488-500. doi:10.1002/path.5275

Han, S. H. (2004). Extrahepatic manifestations of chronic hepatitis B. *Clin Liver Dis, 8*(2), 403-418. doi:10.1016/j.cld.2004.02.003

Hantz, O., Parent, R., Durantel, D., Gripon, P., Guguen-Guillouzo, C., & Zoulim, F. (2009). Persistence of the hepatitis B virus covalently closed circular DNA in HepaRG human hepatocyte-like cells. *J Gen Virol, 90*(Pt 1), 127-135. doi:10.1099/vir.0.004861-0

Happel, N., & Doenecke, D. (2009). Histone H1 and its isoforms: contribution to chromatin structure and function. *Gene, 431*(1-2), 1-12. doi:10.1016/j.gene.2008.11.003

Happel, N., Stoldt, S., Schmidt, B., & Doenecke, D. (2009). M phase-specific phosphorylation of histone H1.5 at threonine 10 by GSK-3. *J Mol Biol, 386*(2), 339-350. doi:10.1016/j.jmb.2008.12.047

Harburger, D. S., & Calderwood, D. A. (2009). Integrin signalling at a glance. *J Cell Sci, 122*(Pt 2), 159-163. doi:10.1242/jcs.018093

Hastings, J. F., Skhinas, J. N., Fey, D., Croucher, D. R., & Cox, T. R. (2019). The extracellular matrix as a key regulator of intracellular signalling networks. *Br J Pharmacol, 176*(1), 82-92. doi:10.1111/bph.14195

Hayes, C. N., Zhang, Y., Makokha, G. N., Hasan, M. Z., Omokoko, M. D., & Chayama, K. (2016). Early events in hepatitis B virus infection: From the cell surface to the nucleus. *J Gastroenterol Hepatol, 31*(2), 302-309. doi:10.1111/jgh.13175

Heermann, K. H., Goldmann, U., Schwartz, W., Seyffarth, T., Baumgarten, H., & Gerlich, W. H. (1984). Large surface proteins of hepatitis B virus containing the pre-s sequence. *J Virol, 52*(2), 396-402. doi:10.1128/JVI.52.2.396-402.1984

Henderson, N. C., & Sheppard, D. (2013). Integrin-mediated regulation of TGFbeta in fibrosis. *Biochim Biophys Acta, 1832*(7), 891-896. doi:10.1016/j.bbadis.2012.10.005

Hensel, K. O., Cantner, F., Bangert, F., Wirth, S., & Postberg, J. (2018). Episomal HBV persistence within transcribed host nuclear chromatin compartments involves HBx. *Epigenetics Chromatin, 11*(1), 34. doi:10.1186/s13072-018-0204-2

Hergeth, S. P., Dundr, M., Tropberger, P., Zee, B. M., Garcia, B. A., Daujat, S., & Schneider, R. (2011). Isoform-specific phosphorylation of human linker histone H1.4 in mitosis by the kinase Aurora B. *J Cell Sci, 124*(Pt 10), 1623-1628. doi:10.1242/jcs.084947

Hergeth, S. P., & Schneider, R. (2015). The H1 linker histones: multifunctional proteins beyond the nucleosomal core particle. *EMBO Rep, 16*(11), 1439-1453. doi:10.15252/embr.201540749

Herrscher, C., Pastor, F., Burlaud-Gaillard, J., Dumans, A., Seigneuret, F., Moreau, A., . . . Blanchard, E. (2020). Hepatitis B virus entry into HepG2-NTCP cells requires clathrin-mediated endocytosis. *Cell Microbiol, 22*(8), e13205. doi:10.1111/cmi.13205

Hilleman, M. R., Buynak, E. B., Roehm, R. R., Tytell, A. A., Bertland, A. U., & Lampson, G. P. (1975). Purified and inactivated human hepatitis B vaccine: progress report. *Am J Med Sci, 270*(2), 401-404. doi:10.1097/00000441-197509000-00025

Ho, C. M., Chang, T. H., Yen, T. L., Hong, K. J., & Huang, S. H. (2021). Collagen type VI regulates the CDK4/6-p-Rb signaling pathway and promotes ovarian cancer invasiveness, stemness, and metastasis. *Am J Cancer Res, 11*(3), 668-690. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/33791147

Hoofnagle, J. H., Doo, E., Liang, T. J., Fleischer, R., & Lok, A. S. (2007). Management of hepatitis B: summary of a clinical research workshop. *Hepatology, 45*(4), 1056-1075. doi:10.1002/hep.21627

Hsu, Y. C., Jun, T., Huang, Y. T., Yeh, M. L., Lee, C. L., Ogawa, S., . . . Tanaka, Y. (2018). Serum M2BPGi level and risk of hepatocellular carcinoma after oral anti-viral therapy in patients with chronic hepatitis B. *Aliment Pharmacol Ther, 48*(10), 1128-1137. doi:10.1111/apt.15006

Hu, J., Lin, Y. Y., Chen, P. J., Watashi, K., & Wakita, T. (2019). Cell and Animal Models for Studying Hepatitis B Virus Infection and Drug Development. *Gastroenterology, 156*(2), 338-354. doi:10.1053/j.gastro.2018.06.093

Hu, J., & Liu, K. (2017). Complete and Incomplete Hepatitis B Virus Particles: Formation, Function, and Application. *Viruses, 9*(3). doi:10.3390/v9030056

Hu, Q., Zhang, F., Duan, L., Wang, B., Ye, Y., Li, P., . . . Chen, W. (2020). E-cadherin Plays a Role in Hepatitis B Virus Entry Through Affecting Glycosylated Sodium-Taurocholate Cotransporting Polypeptide Distribution. *Front Cell Infect Microbiol, 10*, 74. doi:10.3389/fcimb.2020.00074

Huang, G., & Brigstock, D. R. (2012). Regulation of hepatic stellate cells by connective tissue growth factor. *Front Biosci (Landmark Ed), 17*(7), 2495-2507. doi:10.2741/4067

Huang, H. C., Chen, C. C., Chang, W. C., Tao, M. H., & Huang, C. (2012). Entry of hepatitis B virus into immortalized human primary hepatocytes by clathrin-dependent endocytosis. *J Virol, 86*(17), 9443-9453. doi:10.1128/JVI.00873-12

Huang, Q., Cai, D., Yan, R., Li, L., Zong, Y., Guo, L., . . . Colonno, R. (2020). Preclinical Profile and Characterization of the Hepatitis B Virus Core Protein Inhibitor ABI-H0731. *Antimicrob Agents Chemother, 64*(11). doi:10.1128/AAC.01463-20

Huang, W., Zhao, F., Huang, Y., Li, X., Zhu, S., Hu, Q., & Chen, W. (2014). Rapamycin enhances HBV production by inducing cellular autophagy. *Hepat Mon, 14*(10), e20719. doi:10.5812/hepatmon.20719

Ichai, P., & Samuel, D. (2019). Management of Fulminant Hepatitis B. *Curr Infect Dis Rep, 21*(7), 25. doi:10.1007/s11908-019-0682-9

Iimuro, Y., Nishio, T., Morimoto, T., Nitta, T., Stefanovic, B., Choi, S. K., . . . Yamaoka, Y. (2003). Delivery of matrix metalloproteinase-1 attenuates established liver fibrosis in the rat. *Gastroenterology, 124*(2), 445-458. doi:10.1053/gast.2003.50063

Ikeda, Y., Murakami, M., Nakagawa, Y., Tsuji, A., Kitagishi, Y., & Matsuda, S. (2020). Diet induces hepatocyte protection in fatty liver disease via modulation of PTEN signaling. *Biomed Rep, 12*(6), 295-302. doi:10.3892/br.2020.1299

Indolfi, G., Easterbrook, P., Dusheiko, G., Siberry, G., Chang, M. H., Thorne, C., . . . Penazzato, M. (2019). Hepatitis B virus infection in children and adolescents. *Lancet Gastroenterol Hepatol, 4*(6), 466-476. doi:10.1016/S2468-1253(19)30042-1

Irwin, W. A., Bergamin, N., Sabatelli, P., Reggiani, C., Megighian, A., Merlini, L., . . . Bonaldo, P. (2003). Mitochondrial dysfunction and apoptosis in myopathic mice with collagen VI deficiency. *Nat Genet, 35*(4), 367-371. doi:10.1038/ng1270

Itoh, Y., Takai, E., Ohnuma, H., Kitajima, K., Tsuda, F., Machida, A., . . . Mayumi, M. (1986). A synthetic peptide vaccine involving the product of the pre-S(2) region of hepatitis B virus DNA: protective efficacy in chimpanzees. *Proc Natl Acad Sci U S A, 83*(23), 9174-9178. doi:10.1073/pnas.83.23.9174

Iwamoto, M., Cai, D., Sugiyama, M., Suzuki, R., Aizaki, H., Ryo, A., . . . Watashi, K. (2017). Functional association of cellular microtubules with viral capsid assembly supports efficient hepatitis B virus replication. *Sci Rep, 7*(1), 10620. doi:10.1038/s41598-017-11015-4

Iwamoto, M., Saso, W., Nishioka, K., Ohashi, H., Sugiyama, R., Ryo, A., . . . Watashi, K. (2020). The machinery for endocytosis of epidermal growth factor receptor

coordinates the transport of incoming hepatitis B virus to the endosomal network. *J Biol Chem, 295*(3), 800-807. doi:10.1074/jbc.AC119.010366

Iwamoto, M., Saso, W., Sugiyama, R., Ishii, K., Ohki, M., Nagamori, S., . . . Watashi, K. (2019). Epidermal growth factor receptor is a host-entry cofactor triggering hepatitis B virus internalization. *Proc Natl Acad Sci U S A, 116*(17), 8487-8492. doi:10.1073/pnas.1811064116

Jansen, D. T., Dou, Y., de Wilde, J. W., Woltman, A. M., & Buschow, S. I. (2021). Designing the next-generation therapeutic vaccines to cure chronic hepatitis B: focus on antigen presentation, vaccine properties and effect measures. *Clin Transl Immunology, 10*(1), e1232. doi:10.1002/cti2.1232

Jansen, L., Kootstra, N. A., van Dort, K. A., Takkenberg, R. B., Reesink, H. W., & Zaaijer, H. L. (2016). Hepatitis B Virus Pregenomic RNA Is Present in Virions in Plasma and Is Associated With a Response to Pegylated Interferon Alfa-2a and Nucleos(t)ide Analogues. *J Infect Dis, 213*(2), 224-232. doi:10.1093/infdis/jiv397

Jo, J., Tan, A. T., Ussher, J. E., Sandalova, E., Tang, X. Z., Tan-Garcia, A., . . . Bertoletti, A. (2014). Toll-like receptor 8 agonist and bacteria trigger potent activation of innate immune cells in human liver. *PLoS Pathog, 10*(6), e1004210. doi:10.1371/journal.ppat.1004210

Jones, S. A., & Hu, J. (2013a). Hepatitis B virus reverse transcriptase: diverse functions as classical and emerging targets for antiviral intervention. *Emerg Microbes Infect, 2*(9), e56. doi:10.1038/emi.2013.56

Jones, S. A., & Hu, J. (2013b). Protein-primed terminal transferase activity of hepatitis B virus polymerase. *J Virol, 87*(5), 2563-2576. doi:10.1128/JVI.02786-12

Jongewaard, I. N., Lauer, R. M., Behrendt, D. A., Patil, S., & Klewer, S. E. (2002). Beta 1 integrin activation mediates adhesive differences between trisomy 21 and non-trisomic fibroblasts on type VI collagen. *Am J Med Genet, 109*(4), 298-305. doi:10.1002/ajmg.10413

Juhling, F., Hamdane, N., Crouchet, E., Li, S., El Saghire, H., Mukherji, A., . . . Baumert, T. F. (2021). Targeting clinical epigenetic reprogramming for chemoprevention of metabolic and viral hepatocellular carcinoma. *Gut, 70*(1), 157-169. doi:10.1136/gutjnl-2019-318918

Jun, T., Hsu, Y. C., Ogawa, S., Huang, Y. T., Yeh, M. L., Tseng, C. H., . . . Nguyen, M. H. (2019). Mac-2 Binding Protein Glycosylation Isomer as a Hepatocellular Carcinoma Marker in Patients With Chronic Hepatitis B or C Infection. *Hepatol Commun, 3*(4), 493-503. doi:10.1002/hep4.1321

Kammarabutr, J., Mahalapbutr, P., Okumura, H., Wolschann, P., & Rungrotmongkol, T. (2021). Structural dynamics and susceptibility of anti-HIV drugs against HBV reverse transcriptase. *J Biomol Struct Dyn, 39*(7), 2502-2511. doi:10.1080/07391102.2020.1751715

Kan, F., Ye, L., Yan, T., Cao, J., Zheng, J., & Li, W. (2017). Proteomic and transcriptomic studies of HBV-associated liver fibrosis of an AAV-HBV-infected mouse model. *BMC Genomics, 18*(1), 641. doi:10.1186/s12864-017-3984-z

Kann, M., Sodeik, B., Vlachou, A., Gerlich, W. H., & Helenius, A. (1999). Phosphorylation-dependent binding of hepatitis B virus core particles to the nuclear pore complex. *J Cell Biol, 145*(1), 45-55. doi:10.1083/jcb.145.1.45

Kantari-Mimoun, C., Castells, M., Klose, R., Meinecke, A. K., Lemberger, U. J., Rautou, P. E., . . . Stockmann, C. (2015). Resolution of liver fibrosis requires myeloid cell-driven sinusoidal angiogenesis. *Hepatology, 61*(6), 2042-2055. doi:10.1002/hep.27635

Karousou, E., D'Angelo, M. L., Kouvidi, K., Vigetti, D., Viola, M., Nikitovic, D., . . . Passi, A. (2014). Collagen VI and hyaluronan: the common role in breast cancer. *Biomed Res Int, 2014*, 606458. doi:10.1155/2014/606458

Karsdal, M. A., Daniels, S. J., Holm Nielsen, S., Bager, C., Rasmussen, D. G. K., Loomba, R., . . . Schuppan, D. (2020). Collagen biology and non-invasive biomarkers of liver fibrosis. *Liver Int, 40*(4), 736-750. doi:10.1111/liv.14390

Karsdal, M. A., Nielsen, M. J., Sand, J. M., Henriksen, K., Genovese, F., Bay-Jensen, A. C., . . . Leeming, D. J. (2013). Extracellular matrix remodeling: the common denominator in connective tissue diseases. Possibilities for evaluation and current understanding of the matrix as more than a passive architecture, but a key player in tissue failure. *Assay Drug Dev Technol, 11*(2), 70-92. doi:10.1089/adt.2012.474

Katrinli, S., Ozdil, K., Sahin, A., Ozturk, O., Kir, G., Baykal, A. T., . . . Dinler Doganay, G. (2016). Proteomic profiling of HBV infected liver biopsies with different fibrotic stages. *Proteome Sci, 15*, 7. doi:10.1186/s12953-017-0114-4

Kayesh, M. E. H., Amako, Y., Hashem, M. A., Murakami, S., Ogawa, S., Yamamoto, N., . . . Tsukiyama-Kohara, K. (2020). Development of an in vivo delivery system for CRISPR/Cas9-mediated targeting of hepatitis B virus cccDNA. *Virus Res, 290*, 198191. doi:10.1016/j.virusres.2020.198191

Keane, E., Funk, A. L., & Shimakawa, Y. (2016). Systematic review with meta-analysis: the risk of mother-to-child transmission of hepatitis B virus infection in sub-Saharan Africa. *Aliment Pharmacol Ther, 44*(10), 1005-1017. doi:10.1111/apt.13795

Khan, T., Muise, E. S., Iyengar, P., Wang, Z. V., Chandalia, M., Abate, N., . . . Scherer, P. E. (2009). Metabolic dysregulation and adipose tissue fibrosis: role of collagen VI. *Mol Cell Biol, 29*(6), 1575-1591. doi:10.1128/MCB.01300-08

Kikuchi, A., Pradhan-Sundd, T., Singh, S., Nagarajan, S., Loizos, N., & Monga, S. P. (2017). Platelet-Derived Growth Factor Receptor alpha Contributes to Human Hepatic Stellate Cell Proliferation and Migration. *Am J Pathol, 187*(10), 2273-2287. doi:10.1016/j.ajpath.2017.06.009

Kimura, T., Ohno, N., Terada, N., Rokuhara, A., Matsumoto, A., Yagi, S., . . . Maki, N. (2005). Hepatitis B virus DNA-negative dane particles lack core protein but contain a 22-kDa precore protein without C-terminal arginine-rich domain. *J Biol Chem, 280*(23), 21713-21719. doi:10.1074/jbc.M501564200

Kimura, T., Rokuhara, A., Sakamoto, Y., Yagi, S., Tanaka, E., Kiyosawa, K., & Maki, N. (2002). Sensitive enzyme immunoassay for hepatitis B virus core-related antigens and their correlation to virus load. *J Clin Microbiol, 40*(2), 439-445. doi:10.1128/JCM.40.2.439-445.2002

Kinoshita, K., Aono, Y., Azuma, M., Kishi, J., Takezaki, A., Kishi, M., . . . Nishioka, Y. (2013). Antifibrotic effects of focal adhesion kinase inhibitor in bleomycin-induced pulmonary fibrosis in mice. *Am J Respir Cell Mol Biol, 49*(4), 536-543. doi:10.1165/rcmb.2012-0277OC

Kitamura, K., Que, L., Shimadu, M., Koura, M., Ishihara, Y., Wakae, K., . . . Muramatsu, M. (2018). Flap endonuclease 1 is involved in cccDNA formation in the hepatitis B virus. *PLoS Pathog, 14*(6), e1007124. doi:10.1371/journal.ppat.1007124

Knolle, P. A., Huang, L. R., Kosinska, A., Wohlleber, D., & Protzer, U. (2021). Improving Therapeutic Vaccination against Hepatitis B-Insights from Preclinical Models of Immune Therapy against Persistent Hepatitis B Virus Infection. *Vaccines (Basel), 9*(11). doi:10.3390/vaccines9111333

Knupp, C., Pinali, C., Munro, P. M., Gruber, H. E., Sherratt, M. J., Baldock, C., & Squire, J. M. (2006). Structural correlation between collagen VI microfibrils and collagen VI banded aggregates. *J Struct Biol, 154*(3), 312-326. doi:10.1016/j.jsb.2006.03.023

Ko, C., Chakraborty, A., Chou, W. M., Hasreiter, J., Wettengel, J. M., Stadler, D., . . . Protzer, U. (2018). Hepatitis B virus genome recycling and de novo secondary infection events maintain stable cccDNA levels. *J Hepatol, 69*(6), 1231-1241. doi:10.1016/j.jhep.2018.08.012

Kocabayoglu, P., Lade, A., Lee, Y. A., Dragomir, A. C., Sun, X., Fiel, M. I., . . . Friedman, S. L. (2015). beta-PDGF receptor expressed by hepatic stellate cells regulates fibrosis in murine liver injury, but not carcinogenesis. *J Hepatol, 63*(1), 141-147. doi:10.1016/j.jhep.2015.01.036

Kongkavitoon, P., Tangkijvanich, P., Hirankarn, N., & Palaga, T. (2016). Hepatitis B Virus HBx Activates Notch Signaling via Delta-Like 4/Notch1 in Hepatocellular Carcinoma. *PLoS One, 11*(1), e0146696. doi:10.1371/journal.pone.0146696

Koniger, C., Wingert, I., Marsmann, M., Rosler, C., Beck, J., & Nassal, M. (2014). Involvement of the host DNA-repair enzyme TDP2 in formation of the covalently closed circular DNA persistence reservoir of hepatitis B viruses. *Proc Natl Acad Sci U S A, 111*(40), E4244-4253. doi:10.1073/pnas.1409986111

Koop, R., Di Croce, L., & Beato, M. (2003). Histone H1 enhances synergistic activation of the MMTV promoter in chromatin. *EMBO J, 22*(3), 588-599. doi:10.1093/emboj/cdg052

Kordes, C., Bock, H. H., Reichert, D., May, P., & Haussinger, D. (2021). Hepatic stellate cells: current state and open questions. *Biol Chem, 402*(9), 1021-1032. doi:10.1515/hsz-2021-0180

Kramvis, A. (2014). Genotypes and genetic variability of hepatitis B virus. *Intervirology, 57*(3-4), 141-150. doi:10.1159/000360947

Kumada, T., Toyoda, H., Tada, T., Kiriyama, S., Tanikawa, M., Hisanaga, Y., . . . Tanaka, J. (2013). Effect of nucleos(t)ide analogue therapy on hepatocarcinogenesis in chronic hepatitis B patients: a propensity score analysis. *J Hepatol, 58*(3), 427-433. doi:10.1016/j.jhep.2012.10.025

Kuo, H. J., Maslen, C. L., Keene, D. R., & Glanville, R. W. (1997). Type VI collagen anchors endothelial basement membranes by interacting with type IV collagen. *J Biol Chem, 272*(42), 26522-26529. doi:10.1074/jbc.272.42.26522

Kysela, B., Chovanec, M., & Jeggo, P. A. (2005). Phosphorylation of linker histones by DNA-dependent protein kinase is required for DNA ligase IV-dependent ligation in the presence of histone H1. *Proc Natl Acad Sci U S A, 102*(6), 1877-1882. doi:10.1073/pnas.0401179102

Ladner, S. K., Otto, M. J., Barker, C. S., Zaifert, K., Wang, G. H., Guo, J. T., . . . King, R. W. (1997). Inducible expression of human hepatitis B virus (HBV) in stably transfected hepatoblastoma cells: a novel system for screening potential inhibitors of HBV replication. *Antimicrob Agents Chemother, 41*(8), 1715-1720. doi:10.1128/AAC.41.8.1715

Lamande, S. R. (2021). Collagen VI Muscle Disorders: Mutation Types, Pathogenic Mechanisms and Approaches to Therapy. *Adv Exp Med Biol, 1348*, 311-323. doi:10.1007/978-3-030-80614-9_14

Lee, H. W., Lee, J. S., & Ahn, S. H. (2020). Hepatitis B Virus Cure: Targets and Future Therapies. *Int J Mol Sci, 22*(1). doi:10.3390/ijms22010213

Lee, S., Goyal, A., Perelson, A. S., Ishida, Y., Saito, T., & Gale, M., Jr. (2021). Suppression of hepatitis B virus through therapeutic activation of RIG-I and IRF3 signaling in hepatocytes. *iScience, 24*(1), 101969. doi:10.1016/j.isci.2020.101969

Lee, Y. S., Kim, S. Y., Ko, E., Lee, J. H., Yi, H. S., Yoo, Y. J., . . . Byun, K. S. (2017). Exosomes derived from palmitic acid-treated hepatocytes induce fibrotic activation of hepatic stellate cells. *Sci Rep, 7*(1), 3710. doi:10.1038/s41598-017-03389-2

Leeming, D. J., Nielsen, S. H., Vongsuvanh, R., Uchila, P., Nielsen, M. J., Reese-Petersen, A. L., . . . George, J. (2020). Endotrophin, a pro-peptide of Type VI collagen, is a biomarker of survival in cirrhotic patients with hepatocellular carcinoma. *Hepat Oncol, 8*(2), HEP32. doi:10.2217/hep-2020-0030

Lempp, F. A., Mutz, P., Lipps, C., Wirth, D., Bartenschlager, R., & Urban, S. (2016). Evidence that hepatitis B virus replication in mouse cells is limited by the lack of a host cell dependency factor. *J Hepatol, 64*(3), 556-564. doi:10.1016/j.jhep.2015.10.030

Lever, M. A., Th'ng, J. P., Sun, X., & Hendzel, M. J. (2000). Rapid exchange of histone H1.1 on chromatin in living human cells. *Nature, 408*(6814), 873-876. doi:10.1038/35048603

Levrero, M., & Zucman-Rossi, J. (2016). Mechanisms of HBV-induced hepatocellular carcinoma. *J Hepatol, 64*(1 Suppl), S84-S101. doi:10.1016/j.jhep.2016.02.021

Li, H., Zhuang, Q., Wang, Y., Zhang, T., Zhao, J., Zhang, Y., . . . Han, J. (2014). HBV life cycle is restricted in mouse hepatocytes expressing human NTCP. *Cell Mol Immunol, 11*(2), 175-183. doi:10.1038/cmi.2013.66

Li, J., Liu, Y., Wang, Z., Liu, K., Wang, Y., Liu, J., . . . Yuan, Z. (2011). Subversion of cellular autophagy machinery by hepatitis B virus for viral envelopment. *J Virol, 85*(13), 6319-6333. doi:10.1128/JVI.02627-10

Li, X., Zhao, J., Yuan, Q., & Xia, N. (2017). Detection of HBV Covalently Closed Circular DNA. *Viruses, 9*(6). doi:10.3390/v9060139

Liao, R., & Mizzen, C. A. (2017). Site-specific regulation of histone H1 phosphorylation in pluripotent cell differentiation. *Epigenetics Chromatin, 10*, 29. doi:10.1186/s13072-017-0135-3

Liaw, Y. F., Sung, J. J., Chow, W. C., Farrell, G., Lee, C. Z., Yuen, H., . . . Cirrhosis Asian Lamivudine Multicentre Study, G. (2004). Lamivudine for patients with chronic hepatitis B and advanced liver disease. *N Engl J Med, 351*(15), 1521-1531. doi:10.1056/NEJMoa033364

Lim, J. K., Nguyen, M. H., Kim, W. R., Gish, R., Perumalswami, P., & Jacobson, I. M. (2020). Prevalence of Chronic Hepatitis B Virus Infection in the United States. *Am J Gastroenterol, 115*(9), 1429-1438. doi:10.14309/ajg.0000000000000651

Lin, Y., Zhao, Z., Huang, A., & Lu, M. (2020). Interplay between Cellular Autophagy and Hepatitis B Virus Replication: A Systematic Review. *Cells, 9*(9). doi:10.3390/cells9092101

Lindholm, M., Godskesen, L. E., Manon-Jensen, T., Kjeldsen, J., Krag, A., Karsdal, M. A., & Mortensen, J. H. (2021). Endotrophin and C6Ma3, serological biomarkers of type VI collagen remodelling, reflect endoscopic and clinical disease activity in IBD. *Sci Rep, 11*(1), 14713. doi:10.1038/s41598-021-94321-2

Liu, C., Wang, L., Xie, H., Zhang, L., Wang, B., Luo, C., . . . Xie, Y. (2018). The relationship between serum hepatitis B virus DNA level and liver histology in patients with chronic HBV infection. *PLoS One, 13*(11), e0206060. doi:10.1371/journal.pone.0206060

Liu, J., Hu, H. H., Lee, M. H., Korenaga, M., Jen, C. L., Batrla-Utermann, R., . . . Yang, H. I. (2017). Serum Levels of M2BPGi as Short-Term Predictors of Hepatocellular Carcinoma in Untreated Chronic Hepatitis B Patients. *Sci Rep, 7*(1), 14352. doi:10.1038/s41598-017-14747-5

Liu, T., Wang, X., Karsdal, M. A., Leeming, D. J., & Genovese, F. (2012). Molecular serum markers of liver fibrosis. *Biomark Insights, 7*, 105-117. doi:10.4137/BMI.S10009

Liu, X., Huang, H., Remmers, N., & Hollingsworth, M. A. (2014). Loss of E-cadherin and epithelial to mesenchymal transition is not required for cell motility in tissues or for metastasis. *Tissue Barriers, 2*(4), e969112. doi:10.4161/21688362.2014.969112

Liu, Y., & Gao, P. (2014). Modulation of hepatitis B surface antigen secretion by annexin II expressed in hepatitis B virusproducing hepatoma cells. *Mol Med Rep, 10*(6), 3113-3117. doi:10.3892/mmr.2014.2602

Liu, Y., Jiang, M., Xue, J., Yan, H., & Liang, X. (2019). Serum HBV RNA quantification: useful for monitoring natural history of chronic hepatitis B infection. *BMC Gastroenterol, 19*(1), 53. doi:10.1186/s12876-019-0966-4

LiverTox. (2012). In *LiverTox: Clinical and Research Information on Drug-Induced Liver Injury*. Bethesda (MD).

Llovet, J. M., Ricci, S., Mazzaferro, V., Hilgard, P., Gane, E., Blanc, J. F., . . . Group, S. I. S. (2008). Sorafenib in advanced hepatocellular carcinoma. *N Engl J Med, 359*(4), 378-390. doi:10.1056/NEJMoa0708857

Lok, A. S., Heathcote, E. J., & Hoofnagle, J. H. (2001). Management of hepatitis B: 2000-- summary of a workshop. *Gastroenterology, 120*(7), 1828-1853. doi:10.1053/gast.2001.24839

Lok, A. S., Zoulim, F., Dusheiko, G., & Ghany, M. G. (2017a). Hepatitis B cure: From discovery to regulatory approval. *J Hepatol, 67*(4), 847-861. doi:10.1016/j.jhep.2017.05.008

Lok, A. S., Zoulim, F., Dusheiko, G., & Ghany, M. G. (2017b). Hepatitis B cure: From discovery to regulatory approval. *Hepatology, 66*(4), 1296-1313. doi:10.1002/hep.29323

London, W. T., Sutnick, A. I., & Blumberg, B. S. (1969). Australia antigen and acute viral hepatitis. *Ann Intern Med, 70*(1), 55-59. doi:10.7326/0003-4819-70-1-55

Long, Q., Yan, R., Hu, J., Cai, D., Mitra, B., Kim, E. S., . . . Guo, H. (2017). The role of host DNA ligases in hepadnavirus covalently closed circular DNA formation. *PLoS Pathog, 13*(12), e1006784. doi:10.1371/journal.ppat.1006784

Loureiro, D., Castelnau, C., Bed, C. M., & Asselah, T. (2022). [Delta hepatitis: Epidemiology, diagnostic, natural history and treatment]. *Rev Med Interne, 43*(3), 160-169. doi:10.1016/j.revmed.2021.10.329

Lu, A., Zougman, A., Pudelko, M., Bebenek, M., Ziolkowski, P., Mann, M., & Wisniewski, J. R. (2009). Mapping of lysine monomethylation of linker histones in human breast and its cancer. *J Proteome Res, 8*(9), 4207-4215. doi:10.1021/pr9000652

Lu, M., Yao, X., Xu, Y., Lorenz, H., Dahmen, U., Chi, H., . . . Roggendorf, M. (2008). Combination of an antiviral drug and immunomodulation against hepadnaviral infection in the woodchuck model. *J Virol, 82*(5), 2598-2603. doi:10.1128/JVI.01613-07

Lu, X., & Hansen, J. C. (2004). Identification of specific functional subdomains within the linker histone H10 C-terminal domain. *J Biol Chem, 279*(10), 8701-8707. doi:10.1074/jbc.M311348200

Lucifora, J., Arzberger, S., Durantel, D., Belloni, L., Strubin, M., Levrero, M., . . . Protzer, U. (2011). Hepatitis B virus X protein is essential to initiate and maintain virus replication after infection. *J Hepatol, 55*(5), 996-1003. doi:10.1016/j.jhep.2011.02.015

Lucifora, J., Michelet, M., Salvetti, A., & Durantel, D. (2020). Fast Differentiation of HepaRG Cells Allowing Hepatitis B and Delta Virus Infections. *Cells, 9*(10). doi:10.3390/cells9102288

Lucifora, J., Pastor, F., Charles, E., Pons, C., Auclair, H., Fusil, F., . . . Salvetti, A. (2021). Evidence for long-term association of virion-delivered HBV core protein with cccDNA independently of viral protein production. *JHEP Rep, 3*(5), 100330. doi:10.1016/j.jhepr.2021.100330

Lucifora, J., Salvetti, A., Marniquet, X., Mailly, L., Testoni, B., Fusil, F., . . . Durantel, D. (2017). Detection of the hepatitis B virus (HBV) covalently-closed-circular DNA (cccDNA) in mice transduced with a recombinant AAV-HBV vector. *Antiviral Res, 145*, 14-19. doi:10.1016/j.antiviral.2017.07.006

Lucifora, J., Xia, Y., Reisinger, F., Zhang, K., Stadler, D., Cheng, X., . . . Protzer, U. (2014). Specific and nonhepatotoxic degradation of nuclear hepatitis B virus cccDNA. *Science, 343*(6176), 1221-1228. doi:10.1126/science.1243462

Luger, K., Mader, A. W., Richmond, R. K., Sargent, D. F., & Richmond, T. J. (1997). Crystal structure of the nucleosome core particle at 2.8 A resolution. *Nature, 389*(6648), 251-260. doi:10.1038/38444

Luk, A., Jiang, Q., Glavini, K., Triyatni, M., Zhao, N., Racek, T., . . . Grippo, J. F. (2020). A Single and Multiple Ascending Dose Study of Toll-Like Receptor 7 Agonist (RO7020531) in Chinese Healthy Volunteers. *Clin Transl Sci, 13*(5), 985-993. doi:10.1111/cts.12791

Lupberger, J., Croonenborghs, T., Roca Suarez, A. A., Van Renne, N., Juhling, F., Oudot, M. A., . . . Baumert, T. F. (2019). Combined Analysis of Metabolomes, Proteomes, and Transcriptomes of Hepatitis C Virus-Infected Cells and Liver to Identify Pathways Associated With Disease Development. *Gastroenterology, 157*(2), 537-551 e539. doi:10.1053/j.gastro.2019.04.003

Lupberger, J., Duong, F. H., Fofana, I., Zona, L., Xiao, F., Thumann, C., . . . Baumert, T. F. (2013). Epidermal growth factor receptor signaling impairs the antiviral activity of interferon-alpha. *Hepatology, 58*(4), 1225-1235. doi:10.1002/hep.26404

Lupberger, J., Schaedler, S., Peiran, A., & Hildt, E. (2013). Identification and characterization of a novel bipartite nuclear localization signal in the hepatitis B virus polymerase. *World J Gastroenterol, 19*(44), 8000-8010. doi:10.3748/wjg.v19.i44.8000

Lupberger, J., Zeisel, M. B., Xiao, F., Thumann, C., Fofana, I., Zona, L., . . . Baumert, T. F. (2011). EGFR and EphA2 are host factors for hepatitis C virus entry and possible targets for antiviral therapy. *Nat Med, 17*(5), 589-595. doi:10.1038/nm.2341

MacDonald, J. K., & McDonald, J. W. (2007). Natalizumab for induction of remission in Crohn's disease. *Cochrane Database Syst Rev*(1), CD006097. doi:10.1002/14651858.CD006097.pub2

Macovei, A., Radulescu, C., Lazar, C., Petrescu, S., Durantel, D., Dwek, R. A., . . . Nichita, N. B. (2010). Hepatitis B virus requires intact caveolin-1 function for productive infection in HepaRG cells. *J Virol, 84*(1), 243-253. doi:10.1128/JVI.01207-09

Mak, L. Y., To, W. P., Wong, D. K., Fung, J., Liu, F., Seto, W. K., . . . Yuen, M. F. (2019). Serum Mac-2 binding protein glycosylation isomer level predicts hepatocellular carcinoma development in E-negative chronic hepatitis B patients. *World J Gastroenterol, 25*(11), 1398-1408. doi:10.3748/wjg.v25.i11.1398

Makwana, M. V., Muimo, R., & Jackson, R. F. (2018). Advances in development of new tools for the study of phosphohistidine. *Lab Invest, 98*(3), 291-303. doi:10.1038/labinvest.2017.126

Malhotra, V., & Erlmann, P. (2015). The pathway of collagen secretion. *Annu Rev Cell Dev Biol, 31*, 109-124. doi:10.1146/annurev-cellbio-100913-013002

Mani, S. K. K., Yan, B., Cui, Z., Sun, J., Utturkar, S., Foca, A., . . . Andrisani, O. (2020). Restoration of RNA helicase DDX5 suppresses hepatitis B virus (HBV) biosynthesis and Wnt signaling in HBV-related hepatocellular carcinoma. *Theranostics, 10*(24), 10957-10972. doi:10.7150/thno.49629

Marcellin, P., Sung, J., & Piratvisuth, T. (2010). Avoiding and managing lamivudine resistance in chronic hepatitis B: current approaches and potential strategies including pegylated interferon. *Liver Int, 30*(5), 657-668. doi:10.1111/j.1478-3231.2010.02207.x

Marion, P. L., & Robinson, W. S. (1983). Hepadna viruses: hepatitis B and related viruses. *Curr Top Microbiol Immunol, 105*, 99-121. doi:10.1007/978-3-642-69159-1_2

Martin, K., Pritchett, J., Llewellyn, J., Mullan, A. F., Athwal, V. S., Dobie, R., . . . Piper Hanley, K. (2016). PAK proteins and YAP-1 signalling downstream of integrin beta-1 in myofibroblasts promote liver fibrosis. *Nat Commun, 7*, 12502. doi:10.1038/ncomms12502

Masiero, E., Agatea, L., Mammucari, C., Blaauw, B., Loro, E., Komatsu, M., . . . Sandri, M. (2009). Autophagy is required to maintain muscle mass. *Cell Metab, 10*(6), 507-515. doi:10.1016/j.cmet.2009.10.008

Maynard, J. E., Berquist, K. R., Krushak, D. H., & Purcell, R. H. (1972). Experimental infection of chimpanzees with the virus of hepatitis B. *Nature, 237*(5357), 514-515. doi:10.1038/237514a0

McMahon, B. J. (2009). The natural history of chronic hepatitis B virus infection. *Hepatology, 49*(5 Suppl), S45-55. doi:10.1002/hep.22898

Menendez-Arias, L., Alvarez, M., & Pacheco, B. (2014). Nucleoside/nucleotide analog inhibitors of hepatitis B virus polymerase: mechanism of action and resistance. *Curr Opin Virol, 8*, 1-9. doi:10.1016/j.coviro.2014.04.005

Mercado-Irizarry, A., & Torres, E. A. (2016). Cryptogenic cirrhosis: Current knowledge and future directions. *Clin Liver Dis (Hoboken), 7*(4), 69-72. doi:10.1002/cld.539

Meredith, L. W., Hu, K., Cheng, X., Howard, C. R., Baumert, T. F., Balfe, P., . . . McKeating, J. A. (2016). Lentiviral hepatitis B pseudotype entry requires sodium taurocholate co-transporting polypeptide and additional hepatocyte-specific factors. *J Gen Virol, 97*(1), 121-127. doi:10.1099/jgv.0.000317

Mertins, P., Tang, L. C., Krug, K., Clark, D. J., Gritsenko, M. A., Chen, L., . . . Carr, S. A. (2018). Reproducible workflow for multiplexed deep-scale proteome and phosphoproteome analysis of tumor tissues by liquid chromatography-mass spectrometry. *Nat Protoc, 13*(7), 1632-1661. doi:10.1038/s41596-018-0006-9

Mihara, K., Cao, X. R., Yen, A., Chandler, S., Driscoll, B., Murphree, A. L., . . . Fung, Y. K. (1989). Cell cycle-dependent regulation of phosphorylation of the human

retinoblastoma gene product. *Science, 246*(4935), 1300-1303. doi:10.1126/science.2588006

Millman, I., Zavatone, V., Gerstley, B. J., & Blumberg, B. S. (1969). Australia antigen detected in the nuclei of liver cells of patients with viral hepatitis by the fluorescent antibody technic. *Nature, 222*(5189), 181-184. doi:10.1038/222181b0

Misteli, T., Gunjan, A., Hock, R., Bustin, M., & Brown, D. T. (2000). Dynamic binding of histone H1 to chromatin in living cells. *Nature, 408*(6814), 877-881. doi:10.1038/35048610

Mohamed, A. A., El-Toukhy, N. E. R., Said, E. M., Gabal, H. M. R., AbdelAziz, H., Doss, W., . . . Abd-Elsalam, S. (2020). Hepatitis C Virus: Efficacy of New DAAs Regimens. *Infect Disord Drug Targets, 20*(2), 143-149. doi:10.2174/1871526519666190121114003

Moller, S., Henriksen, J. H., & Bendtsen, F. (2014). Extrahepatic complications to cirrhosis and portal hypertension: haemodynamic and homeostatic aspects. *World J Gastroenterol, 20*(42), 15499-15517. doi:10.3748/wjg.v20.i42.15499

Moolla, N., Kew, M., & Arbuthnot, P. (2002). Regulatory elements of hepatitis B virus transcription. *J Viral Hepat, 9*(5), 323-331. doi:10.1046/j.1365-2893.2002.00381.x

Moradpour, D., Penin, F., & Rice, C. M. (2007). Replication of hepatitis C virus. *Nat Rev Microbiol, 5*(6), 453-463. doi:10.1038/nrmicro1645

Moran-Salvador, E., & Mann, J. (2017). Epigenetics and Liver Fibrosis. *Cell Mol Gastroenterol Hepatol, 4*(1), 125-134. doi:10.1016/j.jcmgh.2017.04.007

Murphy, C. M., Xu, Y., Li, F., Nio, K., Reszka-Blanco, N., Li, X., . . . Su, L. (2016). Hepatitis B Virus X Protein Promotes Degradation of SMC5/6 to Enhance HBV Replication. *Cell Rep, 16*(11), 2846-2854. doi:10.1016/j.celrep.2016.08.026

Nair, A., Chauhan, P., Saha, B., & Kubatzky, K. F. (2019). Conceptual Evolution of Cell Signaling. *Int J Mol Sci, 20*(13). doi:10.3390/ijms20133292

Nallagangula, K. S., Nagaraj, S. K., Venkataswamy, L., & Chandrappa, M. (2018). Liver fibrosis: a compilation on the biomarkers status and their significance during disease progression. *Future Sci OA, 4*(1), FSO250. doi:10.4155/fsoa-2017-0083

Nguyen-Lefebvre, A. T., & Horuzsko, A. (2015). Kupffer Cell Metabolism and Function. *J Enzymol Metab, 1*(1). Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/26937490

Nielsen, M. J., Leeming, D. J., Karsdal, M. A., & Krag, A. (2017). Biomarkers of Extracellular Matrix Remodeling in Liver Diseases. In V. B. Patel & V. R. Preedy (Eds.), *Biomarkers in Liver Disease* (pp. 221-246). Dordrecht: Springer Netherlands.

Nikawa, T., Towatari, T., Ike, Y., & Katunuma, N. (1989). Studies on the reactive site of the cystatin superfamily using recombinant cystatin A mutants. Evidence that the QVVAG region is not essential for cysteine proteinase inhibitory activities. *FEBS Lett, 255*(2), 309-314. doi:10.1016/0014-5793(89)81112-3

Nishimura, S. L. (2009). Integrin-mediated transforming growth factor-beta activation, a potential therapeutic target in fibrogenic disorders. *Am J Pathol, 175*(4), 1362-1370. doi:10.2353/ajpath.2009.090393

Niu, C., Li, L., Daffis, S., Lucifora, J., Bonnin, M., Maadadi, S., . . . Fletcher, S. P. (2018). Toll-like receptor 7 agonist GS-9620 induces prolonged inhibition of HBV via a type I interferon-dependent mechanism. *J Hepatol, 68*(5), 922-931. doi:10.1016/j.jhep.2017.12.007

O'Brien, S. K., Cao, H., Nathans, R., Ali, A., & Rana, T. M. (2010). P-TEFb kinase complex phosphorylates histone H1 to regulate expression of cellular and HIV-1 genes. *J Biol Chem, 285*(39), 29713-29720. doi:10.1074/jbc.M110.125997

Okochi, K., & Murakami, S. (1968). Observations on Australia antigen in Japanese. *Vox Sang, 15*(5), 374-385. doi:10.1111/j.1423-0410.1968.tb04078.x

Orasan, O. H., Ciulei, G., Cozma, A., Sava, M., & Dumitrascu, D. L. (2016). Hyaluronic acid as a biomarker of fibrosis in chronic liver diseases of different etiologies. *Clujul Med, 89*(1), 24-31. doi:10.15386/cjmed-554

Osseman, Q., Gallucci, L., Au, S., Cazenave, C., Berdance, E., Blondot, M. L., . . . Kann, M. (2018). The chaperone dynein LL1 mediates cytoplasmic transport of empty and

mature hepatitis B virus capsids. *J Hepatol, 68*(3), 441-448. doi:10.1016/j.jhep.2017.10.032

Oswald, A., Chakraborty, A., Ni, Y., Wettengel, J. M., Urban, S., & Protzer, U. (2021). Concentration of Na(+)-taurocholate-cotransporting polypeptide expressed after in vitro-transcribed mRNA transfection determines susceptibility of hepatoma cells for hepatitis B virus. *Sci Rep, 11*(1), 19799. doi:10.1038/s41598-021-99263-3

Otsuka, M., & Koike, K. (2020). Should Level of HBV RNA be Used to Determine When Patients Should Stop Treatment With Nucleos(t)ide Analogues. *Clin Gastroenterol Hepatol, 18*(3), 551-552. doi:10.1016/j.cgh.2019.08.044

Papavramidou, N., Fee, E., & Christopoulou-Aletra, H. (2007). Jaundice in the Hippocratic Corpus. *J Gastrointest Surg, 11*(12), 1728-1731. doi:10.1007/s11605-007-0281-1

Parent, R., Marion, M. J., Furio, L., Trepo, C., & Petit, M. A. (2004). Origin and characterization of a human bipotent liver progenitor cell line. *Gastroenterology, 126*(4), 1147-1156. doi:10.1053/j.gastro.2004.01.002

Parikh, P., Ryan, J. D., & Tsochatzis, E. A. (2017). Fibrosis assessment in patients with chronic hepatitis B virus (HBV) infection. *Ann Transl Med, 5*(3), 40. doi:10.21037/atm.2017.01.28

Park, J., & Scherer, P. E. (2012). Adipocyte-derived endotrophin promotes malignant tumor progression. *J Clin Invest, 122*(11), 4243-4256. doi:10.1172/JCI63930

Pellicoro, A., Ramachandran, P., Iredale, J. P., & Fallowfield, J. A. (2014). Liver fibrosis and repair: immune regulation of wound healing in a solid organ. *Nat Rev Immunol, 14*(3), 181-194. doi:10.1038/nri3623

Peneau, C., Imbeaud, S., La Bella, T., Hirsch, T. Z., Caruso, S., Calderaro, J., . . . Zucman-Rossi, J. (2022). Hepatitis B virus integrations promote local and distant oncogenic driver alterations in hepatocellular carcinoma. *Gut, 71*(3), 616-626. doi:10.1136/gutjnl-2020-323153

Peng, W. C., Kraaier, L. J., & Kluiver, T. A. (2021). Hepatocyte organoids and cell transplantation: What the future holds. *Exp Mol Med, 53*(10), 1512-1528. doi:10.1038/s12276-021-00579-x

Perez-Montero, S., Carbonell, A., & Azorin, F. (2016). Germline-specific H1 variants: the "sexy" linker histones. *Chromosoma, 125*(1), 1-13. doi:10.1007/s00412-015-0517-x

Pfaff, M., Aumailley, M., Specks, U., Knolle, J., Zerwes, H. G., & Timpl, R. (1993). Integrin and Arg-Gly-Asp dependence of cell adhesion to the native and unfolded triple helix of collagen type VI. *Exp Cell Res, 206*(1), 167-176. doi:10.1006/excr.1993.1134

Pino, L. K., Rose, J., O'Broin, A., Shah, S., & Schilling, B. (2020). Emerging mass spectrometry-based proteomics methodologies for novel biomedical applications. *Biochem Soc Trans, 48*(5), 1953-1966. doi:10.1042/BST20191091

Poisson, J., Lemoinne, S., Boulanger, C., Durand, F., Moreau, R., Valla, D., & Rautou, P. E. (2017). Liver sinusoidal endothelial cells: Physiology and role in liver diseases. *J Hepatol, 66*(1), 212-227. doi:10.1016/j.jhep.2016.07.009

Pollicino, T., Belloni, L., Raffa, G., Pediconi, N., Squadrito, G., Raimondo, G., & Levrero, M. (2006). Hepatitis B virus replication is regulated by the acetylation status of hepatitis B virus cccDNA-bound H3 and H4 histones. *Gastroenterology, 130*(3), 823-837. doi:10.1053/j.gastro.2006.01.001

Poltavets, V., Kochetkova, M., Pitson, S. M., & Samuel, M. S. (2018). The Role of the Extracellular Matrix and Its Molecular and Cellular Regulators in Cancer Cell Plasticity. *Front Oncol, 8*, 431. doi:10.3389/fonc.2018.00431

Pouri, A. A., Ghojazadeh, M., Baiaz, B., Hamzavi, F. S., Pourasghari, B., & Somi, M. H. (2020). Prevalence of hepatitis D virus among HBsAg-positive individuals, 2015-2016: Azar cohort study. *Health Promot Perspect, 10*(1), 38-42. doi:10.15171/hpp.2020.07

Prendergast, L., & Reinberg, D. (2021). The missing linker: emerging trends for H1 variant-specific functions. *Genes Dev, 35*(1-2), 40-58. doi:10.1101/gad.344531.120

Prince, A. M. (1968). An antigen detected in the blood during the incubation period of serum hepatitis. *Proc Natl Acad Sci U S A, 60*(3), 814-821. doi:10.1073/pnas.60.3.814

Puche, J. E., Saiman, Y., & Friedman, S. L. (2013). Hepatic stellate cells and liver fibrosis. *Compr Physiol, 3*(4), 1473-1492. doi:10.1002/cphy.c120035

Purcell, R. H., & Gerin, J. L. (1975). Hepatitis B subunit vaccine: a preliminary report of safety and efficacy tests in chimpanzees. *Am J Med Sci, 270*(2), 395-399. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/828832

Qi, Y., Gao, Z., Xu, G., Peng, B., Liu, C., Yan, H., . . . Li, W. (2016). DNA Polymerase kappa Is a Key Cellular Factor for the Formation of Covalently Closed Circular DNA of Hepatitis B Virus. *PLoS Pathog, 12*(10), e1005893. doi:10.1371/journal.ppat.1005893

Quarleri, J. (2014). Core promoter: a critical region where the hepatitis B virus makes decisions. *World J Gastroenterol, 20*(2), 425-435. doi:10.3748/wjg.v20.i2.425

Rabe, B., Glebe, D., & Kann, M. (2006). Lipid-mediated introduction of hepatitis B virus capsids into nonsusceptible cells allows highly efficient replication and facilitates the study of early infection events. *J Virol, 80*(11), 5465-5473. doi:10.1128/JVI.02303-05

Rabe, B., Vlachou, A., Pante, N., Helenius, A., & Kann, M. (2003). Nuclear import of hepatitis B virus capsids and release of the viral genome. *Proc Natl Acad Sci U S A, 100*(17), 9849-9854. doi:10.1073/pnas.1730940100

Raffetti, E., Fattovich, G., & Donato, F. (2016). Incidence of hepatocellular carcinoma in untreated subjects with chronic hepatitis B: a systematic review and meta-analysis. *Liver Int, 36*(9), 1239-1251. doi:10.1111/liv.13142

Rawat, S., & Bouchard, M. J. (2015). The hepatitis B virus (HBV) HBx protein activates AKT to simultaneously regulate HBV replication and hepatocyte survival. *J Virol, 89*(2), 999-1012. doi:10.1128/JVI.02440-14

Ren, L., Li, C., Wang, Y., Teng, Y., Sun, H., Xing, B., . . . He, F. (2018). In Vivo Phosphoproteome Analysis Reveals Kinome Reprogramming in Hepatocellular Carcinoma. *Mol Cell Proteomics, 17*(6), 1067-1083. doi:10.1074/mcp.RA117.000421

Rigberg, D. A., Kim, F. S., Sebastian, J. L., Kazanjian, K. K., & McFadden, D. W. (1999). Hypophosphorylated retinoblastoma protein is associated with G2 arrest in esophageal squamous cell carcinoma. *J Surg Res, 84*(1), 101-105. doi:10.1006/jsre.1999.5617

Rizzetto, M., Canese, M. G., Arico, S., Crivelli, O., Trepo, C., Bonino, F., & Verme, G. (1977). Immunofluorescence detection of new antigen-antibody system (delta/anti-delta) associated to hepatitis B virus in liver and in serum of HBsAg carriers. *Gut, 18*(12), 997-1003. doi:10.1136/gut.18.12.997

Rockey, D. C. (2016). Liver Fibrosis Reversion After Suppression of Hepatitis B Virus. *Clin Liver Dis, 20*(4), 667-679. doi:10.1016/j.cld.2016.06.003

Rodbell, M. (1980). The role of hormone receptors and GTP-regulatory proteins in membrane transduction. *Nature, 284*(5751), 17-22. doi:10.1038/284017a0

Rodriguez, M. A., Del Rio Barquero, L. M., Ortez, C. I., Jou, C., Vigo, M., Medina, J., . . . Jimenez-Mallebrera, C. (2017). Differences in Adipose Tissue and Lean Mass Distribution in Patients with Collagen VI Related Myopathies Are Associated with Disease Severity and Physical Ability. *Front Aging Neurosci, 9*, 268. doi:10.3389/fnagi.2017.00268

Roehlen, N., Crouchet, E., & Baumert, T. F. (2020). Liver Fibrosis: Mechanistic Concepts and Therapeutic Perspectives. *Cells, 9*(4). doi:10.3390/cells9040875

Roggendorf, M., Schulte, I., Xu, Y., & Lu, M. (2007). Therapeutic vaccination in chronic hepatitis B: preclinical studies in the woodchuck model. *J Viral Hepat, 14 Suppl 1*, 51-57. doi:10.1111/j.1365-2893.2007.00914.x

Rokuhara, A., Matsumoto, A., Tanaka, E., Umemura, T., Yoshizawa, K., Kimura, T., . . . Kiyosawa, K. (2006). Hepatitis B virus RNA is measurable in serum and can be a new marker for monitoring lamivudine therapy. *J Gastroenterol, 41*(8), 785-790. doi:10.1007/s00535-006-1856-4

Ronnow, S. R., Langholm, L. L., Karsdal, M. A., Manon-Jensen, T., Tal-Singer, R., Miller, B. E., . . . Sand, J. M. B. (2020). Endotrophin, an extracellular hormone, in combination with neoepitope markers of von Willebrand factor improves prediction of mortality in

the ECLIPSE COPD cohort. *Respir Res, 21*(1), 202. doi:10.1186/s12931-020-01461-6

Roque, A., Ponte, I., Arrondo, J. L., & Suau, P. (2008). Phosphorylation of the carboxy-terminal domain of histone H1: effects on secondary structure and DNA condensation. *Nucleic Acids Res, 36*(14), 4719-4726. doi:10.1093/nar/gkn440

Roudot-Thoraval, F. (2021). Epidemiology of hepatitis C virus infection. *Clin Res Hepatol Gastroenterol, 45*(3), 101596. doi:10.1016/j.clinre.2020.101596

Ruan, P., Yang, C., Su, J., Cao, J., Ou, C., Luo, C., . . . Li, Y. (2013). Histopathological changes in the liver of tree shrew (Tupaia belangeri chinensis) persistently infected with hepatitis B virus. *Virol J, 10*, 333. doi:10.1186/1743-422X-10-333

Ruhl, M., Sahin, E., Johannsen, M., Somasundaram, R., Manski, D., Riecken, E. O., & Schuppan, D. (1999). Soluble collagen VI drives serum-starved fibroblasts through S phase and prevents apoptosis via down-regulation of Bax. *J Biol Chem, 274*(48), 34361-34368. doi:10.1074/jbc.274.48.34361

Rumin, S., Gripon, P., Le Seyec, J., Corral-Debrinski, M., & Guguen-Guillouzo, C. (1996). Long-term productive episomal hepatitis B virus replication in primary cultures of adult human hepatocytes infected in vitro. *J Viral Hepat, 3*(5), 227-238. doi:10.1111/j.1365-2893.1996.tb00048.x

Saha, A., Seward, C. H., Stubbs, L., & Mizzen, C. A. (2020). Site-Specific Phosphorylation of Histone H1.4 Is Associated with Transcription Activation. *Int J Mol Sci, 21*(22). doi:10.3390/ijms21228861

Sarg, B., Helliger, W., Talasz, H., Forg, B., & Lindner, H. H. (2006). Histone H1 phosphorylation occurs site-specifically during interphase and mitosis: identification of a novel phosphorylation site on histone H1. *J Biol Chem, 281*(10), 6573-6580. doi:10.1074/jbc.M508957200

Saviano, A., Roehlen, N., Virzi, A., Roca Suarez, A. A., Hoshida, Y., Lupberger, J., & Baumert, T. F. (2019). Stromal and Immune Drivers of Hepatocarcinogenesis. In Y. Hoshida (Ed.), *Hepatocellular Carcinoma: Translational Precision Medicine Approaches* (pp. 317-331). Cham (CH).

Schiller, H. B., Fernandez, I. E., Burgstaller, G., Schaab, C., Scheltema, R. A., Schwarzmayr, T., . . . Mann, M. (2015). Time- and compartment-resolved proteome profiling of the extracellular niche in lung injury and repair. *Mol Syst Biol, 11*(7), 819. doi:10.15252/msb.20156123

Schnoor, M., Cullen, P., Lorkowski, J., Stolle, K., Robenek, H., Troyer, D., . . . Lorkowski, S. (2008). Production of type VI collagen by human macrophages: a new dimension in macrophage functional heterogeneity. *J Immunol, 180*(8), 5707-5719. doi:10.4049/jimmunol.180.8.5707

Schubeler, D. (2015). Function and information content of DNA methylation. *Nature, 517*(7534), 321-326. doi:10.1038/nature14192

Seck, A., Ndiaye, F., Maylin, S., Ndiaye, B., Simon, F., Funk, A. L., . . . Shimakawa, Y. (2018). Poor Sensitivity of Commercial Rapid Diagnostic Tests for Hepatitis B e Antigen in Senegal, West Africa. *Am J Trop Med Hyg, 99*(2), 428-434. doi:10.4269/ajtmh.18-0116

Seeff, L. B., Wright, E. C., Zimmerman, H. J., Alter, H. J., Dietz, A. A., Felsher, B. F., . . . Zimmon, D. S. (1978). Type B hepatitis after needle-stick exposure: prevention with hepatitis B immune globulin. Final report of the Veterans Administration Cooperative Study. *Ann Intern Med, 88*(3), 285-293. doi:10.7326/0003-4819-88-3-285

Seeger, C., Ganem, D., & Varmus, H. E. (1986). Biochemical and genetic evidence for the hepatitis B virus replication strategy. *Science, 232*(4749), 477-484. doi:10.1126/science.3961490

Seifer, M., & Standring, D. N. (1995). Ribonucleoprotein complex formation by the human hepatitis B virus polymerase. *Intervirology, 38*(5), 295-303. doi:10.1159/000150454

Sells, M. A., Chen, M. L., & Acs, G. (1987). Production of hepatitis B virus particles in Hep G2 cells transfected with cloned hepatitis B virus DNA. *Proc Natl Acad Sci U S A, 84*(4), 1005-1009. doi:10.1073/pnas.84.4.1005

Shahin, M., Schuppan, D., Waldherr, R., Risteli, J., Risteli, L., Savolainen, E. R., . . . et al. (1992). Serum procollagen peptides and collagen type VI for the assessment of activity and degree of hepatic fibrosis in schistosomiasis and alcoholic liver disease. *Hepatology, 15*(4), 637-644. doi:10.1002/hep.1840150414

Shimura, S., Watashi, K., Fukano, K., Peel, M., Sluder, A., Kawai, F., . . . Wakita, T. (2017). Cyclosporin derivatives inhibit hepatitis B virus entry without interfering with NTCP transporter activity. *J Hepatol, 66*(4), 685-692. doi:10.1016/j.jhep.2016.11.009

Slack, R. J., Macdonald, S. J. F., Roper, J. A., Jenkins, R. G., & Hatley, R. J. D. (2022). Emerging therapeutic opportunities for integrin inhibitors. *Nat Rev Drug Discov, 21*(1), 60-78. doi:10.1038/s41573-021-00284-4

Smolders, E. J., Burger, D. M., Feld, J. J., & Kiser, J. J. (2020). Review article: clinical pharmacology of current and investigational hepatitis B virus therapies. *Aliment Pharmacol Ther, 51*(2), 231-243. doi:10.1111/apt.15581

Sonderup, M. W., & Spearman, C. W. (2022). Global Disparities in Hepatitis B Elimination-A Focus on Africa. *Viruses, 14*(1). doi:10.3390/v14010082

Soyer, O. S., & Bonhoeffer, S. (2006). Evolution of complexity in signaling pathways. *Proc Natl Acad Sci U S A, 103*(44), 16337-16342. doi:10.1073/pnas.0604449103

Specks, U., Mayer, U., Nischt, R., Spissinger, T., Mann, K., Timpl, R., . . . Chu, M. L. (1992). Structure of recombinant N-terminal globule of type VI collagen alpha 3 chain and its binding to heparin and hyaluronan. *EMBO J, 11*(12), 4281-4290. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/1425570

Specks, U., Nerlich, A., Colby, T. V., Wiest, I., & Timpl, R. (1995). Increased expression of type VI collagen in lung fibrosis. *Am J Respir Crit Care Med, 151*(6), 1956-1964. doi:10.1164/ajrccm.151.6.7767545

Stallcup, W. B., Dahlin, K., & Healy, P. (1990). Interaction of the NG2 chondroitin sulfate proteoglycan with type VI collagen. *J Cell Biol, 111*(6 Pt 2), 3177-3188. doi:10.1083/jcb.111.6.3177

Sterling, R. K., Lissen, E., Clumeck, N., Sola, R., Correa, M. C., Montaner, J., . . . Investigators, A. C. (2006). Development of a simple noninvasive index to predict significant fibrosis in patients with HIV/HCV coinfection. *Hepatology, 43*(6), 1317-1325. doi:10.1002/hep.21178

Stickel, F., Urbaschek, R., Schuppan, D., Poeschl, G., Oesterling, C., Conradt, C., . . . Seitz, H. K. (2001). Serum collagen type VI and XIV and hyaluronic acid as early indicators for altered connective tissue turnover in alcoholic liver disease. *Dig Dis Sci, 46*(9), 2025-2032. doi:10.1023/a:1010616021659

Summers, J., O'Connell, A., & Millman, I. (1975). Genome of hepatitis B virus: restriction enzyme cleavage and structure of DNA extracted from Dane particles. *Proc Natl Acad Sci U S A, 72*(11), 4597-4601. doi:10.1073/pnas.72.11.4597

Sun, K., Park, J., Gupta, O. T., Holland, W. L., Auerbach, P., Zhang, N., . . . Scherer, P. E. (2014). Endotrophin triggers adipose tissue fibrosis and metabolic dysfunction. *Nat Commun, 5*, 3485. doi:10.1038/ncomms4485

Sun, Y., Wu, X., Zhou, J., Meng, T., Wang, B., Chen, S., . . . You, H. (2020). Persistent Low Level of Hepatitis B Virus Promotes Fibrosis Progression During Therapy. *Clin Gastroenterol Hepatol, 18*(11), 2582-2591 e2586. doi:10.1016/j.cgh.2020.03.001

Sun, Y., Zhou, J., Wu, X., Chen, Y., Piao, H., Lu, L., . . . You, H. (2018). Quantitative assessment of liver fibrosis (qFibrosis) reveals precise outcomes in Ishak "stable" patients on anti-HBV therapy. *Sci Rep, 8*(1), 2989. doi:10.1038/s41598-018-21179-2

Sunbul, M. (2014). Hepatitis B virus genotypes: global distribution and clinical importance. *World J Gastroenterol, 20*(18), 5427-5434. doi:10.3748/wjg.v20.i18.5427

Sureau, C., Romet-Lemonne, J. L., Mullins, J. I., & Essex, M. (1986). Production of hepatitis B virus by a differentiated human hepatoma cell line after transfection with cloned circular HBV DNA. *Cell, 47*(1), 37-47. doi:10.1016/0092-8674(86)90364-8

Sureau, C., & Salisse, J. (2013). A conformational heparan sulfate binding site essential to infectivity overlaps with the conserved hepatitis B virus a-determinant. *Hepatology, 57*(3), 985-994. doi:10.1002/hep.26125

Suslov, A., Boldanova, T., Wang, X., Wieland, S., & Heim, M. H. (2018). Hepatitis B Virus Does Not Interfere With Innate Immune Responses in the Human Liver. *Gastroenterology, 154*(6), 1778-1790. doi:10.1053/j.gastro.2018.01.034

Sutherland, F., & Harris, J. (2002). Claude Couinaud: a passion for the liver. *Arch Surg, 137*(11), 1305-1310. doi:10.1001/archsurg.137.11.1305

Sutnick, A. I., Millman, I., London, W. T., & Blumberg, B. S. (1972). The role of Australia antigen in viral hepatitis and other diseases. *Annu Rev Med, 23*, 161-176. doi:10.1146/annurev.me.23.020172.001113

Suzuki, F., Miyakoshi, H., Kobayashi, M., & Kumada, H. (2009). Correlation between serum hepatitis B virus core-related antigen and intrahepatic covalently closed circular DNA in chronic hepatitis B patients. *J Med Virol, 81*(1), 27-33. doi:10.1002/jmv.21339

Szmuness, W., Stevens, C. E., Zang, E. A., Harley, E. J., & Kellner, A. (1981). A controlled clinical trial of the efficacy of the hepatitis B vaccine (Heptavax B): a final report. *Hepatology, 1*(5), 377-385. doi:10.1002/hep.1840010502

Tada, T., Kumada, T., Toyoda, H., Kobayashi, N., Akita, T., & Tanaka, J. (2018). Hepatitis B virus core-related antigen levels predict progression to liver cirrhosis in hepatitis B carriers. *J Gastroenterol Hepatol, 33*(4), 918-925. doi:10.1111/jgh.13989

Takahara, T., Furui, K., Yata, Y., Jin, B., Zhang, L. P., Nambu, S., . . . Watanabe, A. (1997). Dual expression of matrix metalloproteinase-2 and membrane-type 1-matrix metalloproteinase in fibrotic human livers. *Hepatology, 26*(6), 1521-1529. doi:10.1002/hep.510260620

Takahara, T., Sollberg, S., Muona, P., & Uitto, J. (1995). Type VI collagen gene expression in experimental liver fibrosis: quantitation and spatial distribution of mRNAs, and immunodetection of the protein. *Liver, 15*(2), 78-86. doi:10.1111/j.1600-0676.1995.tb00111.x

Takahashi, K., Machida, A., Funatsu, G., Nomura, M., Usuda, S., Aoyagi, S., . . . Mayumi, M. (1983). Immunochemical structure of hepatitis B e antigen in the serum. *J Immunol, 130*(6), 2903-2907. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/6189903

Tan, Q., Chen, J., Gao, G., Yizhang, Chen, X., Yu, Y., . . . Tang, Z. (2022). Adenovirus vector encoding TPPII ignites HBV-specific CTL response by activating autophagy in CD8+ T cell. *J Viral Hepat, 29*(3), 178-188. doi:10.1111/jvh.13638

Tan, Z., Liu, Q., Jiang, R., Lv, L., Shoto, S. S., Maillet, I., . . . Ryffel, B. (2018). Interleukin-33 drives hepatic fibrosis through activation of hepatic stellate cells. *Cell Mol Immunol, 15*(4), 388-398. doi:10.1038/cmi.2016.63

Tang, H., & Xue, G. (2017). Major physiological signaling pathways in the regulation of cell proliferation and survival. In *Mechanisms of Drug Resistance in Cancer Therapy* (pp. 13-30): Springer.

Tang, L., Sheraz, M., McGrane, M., Chang, J., & Guo, J. T. (2019). DNA Polymerase alpha is essential for intracellular amplification of hepatitis B virus covalently closed circular DNA. *PLoS Pathog, 15*(4), e1007742. doi:10.1371/journal.ppat.1007742

Tantiwetrueangdet, A., Panvichian, R., Sornmayura, P., Sueangoen, N., & Leelaudomlipi, S. (2018). Reduced HBV cccDNA and HBsAg in HBV-associated hepatocellular carcinoma tissues. *Med Oncol, 35*(10), 127. doi:10.1007/s12032-018-1191-7

Tao, Y., Wu, D., Zhou, L., Chen, E., Liu, C., Tang, X., . . . Tang, H. (2020). Present and Future Therapies for Chronic Hepatitis B. *Adv Exp Med Biol, 1179*, 137-186. doi:10.1007/978-981-13-9151-4_6

Terrault, N. A., Lok, A. S. F., McMahon, B. J., Chang, K. M., Hwang, J. P., Jonas, M. M., . . . Wong, J. B. (2018). Update on prevention, diagnosis, and treatment of chronic hepatitis B: AASLD 2018 hepatitis B guidance. *Hepatology, 67*(4), 1560-1599. doi:10.1002/hep.29800

Teshale, E., Lu, M., Rupp, L. B., Holmberg, S. D., Moorman, A. C., Spradling, P., . . . Investigators, C. H. (2014). APRI and FIB-4 are good predictors of the stage of liver fibrosis in chronic hepatitis B: the Chronic Hepatitis Cohort Study (CHeCS). *J Viral Hepat, 21*(12), 917-920. doi:10.1111/jvh.12279

Testoni, B., Lebosse, F., Scholtes, C., Berby, F., Miaglia, C., Subic, M., . . . Zoulim, F. (2019). Serum hepatitis B core-related antigen (HBcrAg) correlates with covalently closed circular DNA transcriptional activity in chronic hepatitis B patients. *J Hepatol, 70*(4), 615-625. doi:10.1016/j.jhep.2018.11.030

Thimme, R., Wieland, S., Steiger, C., Ghrayeb, J., Reimann, K. A., Purcell, R. H., & Chisari, F. V. (2003). CD8(+) T cells mediate viral clearance and disease pathogenesis during acute hepatitis B virus infection. *J Virol, 77*(1), 68-76. doi:10.1128/jvi.77.1.68-76.2003

Thomas, D. L. (2019). Global Elimination of Chronic Hepatitis. *N Engl J Med, 380*(21), 2041-2050. doi:10.1056/NEJMra1810477

Thomas, E., Yoneda, M., & Schiff, E. R. (2015). Viral hepatitis: past and future of HBV and HDV. *Cold Spring Harb Perspect Med, 5*(2), a021345. doi:10.1101/cshperspect.a021345

Thomas, R. E., Lorenzetti, D. L., & Spragins, W. (2013). Mortality and morbidity among military personnel and civilians during the 1930s and World War II from transmission of hepatitis during yellow fever vaccination: systematic review. *Am J Public Health, 103*(3), e16-29. doi:10.2105/AJPH.2012.301158

Tillet, E., Gential, B., Garrone, R., & Stallcup, W. B. (2002). NG2 proteoglycan mediates beta1 integrin-independent cell adhesion and spreading on collagen VI. *J Cell Biochem, 86*(4), 726-736. doi:10.1002/jcb.10268

Tillet, E., Wiedemann, H., Golbik, R., Pan, T. C., Zhang, R. Z., Mann, K., . . . Timpl, R. (1994). Recombinant expression and structural and binding properties of alpha 1(VI) and alpha 2(VI) chains of human collagen type VI. *Eur J Biochem, 221*(1), 177-185. doi:10.1111/j.1432-1033.1994.tb18727.x

Torresi, J., Tran, B. M., Christiansen, D., Earnest-Silveira, L., Schwab, R. H. M., & Vincan, E. (2019). HBV-related hepatocarcinogenesis: the role of signalling pathways and innovative ex vivo research models. *BMC Cancer, 19*(1), 707. doi:10.1186/s12885-019-5916-6

Tout, I., Lampertico, P., Berg, T., & Asselah, T. (2021). Perspectives on stopping nucleos(t)ide analogues therapy in patients with chronic hepatitis B. *Antiviral Res, 185*, 104992. doi:10.1016/j.antiviral.2020.104992

Tout, I., Loureiro, D., & Asselah, T. (2021). The Changing Demographics of Hepatitis B Virus Infection. *Clin Liver Dis, 25*(4), 673-687. doi:10.1016/j.cld.2021.06.001

Tout, I., Loureiro, D., Mansouri, A., Soumelis, V., Boyer, N., & Asselah, T. (2020). Hepatitis B surface antigen seroclearance: Immune mechanisms, clinical impact, importance for drug development. *J Hepatol, 73*(2), 409-422. doi:10.1016/j.jhep.2020.04.013

Tropberger, P., Mercier, A., Robinson, M., Zhong, W., Ganem, D. E., & Holdorf, M. (2015). Mapping of histone modifications in episomal HBV cccDNA uncovers an unusual chromatin organization amenable to epigenetic manipulation. *Proc Natl Acad Sci U S A, 112*(42), E5715-5724. doi:10.1073/pnas.1518090112

Tsai, E. (2021). Review of Current and Potential Treatments for Chronic Hepatitis B Virus Infection. *Gastroenterol Hepatol (N Y), 17*(8), 367-376. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/34602899

Tsai, W. L., & Chung, R. T. (2010). Viral hepatocarcinogenesis. *Oncogene, 29*(16), 2309-2324. doi:10.1038/onc.2010.36

Tsounis, E. P., Tourkochristou, E., Mouzaki, A., & Triantos, C. (2021). Toward a new era of hepatitis B virus therapeutics: The pursuit of a functional cure. *World J Gastroenterol, 27*(21), 2727-2757. doi:10.3748/wjg.v27.i21.2727

Tsuchida, T., & Friedman, S. L. (2017). Mechanisms of hepatic stellate cell activation. *Nat Rev Gastroenterol Hepatol, 14*(7), 397-411. doi:10.1038/nrgastro.2017.38

Tsukuda, S., & Watashi, K. (2020). Hepatitis B virus biology and life cycle. *Antiviral Res, 182*, 104925. doi:10.1016/j.antiviral.2020.104925

Tu, T., Zehnder, B., Qu, B., & Urban, S. (2021). D e novo synthesis of hepatitis B virus nucleocapsids is dispensable for the maintenance and transcriptional regulation of cccDNA. *JHEP Rep, 3*(1), 100195. doi:10.1016/j.jhepr.2020.100195

Tulla, M., Pentikainen, O. T., Viitasalo, T., Kapyla, J., Impola, U., Nykvist, P., . . . Heino, J. (2001). Selective binding of collagen subtypes by integrin alpha 1I, alpha 2I, and alpha 10I domains. *J Biol Chem, 276*(51), 48206-48212. doi:10.1074/jbc.M104058200

Turker, M. S. (2002). Gene silencing in mammalian cells and the spread of DNA methylation. *Oncogene, 21*(35), 5388-5393. doi:10.1038/sj.onc.1205599

Turner, A. L., Watson, M., Wilkins, O. G., Cato, L., Travers, A., Thomas, J. O., & Stott, K. (2018). Highly disordered histone H1-DNA model complexes and their condensates. *Proc Natl Acad Sci U S A, 115*(47), 11964-11969. doi:10.1073/pnas.1805943115

Turtoi, A., Blomme, A., Bianchi, E., Maris, P., Vannozzi, R., Naccarato, A. G., . . . Castronovo, V. (2014). Accessibilome of human glioblastoma: collagen-VI-alpha-1 is a new target and a marker of poor outcome. *J Proteome Res, 13*(12), 5660-5669. doi:10.1021/pr500657w

Tuttleman, J. S., Pourcel, C., & Summers, J. (1986). Formation of the pool of covalently closed circular viral DNA in hepadnavirus-infected cells. *Cell, 47*(3), 451-460. doi:10.1016/0092-8674(86)90602-1

Vallet-Pichard, A., Mallet, V., Nalpas, B., Verkarre, V., Nalpas, A., Dhalluin-Venier, V., . . . Pol, S. (2007). FIB-4: an inexpensive and accurate marker of fibrosis in HCV infection. comparison with liver biopsy and fibrotest. *Hepatology, 46*(1), 32-36. doi:10.1002/hep.21669

van de Klundert, M. A., Zaaijer, H. L., & Kootstra, N. A. (2016). Identification of FDA-approved drugs that target hepatitis B virus transcription. *J Viral Hepat, 23*(3), 191-201. doi:10.1111/jvh.12479

van den Berg, F., Limani, S. W., Mnyandu, N., Maepa, M. B., Ely, A., & Arbuthnot, P. (2020). Advances with RNAi-Based Therapy for Hepatitis B Virus Infection. *Viruses, 12*(8). doi:10.3390/v12080851

Van Renne, N., Roca Suarez, A. A., Duong, F. H. T., Gondeau, C., Calabrese, D., Fontaine, N., . . . Lupberger, J. (2018). miR-135a-5p-mediated downregulation of protein tyrosine phosphatase receptor delta is a candidate driver of HCV-associated hepatocarcinogenesis. *Gut, 67*(5), 953-962. doi:10.1136/gutjnl-2016-312270

Veidal, S. S., Karsdal, M. A., Vassiliadis, E., Nawrocki, A., Larsen, M. R., Nguyen, Q. H., . . . Leeming, D. J. (2011). MMP mediated degradation of type VI collagen is highly associated with liver fibrosis--identification and validation of a novel biochemical marker assay. *PLoS One, 6*(9), e24753. doi:10.1371/journal.pone.0024753

Vermeulen, K., Van Bockstaele, D. R., & Berneman, Z. N. (2003). The cell cycle: a review of regulation, deregulation and therapeutic targets in cancer. *Cell Prolif, 36*(3), 131-149. doi:10.1046/j.1365-2184.2003.00266.x

Vernon, H., Wehrle, C. J., & Kasi, A. (2022). Anatomy, Abdomen and Pelvis, Liver. In *StatPearls*. Treasure Island (FL).

Verrier, E. R., Colpitts, C. C., Bach, C., Heydmann, L., Weiss, A., Renaud, M., . . . Baumert, T. F. (2016). A targeted functional RNA interference screen uncovers glypican 5 as an entry factor for hepatitis B and D viruses. *Hepatology, 63*(1), 35-48. doi:10.1002/hep.28013

Verrier, E. R., Colpitts, C. C., Schuster, C., Zeisel, M. B., & Baumert, T. F. (2016). Cell Culture Models for the Investigation of Hepatitis B and D Virus Infection. *Viruses, 8*(9). doi:10.3390/v8090261

Verrier, E. R., Yim, S. A., Heydmann, L., El Saghire, H., Bach, C., Turon-Lagot, V., . . . Baumert, T. F. (2018). Hepatitis B Virus Evasion From Cyclic Guanosine Monophosphate-Adenosine Monophosphate Synthase Sensing in Human Hepatocytes. *Hepatology, 68*(5), 1695-1709. doi:10.1002/hep.30054

Vicent, G. P., Nacht, A. S., Font-Mateu, J., Castellano, G., Gaveglia, L., Ballare, C., & Beato, M. (2011). Four enzymes cooperate to displace histone H1 during the first minute of hormonal gene activation. *Genes Dev, 25*(8), 845-862. doi:10.1101/gad.621811

Virzi, A., Gonzalez-Motos, V., Tripon, S., Baumert, T. F., & Lupberger, J. (2021). Profibrotic Signaling and HCC Risk during Chronic Viral Hepatitis: Biomarker Development. *J Clin Med, 10*(5). doi:10.3390/jcm10050977

Virzi, A., Roca Suarez, A. A., Baumert, T. F., & Lupberger, J. (2020). Rewiring Host Signaling: Hepatitis C Virus in Liver Pathogenesis. *Cold Spring Harb Perspect Med, 10*(1). doi:10.1101/cshperspect.a037366

Vivekanandan, P., Daniel, H. D., Kannangai, R., Martinez-Murillo, F., & Torbenson, M. (2010). Hepatitis B virus replication induces methylation of both host and viral DNA. *J Virol, 84*(9), 4321-4329. doi:10.1128/JVI.02280-09

Vivekanandan, P., Thomas, D., & Torbenson, M. (2009). Methylation regulates hepatitis B viral protein expression. *J Infect Dis, 199*(9), 1286-1291. doi:10.1086/597614

Wagai, N., Yamaguchi, F., Sekiguchi, M., & Tawara, K. (1990). Phototoxic potential of quinolone antibacterial agents in Balb/c mice. *Toxicol Lett, 54*(2-3), 299-308. doi:10.1016/0378-4274(90)90197-t

Wai, C. T., Greenson, J. K., Fontana, R. J., Kalbfleisch, J. D., Marrero, J. A., Conjeevaram, H. S., & Lok, A. S. (2003). A simple noninvasive index can predict both significant fibrosis and cirrhosis in patients with chronic hepatitis C. *Hepatology, 38*(2), 518-526. doi:10.1053/jhep.2003.50346

Wake, K. (1971). "Sternzellen" in the liver: perisinusoidal cells with special reference to storage of vitamin A. *Am J Anat, 132*(4), 429-462. doi:10.1002/aja.1001320404

Walter, E., Keist, R., Niederost, B., Pult, I., & Blum, H. E. (1996). Hepatitis B virus infection of tupaia hepatocytes in vitro and in vivo. *Hepatology, 24*(1), 1-5. doi:10.1002/hep.510240101

Wang, J., Huang, H., Liu, Y., Chen, R., Yan, Y., Shi, S., . . . Lu, F. (2020). HBV Genome and Life Cycle. *Adv Exp Med Biol, 1179*, 17-37. doi:10.1007/978-981-13-9151-4_2

Wang, J., Shen, T., Huang, X., Kumar, G. R., Chen, X., Zeng, Z., . . . Lu, F. (2016). Serum hepatitis B virus RNA is encapsidated pregenome RNA that may be associated with persistence of viral infection and rebound. *J Hepatol, 65*(4), 700-710. doi:10.1016/j.jhep.2016.05.029

Wang, M., Gong, Q., Zhang, J., Chen, L., Zhang, Z., Lu, L., . . . Zhang, X. (2017). Characterization of gene expression profiles in HBV-related liver fibrosis patients and identification of ITGBL1 as a key regulator of fibrogenesis. *Sci Rep, 7*, 43446. doi:10.1038/srep43446

Wang, T., Zhao, R., Wu, Y., Kong, D., Zhang, L., Wu, D., . . . Jin, X. (2011). Hepatitis B virus induces G1 phase arrest by regulating cell cycle genes in HepG2.2.15 cells. *Virol J, 8*, 231. doi:10.1186/1743-422X-8-231

Wang, X., Bie, L., & Gao, J. (2022). Structural Insights into the Cofactor Role of Heparin/Heparan Sulfate in Binding between the SARS-CoV-2 Spike Protein and Host Angiotensin-Converting Enzyme II. *J Chem Inf Model, 62*(3), 656-667. doi:10.1021/acs.jcim.1c01484

Wang, X., Lin, Y., Kemper, T., Chen, J., Yuan, Z., Liu, S., . . . Lu, M. (2020). AMPK and Akt/mTOR signalling pathways participate in glucose-mediated regulation of hepatitis B virus replication and cellular autophagy. *Cell Microbiol, 22*(2), e13131. doi:10.1111/cmi.13131

Wang, X., Wei, Z., Jiang, Y., Meng, Z., & Lu, M. (2021). mTOR Signaling: The Interface Linking Cellular Metabolism and Hepatitis B Virus Replication. *Virol Sin, 36*(6), 1303-1314. doi:10.1007/s12250-021-00450-3

Wang, Y., Li, Y., Zai, W., Hu, K., Zhu, Y., Deng, Q., . . . Yuan, Z. (2021). HBV covalently closed circular DNA minichromosomes in distinct epigenetic transcriptional states differ in their vulnerability to damage. *Hepatology*. doi:10.1002/hep.32245

Wang, Y., & Tao, Q. (1990). [Function of direct repeat sequences in DNA replication of hepatitis B virus]. *Zhonghua Yi Xue Za Zhi, 70*(11), 604-607, 642. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/1963348

Wang, Z., Wang, W., & Wang, L. (2020). Epigenetic regulation of covalently closed circular DNA minichromosome in hepatitis B virus infection. *Biophysics Reports, 6*(4), 115-126. doi:10.1007/s41048-020-00112-z

Wei, L., & Ploss, A. (2021). Hepatitis B virus cccDNA is formed through distinct repair processes of each strand. *Nat Commun, 12*(1), 1591. doi:10.1038/s41467-021-21850-9

Wei, Z. Q., Zhang, Y. H., Ke, C. Z., Chen, H. X., Ren, P., He, Y. L., . . . Meng, Z. J. (2017). Curcumin inhibits hepatitis B virus infection by down-regulating cccDNA-bound histone acetylation. *World J Gastroenterol, 23*(34), 6252-6260. doi:10.3748/wjg.v23.i34.6252

Weiskirchen, R., & Gressner, A. M. (2005). Isolation and culture of hepatic stellate cells. *Methods Mol Med, 117*, 99-113. doi:10.1385/1-59259-940-0:099

Weng, Y., Lieberthal, T. J., Zhou, V. X., Lopez-Ichikawa, M., Armas-Phan, M., Bond, T. K., . . . Chang, T. T. (2020). Liver epithelial focal adhesion kinase modulates fibrogenesis and hedgehog signaling. *JCI Insight, 5*(20). doi:10.1172/jci.insight.141217

Werner, E., & Werb, Z. (2002). Integrins engage mitochondrial function for signal transduction by a mechanism dependent on Rho GTPases. *J Cell Biol, 158*(2), 357-368. doi:10.1083/jcb.200111028

Whitefield, D. B., Spagnol, S. T., Armiger, T. J., Lan, L., & Dahl, K. N. (2018). Quantifying site-specific chromatin mechanics and DNA damage response. *Sci Rep, 8*(1), 18084. doi:10.1038/s41598-018-36343-x

Whittaker, S., Marais, R., & Zhu, A. X. (2010). The role of signaling pathways in the development and treatment of hepatocellular carcinoma. *Oncogene, 29*(36), 4989-5005. doi:10.1038/onc.2010.236

WHO. (2015). *Guidelines for the prevention care and treatment of persons with chronic hepatitis B infection: Mar-15*: World Health Organization.

WHO. (2017). *Global hepatitis report 2017*: World Health Organization.

WHO. (2021). Global progress report on HIV, viral hepatitis and sexually transmitted infections, 2021: accountability for the global health sector strategies 2016–2021: actions for impact: web annex 2: data methods.

Wieland, S., Thimme, R., Purcell, R. H., & Chisari, F. V. (2004). Genomic analysis of the host response to hepatitis B virus infection. *Proc Natl Acad Sci U S A, 101*(17), 6669-6674. doi:10.1073/pnas.0401771101

Williams, L., Layton, T., Yang, N., Feldmann, M., & Nanchahal, J. (2021). Collagen VI as a driver and disease biomarker in human fibrosis. *FEBS J*. doi:10.1111/febs.16039

Williams, L. M., McCann, F. E., Cabrita, M. A., Layton, T., Cribbs, A., Knezevic, B., . . . Nanchahal, J. (2020). Identifying collagen VI as a target of fibrotic diseases regulated by CREBBP/EP300. *Proc Natl Acad Sci U S A, 117*(34), 20753-20763. doi:10.1073/pnas.2004281117

Willumsen, N., Bager, C., & Karsdal, M. A. (2019). Matrix Metalloprotease Generated Fragments of Type VI Collagen Have Serum Biomarker Potential in Cancer - A Proof of Concept Study. *Transl Oncol, 12*(5), 693-698. doi:10.1016/j.tranon.2019.02.004

Wishart, A. L., Conner, S. J., Guarin, J. R., Fatherree, J. P., Peng, Y., McGinn, R. A., . . . Oudin, M. J. (2020). Decellularized extracellular matrix scaffolds identify full-length collagen VI as a driver of breast cancer cell invasion in obesity and metastasis. *Sci Adv, 6*(43). doi:10.1126/sciadv.abc3175

Wong, D. K., Cheung, A. M., O'Rourke, K., Naylor, C. D., Detsky, A. S., & Heathcote, J. (1993). Effect of alpha-interferon treatment in patients with hepatitis B e antigen-positive chronic hepatitis B. A meta-analysis. *Ann Intern Med, 119*(4), 312-323. doi:10.7326/0003-4819-119-4-199308150-00011

Wong, G. L. (2018). Management of chronic hepatitis B patients in immunetolerant phase: what latest guidelines recommend. *Clin Mol Hepatol, 24*(2), 108-113. doi:10.3350/cmh.2017.0068

World Health, O. (2019). Hepatitis B vaccines: WHO position paper, July 2017 - Recommendations. *Vaccine, 37*(2), 223-225. doi:10.1016/j.vaccine.2017.07.046

Wose Kinge, C. N., Bhoola, N. H., & Kramvis, A. (2020). In Vitro Systems for Studying Different Genotypes/Sub-Genotypes of Hepatitis B Virus: Strengths and Limitations. *Viruses, 12*(3). doi:10.3390/v12030353

Wu, S., Chen, X., Tang, Y., Zhang, Y., Li, D., Chen, J., . . . Yu, Y. (2018). Delivery of Tapasin-modified CTL epitope peptide via cytoplasmic transduction peptide induces CTLs by JAK/STAT signaling pathway in vivo. *Acta Biochim Biophys Sin (Shanghai), 50*(2), 181-190. doi:10.1093/abbs/gmx133

WuDunn, D., & Spear, P. G. (1989). Initial interaction of herpes simplex virus with cells is binding to heparan sulfate. *J Virol, 63*(1), 52-58. doi:10.1128/JVI.63.1.52-58.1989

Xia, Y., Cheng, X., Li, Y., Valdez, K., Chen, W., & Liang, T. J. (2018). Hepatitis B Virus Deregulates the Cell Cycle To Promote Viral Replication and a Premalignant Phenotype. *J Virol, 92*(19). doi:10.1128/JVI.00722-18

Xia, Y., Stadler, D., Lucifora, J., Reisinger, F., Webb, D., Hosel, M., . . . Protzer, U. (2016). Interferon-gamma and Tumor Necrosis Factor-alpha Produced by T Cells Reduce the HBV Persistence Form, cccDNA, Without Cytolysis. *Gastroenterology, 150*(1), 194-205. doi:10.1053/j.gastro.2015.09.026

Xiang, K., & Wang, B. (2018). Role of the PI3KAKTmTOR pathway in hepatitis B virus infection and replication. *Mol Med Rep, 17*(3), 4713-4719. doi:10.3892/mmr.2018.8395

Xie, G., & Diehl, A. M. (2013). Evidence for and against epithelial-to-mesenchymal transition in the liver. *Am J Physiol Gastrointest Liver Physiol, 305*(12), G881-890. doi:10.1152/ajpgi.00289.2013

Xie, N., Yuan, K., Zhou, L., Wang, K., Chen, H. N., Lei, Y., . . . Huang, C. (2016). PRKAA/AMPK restricts HBV replication through promotion of autophagic degradation. *Autophagy, 12*(9), 1507-1520. doi:10.1080/15548627.2016.1191857

Xu, C., Chen, J., & Chen, X. (2021). Host Innate Immunity Against Hepatitis Viruses and Viral Immune Evasion. *Front Microbiol, 12*, 740464. doi:10.3389/fmicb.2021.740464

Yan, H., Zhong, G., Xu, G., He, W., Jing, Z., Gao, Z., . . . Li, W. (2012). Sodium taurocholate cotransporting polypeptide is a functional receptor for human hepatitis B and D virus. *Elife, 1*, e00049. doi:10.7554/eLife.00049

Yan, Y., Qiu, Y., Davgadorj, C., & Zheng, C. (2022). Novel Molecular Therapeutics Targeting Signaling Pathway to Control Hepatitis B Viral Infection. *Front Cell Infect Microbiol, 12*, 847539. doi:10.3389/fcimb.2022.847539

Yang, C., Ruan, P., Ou, C., Su, J., Cao, J., Luo, C., . . . Li, Y. (2015). Chronic hepatitis B virus infection and occurrence of hepatocellular carcinoma in tree shrews (Tupaia belangeri chinensis). *Virol J, 12*, 26. doi:10.1186/s12985-015-0256-x

Yang, G., Feng, J., Liu, Y., Zhao, M., Yuan, Y., Yuan, H., . . . Zhang, X. (2019). HAT1 signaling confers to assembly and epigenetic regulation of HBV cccDNA minichromosome. *Theranostics, 9*(24), 7345-7358. doi:10.7150/thno.37173

Yang, H. I., Yeh, S. H., Chen, P. J., Iloeje, U. H., Jen, C. L., Su, J., . . . Group, R.-H. S. (2008). Associations between hepatitis B virus genotype and mutants and the risk of hepatocellular carcinoma. *J Natl Cancer Inst, 100*(16), 1134-1143. doi:10.1093/jnci/djn243

Yang, L., Kwon, J., Popov, Y., Gajdos, G. B., Ordog, T., Brekken, R. A., . . . Shah, V. H. (2014). Vascular endothelial growth factor promotes fibrosis resolution and repair in mice. *Gastroenterology, 146*(5), 1339-1350 e1331. doi:10.1053/j.gastro.2014.01.061

Yang, S., Wang, D., Zhang, Y., Yu, C., Ren, J., Xu, K., . . . Li, L. (2015). Transmission of Hepatitis B and C Virus Infection Through Body Piercing: A Systematic Review and Meta-Analysis. *Medicine (Baltimore), 94*(47), e1893. doi:10.1097/MD.0000000000001893

Yardeni, D., & Koh, C. (2021). Bulevirtide for HBV and HDV infections. *Drugs Today (Barc), 57*(7), 433-448. doi:10.1358/dot.2021.57.7.3283861

Ye, J., & Chen, J. (2021). Interferon and Hepatitis B: Current and Future Perspectives. *Front Immunol, 12*, 733364. doi:10.3389/fimmu.2021.733364

Yeverino-Gutierrez, M. L., Gonzalez-Gonzalez, M. D. R., & Gonzalez-Santiago, O. (2020). Mortality From Alcohol-Related Liver Cirrhosis in Mexico (2000-2017). *Front Public Health, 8*, 524356. doi:10.3389/fpubh.2020.524356

Ying, H. Z., Chen, Q., Zhang, W. Y., Zhang, H. H., Ma, Y., Zhang, S. Z., . . . Yu, C. H. (2017). PDGF signaling pathway in hepatic fibrosis pathogenesis and therapeutics (Review). *Mol Med Rep, 16*(6), 7879-7889. doi:10.3892/mmr.2017.7641

Yip, H. Y. K., & Papa, A. (2021). Signaling Pathways in Cancer: Therapeutic Targets, Combinatorial Treatments, and New Developments. *Cells, 10*(3). doi:10.3390/cells10030659

Yokosaki, Y., & Nishimichi, N. (2021). New Therapeutic Targets for Hepatic Fibrosis in the Integrin Family, alpha8beta1 and alpha11beta1, Induced Specifically on Activated Stellate Cells. *Int J Mol Sci, 22*(23). doi:10.3390/ijms222312794

Yoshida, K., Desbiolles, A., Feldman, S. F., Ahn, S. H., Alidjinou, E. K., Atsukawa, M., . . . Shimakawa, Y. (2021). Hepatitis B Core-Related Antigen to Indicate High Viral Load: Systematic Review and Meta-Analysis of 10,397 Individual Participants. *Clin Gastroenterol Hepatol, 19*(1), 46-60 e48. doi:10.1016/j.cgh.2020.04.045

Zeisel, M. B., Guerrieri, F., & Levrero, M. (2021). Host Epigenetic Alterations and Hepatitis B Virus-Associated Hepatocellular Carcinoma. *J Clin Med, 10*(8). doi:10.3390/jcm10081715

Zhang, X., Wang, X., Wu, M., Ghildyal, R., & Yuan, Z. (2021). Animal Models for the Study of Hepatitis B Virus Pathobiology and Immunity: Past, Present, and Future. *Front Microbiol, 12*, 715450. doi:10.3389/fmicb.2021.715450

Zhang, Y., Mao, R., Yan, R., Cai, D., Zhang, Y., Zhu, H., . . . Zhang, J. (2014). Transcription of hepatitis B virus covalently closed circular DNA is regulated by CpG methylation during chronic infection. *PLoS One, 9*(10), e110442. doi:10.1371/journal.pone.0110442

Zhao, F., Xie, X., Tan, X., Yu, H., Tian, M., Lv, H., . . . Zhu, Q. (2021). The Functions of Hepatitis B Virus Encoding Proteins: Viral Persistence and Liver Pathogenesis. *Front Immunol, 12*, 691766. doi:10.3389/fimmu.2021.691766

Zhao, P., Malik, S., & Xing, S. (2021). Epigenetic Mechanisms Involved in HCV-Induced Hepatocellular Carcinoma (HCC). *Front Oncol, 11*, 677926. doi:10.3389/fonc.2021.677926

Zhao, X., Sun, L., Mu, T., Yi, J., Ma, C., Xie, H., . . . Tang, H. (2020). An HBV-encoded miRNA activates innate immunity to restrict HBV replication. *J Mol Cell Biol, 12*(4), 263-276. doi:10.1093/jmcb/mjz104

Zhao, X. K., Yu, L., Cheng, M. L., Che, P., Lu, Y. Y., Zhang, Q., . . . Ding, Q. (2017). Focal Adhesion Kinase Regulates Hepatic Stellate Cell Activation and Liver Fibrosis. *Sci Rep, 7*(1), 4032. doi:10.1038/s41598-017-04317-0

Zheng, Y., John, S., Pesavento, J. J., Schultz-Norton, J. R., Schiltz, R. L., Baek, S., . . . Mizzen, C. A. (2010). Histone H1 phosphorylation is associated with transcription by RNA polymerases I and II. *J Cell Biol, 189*(3), 407-415. doi:10.1083/jcb.201001148

Zhou, B. R., Feng, H., Kale, S., Fox, T., Khant, H., de Val, N., . . . Bai, Y. (2021). Distinct Structures and Dynamics of Chromatosomes with Different Human Linker Histone Isoforms. *Mol Cell, 81*(1), 166-182 e166. doi:10.1016/j.molcel.2020.10.038

Zhou, Z., Xu, M. J., & Gao, B. (2016). Hepatocytes: a key cell type for innate immunity. *Cell Mol Immunol, 13*(3), 301-315. doi:10.1038/cmi.2015.97

Zhu, C. L., Li, W. T., Li, Y., & Gao, R. T. (2012). Serum levels of tissue inhibitor of metalloproteinase-1 are correlated with liver fibrosis in patients with chronic hepatitis B. *J Dig Dis, 13*(11), 558-563. doi:10.1111/j.1751-2980.2012.00629.x

Zoulim, F., Fournier, C., Habersetzer, F., Sprinzl, M., Pol, S., Coffin, C. S., . . . Inchauspe, G. (2020). Safety and immunogenicity of the therapeutic vaccine TG1050 in chronic hepatitis B patients: a phase 1b placebo-controlled trial. *Hum Vaccin Immunother, 16*(2), 388-399. doi:10.1080/21645515.2019.1651141

Zoulim, F., Lenz, O., Vandenbossche, J. J., Talloen, W., Verbinnen, T., Moscalu, I., . . . Yogaratnam, J. Z. (2020). JNJ-56136379, an HBV Capsid Assembly Modulator, Is

Well-Tolerated and Has Antiviral Activity in a Phase 1 Study of Patients With Chronic Infection. *Gastroenterology, 159*(2), 521-533 e529. doi:10.1053/j.gastro.2020.04.036

ANNEX

My PhD fellowship additionally contributed to the publication of three scientific articles, three reviews, two book chapters, and one editorial. For clarity reasons, my personal contribution to each additional project will first be highlighted separately.

Annex I: Impaired hepatic protein tyrosine phosphatase delta is a driver of metabolic syndrome

Research article (manuscript in preparation, not attached).

Roca Suarez AA, Mailly L, **Virzì A,** Brignon N, Jühling F, Thumann C, Durand S, Oudot M, Schaeffer E, Martin R, Heydmann L, Bach C, Jamey C, Brumaru D, Dali-Youcef N, Felli E, Pessaux P, Schuster C, Mukherji A, Baumert TF, Lupberger J.

Throughout this project, my team and I aim to reveal the role of the protein phosphatase PTPRD in liver disease progression and metabolic liver disease. Impaired PTPRD expression in mice were correlated with alteration of signaling involved in glucose and lipid metabolism. Especially, impaired PTPRD expression caused STAT3 hyperphosphorylation which in turn is responsible for accumulation of free fatty acids (FFAs) and insulin resistance.

In this context, **my contribution** was to demonstrate that FFAs accumulation strongly impairs Akt signaling pathway activation, which is physiologically triggered by insulin and it is responsible for glucose and lipid homeostasis. At the same time, my team further observed a reduction of Akt phosphorylation following silencing of PTPRD. In this context, these results led to the conclusion that impaired PTPRD expression caused by HCV infection as previously demonstrated by my team (Van Renne et al., 2018), leads to upregulation of STAT3 signaling and consequent FFA accumulation, which in turn damages Akt pathway with consequent development of insulin resistance. The restauration of PTPRD functions within the liver may be crucial for the re-establishment of a functional STAT3 signaling axis and the design of therapeutic strategies against HCV chronic infection and metabolic disease such as NASH and NAFLD.

Annex II: GOLT1B Activation in Hepatitis C Virus-Infected Hepatocytes Links ER Trafficking and Viral Replication

Research article

Butterworth J, Gregoire D, Peter M, Roca Suarez AA, Desandré G, Simonin Y, **Virzì A**, Zine El Aabidine A, Guivarch M, Andrau JC, Bertrand E, Assenat E, Lupberger J, Hibner U.

Using clusters selection of HCV-positive and HCV-negative hepatocytes by single molecule fluorescent in situ hybridization methodology (smiFISH) in liver sections of a HCV patient with HCC, followed by RNAseq sequencing, the team identified a set of genes potentially deregulated in advanced liver disease (Butterworth et al., 2021). This study highlights the proviral role of GOLT1B, which in addition was found to be induced by endoplasmic reticulum (ER) stress and unfolded protein response (UPR).

In this context, **my contribution** was to study the impact GOLT1B at clinical level. Indeed, I demonstrated that *GOLT1B* is upregulated in tumor area of HBV-associated HCC and HCV-associated HCC. Moreover, *GOLT1B* expression appeared higher in HBV patients with severe fibrosis than in those with mild fibrosis (Fig. 5E of the research article) (Butterworth et al., 2021). These findings opened new perspective for the discovery of the specific role of GOLT1B in HCV infection and liver-associated diseases.

To conclude, I would like to thank Dr. Urszula Hibner and the team of the Institut de Génétique Moléculaire of the University of Montpellier for giving me the opportunity to collaborate with them.

GOLT1B Activation in Hepatitis C Virus-Infected Hepatocytes Links ER Trafficking and Viral Replication

Jacqueline Butterworth [1,†], Damien Gregoire [1,*,†], Marion Peter [1], Armando Andres Roca Suarez [2,3], Guillaume Desandré [1], Yannick Simonin [1], Alessia Virzì [2,3], Amal Zine El Aabidine [1], Marine Guivarch [2,3], Jean-Christophe Andrau [1], Edouard Bertrand [1], Eric Assenat [1,4], Joachim Lupberger [2,3,‡] and Urszula Hibner [1,*,‡]

[1] Institut de Génétique Moléculaire de Montpellier, University of Montpellier, CNRS, 34293 Montpellier, France; jackie@wordperfectscience.com (J.B.); marion.peter@inserm.fr (M.P.); guillaume.desandre@igmm.cnrs.fr (G.D.); yannick.simonin@umontpellier.fr (Y.S.); amal.makrini@igmm.cnrs.fr (A.Z.E.A.); jean-christophe.andrau@igmm.cnrs.fr (J.-C.A.); Edouard.bertrand@igh.cnrs.fr (E.B.); e-assenat@chu-montpellier.fr (E.A.)
[2] Inserm, U1110, Institut de Recherche sur les Maladies Virales et Hépatiques, 67000 Strasbourg, France; armando-andres.roca-suarez@inserm.fr (A.A.R.S.); virzi@unistra.fr (A.V.); marine.guivarch@etu.unistra.fr (M.G.); joachim.lupberger@unistra.fr (J.L.)
[3] Université de Strasbourg, 67000 Strasbourg, France
[4] Department of Hepatogastroenterology, Hepatology and Liver Transplantation Unit, Saint Eloi Hospital, University of Montpellier, 34000 Montpellier, France
* Correspondence: damien.gregoire@igmm.cnrs.fr (D.G.); ula.hibner@igmm.cnrs.fr (U.H.)
† These authors contributed equally to this work.
‡ These authors contributed equally to this work.

Abstract: Chronic hepatitis C carries a high risk of development of hepatocellular carcinoma (HCC), triggered by both direct and indirect effects of the virus. We examined cell-autonomous alterations in gene expression profiles associated with hepatitis C viral presence. Highly sensitive single molecule fluorescent in situ hybridization applied to frozen tissue sections of a hepatitis C patient allowed the delineation of clusters of infected hepatocytes. Laser microdissection followed by RNAseq analysis of hepatitis C virus (HCV)-positive and -negative regions from the tumoral and non-tumoral tissues from the same patient revealed HCV-related deregulation of expression of genes in the tumor and in the non-tumoral tissue. However, there was little overlap between both gene sets. Our interest in alterations that increase the probability of tumorigenesis prompted the examination of genes whose expression was increased by the virus in the non-transformed cells and whose level remained high in the tumor. This strategy led to the identification of a novel HCV target gene: GOLT1B, which encodes a protein involved in ER-Golgi trafficking. We further show that GOLT1B expression is induced during the unfolded protein response, that its presence is essential for efficient viral replication, and that its expression is correlated with poor outcome in HCC.

Keywords: hepatitis C; transcriptomic profiling; hepatocellular carcinoma; smiFISH

1. Introduction

Chronic hepatitis C predisposes patients to life-threatening pathologies, including hepatocellular carcinoma (HCC) [1]. The persistence of the virus and the accompanying inflammation-driven liver disease are major contributors to the progression from steatosis to fibrosis, cirrhosis, and ultimately HCC. In addition, cell-autonomous effects of the virus that disturb cellular homeostasis and generate a pro-oncogenic environment via deregulation of hepatic metabolism and signal transduction further increase the risk of tumorigenesis (for review see [2–4]).

In the case of hepatitis C virus (HCV), the distinction between direct and indirect effects of the virus on the host cell is not clear-cut. Indeed, HCV-infected hepatocytes secrete a number of active molecules that impact the liver physiopathology through both autocrine

and paracrine signaling. In addition to the well-studied cases of class I interferon [5], TGFβ and VEGF [6,7], lymphotoxin β, and wnt-mediated signaling that originate from the HCV-harboring cells also belong to this group [8,9]. An unbiased genome-wide proteogenomic approach highlighted major HCC hallmarks that are induced by HCV infection, including EGFR, STAT3, epithelial–mesenchymal transition (EMT), and perturbations in liver metabolism and DNA repair [10,11]. Interestingly, key HCV-dysregulated signaling pathways are also major players in the tight regulation of liver regeneration [12]. Importantly, even when viral infection is cleared, an epigenetic imprinting prevents a full recovery and contributes to the elevated HCC risk observed in HCV cured patients [13–15]. Despite recent progress in our understanding of the virus–host interactions, much remains to be learned about subtle virus-driven cell-autonomous alterations that may escape detection in a bulk analysis of the "omics" landscape.

In order to identify HCV-positive and HCV-negative hepatocytes, we used an improved version of single molecule fluorescent in situ hybridization methodology (smiFISH) [16] for frozen tissue sections from a chronic hepatitis C patient. Mapping of virus-infected cells was followed by tissue microdissection and transcriptomic profiling, leading to the identification of candidate gene products deregulated by HCV. One such deregulated gene discovered in our study is GOLT1B, encoding a so far poorly-studied protein involved in endoplasmic reticulum (ER)-Golgi trafficking [17,18]. We further show that GOLT1B is involved in the unfolded protein response (UPR) and is essential for HCV replication.

2. Results

2.1. HCV RNA Detection by Single Molecule In Situ Hybridization

Single molecule fluorescent in situ hybridization (smFISH) has previously been used to detect and quantify HCV viral RNA within infected hepatocytes in frozen liver sections of patients with hepatitis C [19]. However, the initial procedure is both time-consuming and expensive; indeed, the technique requires the use of long (~50 bp) specific probes covering about 1 kb of the target RNA sequence [20]. Given the high sequence divergence in different HCV subtypes [21], each patient's viral isolate needs to be sequenced prior to probe design. Patient-specific sets of fluorescent probes are then synthesized and used for FISH experiments. In contrast, in the recently developed highly sensitive and versatile version of single molecule FISH, the smiFISH [16], the target-specific sequence of each probe is shorter (26–32 nucleotides) and a single secondary detection probe is used, thus considerably lowering the cost of the experiment and allowing the use of a large number of probes for the targeted RNA (reviewed in [22]). We reasoned that it should be possible to design a set of probes with a broader specificity, recognizing, if not all HCV isolates, then at least all viral RNAs within a given genotype. In this work, we used a consensus sequence derived from 20,000 genotype 1b sequences deposited in the euHCVdb database (http://euhcvdb.lyon.inserm.fr/euHCVdb/; accessed on 26 March 2016) to design 61 probes, the locations of which are shown in Figure 1A (and Supplementary Materials Table S1). We predict that every viral genome should be recognized by a minimum of 40 probes.

The sensitivity and specificity of the pan-genotype 1b HCV probes were first tested on Huh7 Nneo/c-5B cells that harbor the full-length genotype 1b replicon [23]. As shown in Figure 1B, a strong signal was detected in the replicon cells, but not in the parental Huh7 line. The Nneo/c-5B replicon cells contain a high concentration of HCV RNA, visualized by hundreds of dots upon smiFISH analysis (Figure 1B). The control probe set, specific for the Firefly luciferase mRNA, gave no signal in either cell line (Figure 1C).

In order to further test the sensitivity of our detection procedure, we next explored detection of the signal in frozen liver sections from a HCV transgenic FL-N/35 mouse model, expressing very low levels of viral RNA [24] and thus reminiscent of the clinically relevant situation found in livers of chronically infected hepatitis C patients. Cytoplasmic HCV RNA was clearly detectable in transgenic mouse livers and absent from the control wild type livers (Figure 1D).
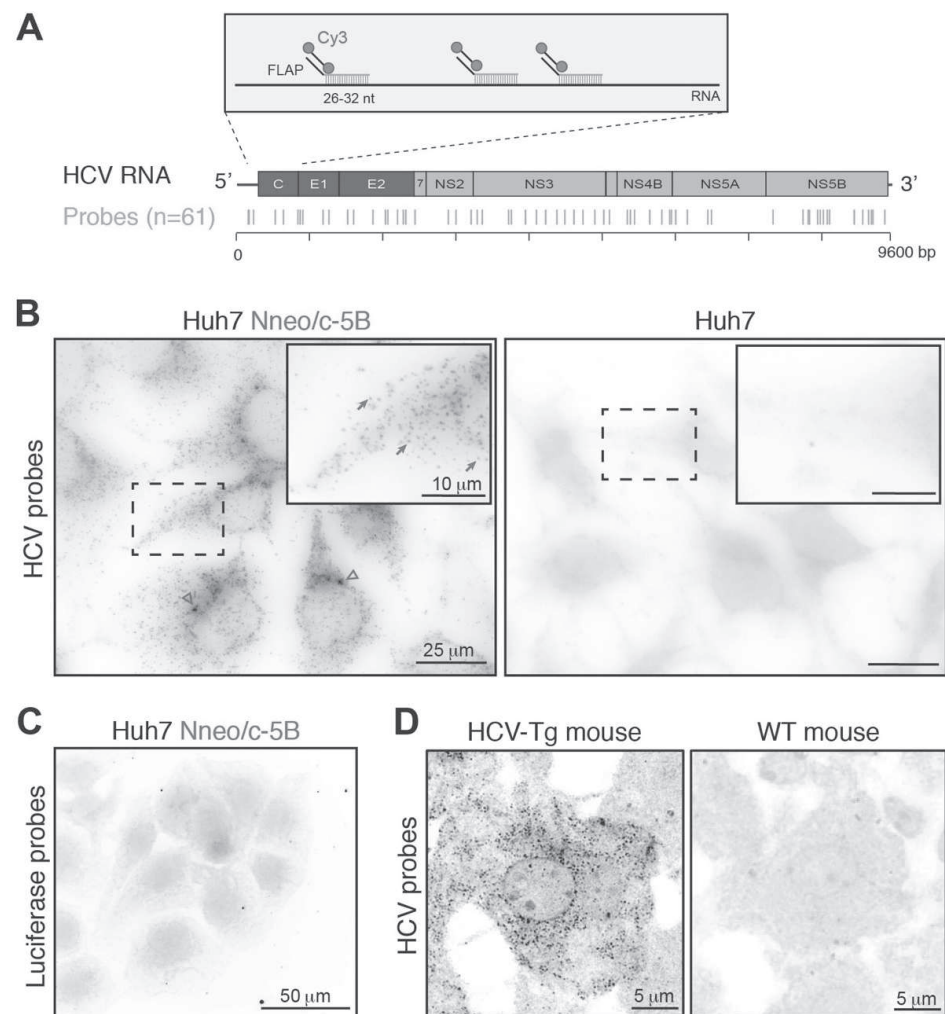
Figure 1. Detection of HCV RNA by smiFISH. (**A**) Principle of smiFISH. Localization of the 61 probes along viral RNA is indicated. (**B**) HCV RNA detection by smiFISH performed on Huh7-Nneo/c-5B replicon and Huh7 control cells. Arrows indicate single RNA molecules, arrowheads point to aggregates likely to correspond to replication sites (**C**) SmiFISH with luciferase control probe set on Huh7 Nneo/c-5B replicon cells (**D**) SmiFISH detection of HCV RNA on liver sections from HCV FLN-35 transgenic and control WT mice.

2.2. Detection of HCV-Infected Cells in Human Liver Sections

High variability of the viral load and of the fraction of infected hepatocytes in livers of hepatitis C patients has been reported [19,25]. Tumors arising in the context of hepatitis C have been described to have low viral levels [26–28], in coherence with the diminished expression of miR-122, an essential co-factor of viral replication (reviewed in [29]). To further investigate this point, we designed pan-genotype HCV primers to compare the abundance of viral RNA in tumoral and peritumoral samples of a small cohort of 20 patients (Figure 2A). As expected, our data show important inter-patient variation and confirm a lower viral load in the majority of tumors, as compared to corresponding non-tumoral tissue. Interestingly however, in at least four out of 20 tumors, the viral load was higher than in the surrounding tissue. We have chosen to concentrate further analysis on patient #8, who displayed high viral abundance (genotype 1b) both within the tumor and in the non-tumoral tissue.
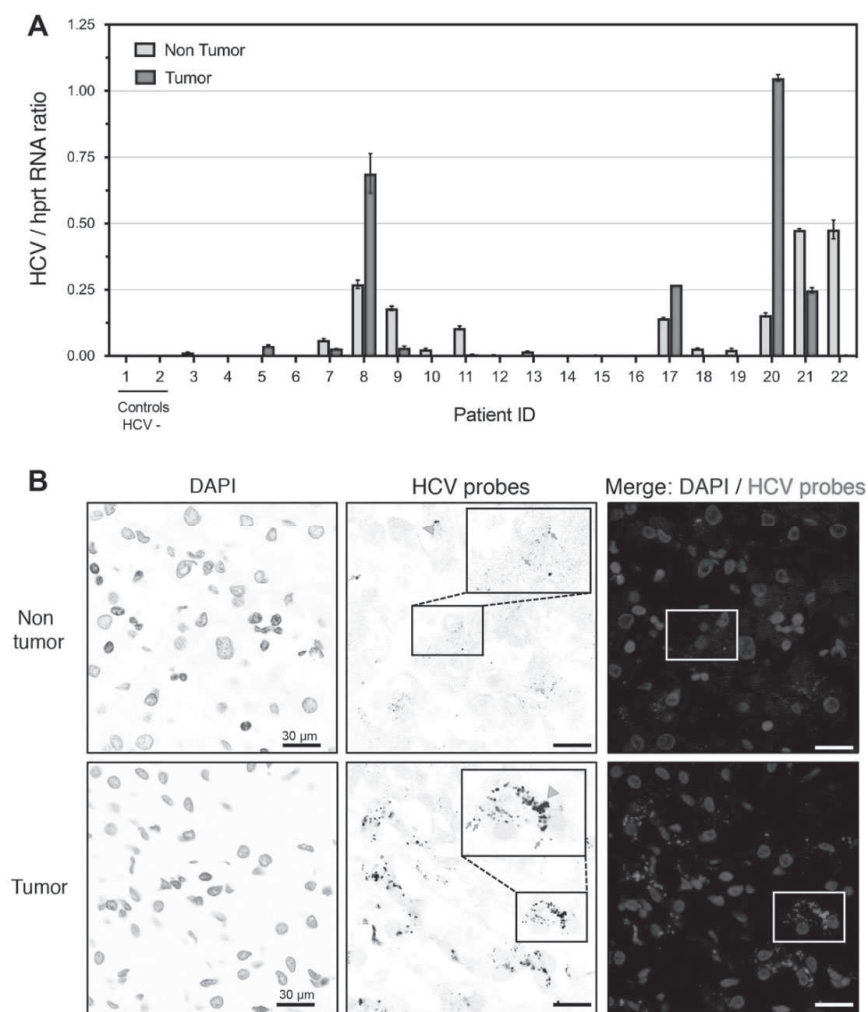
Figure 2. Detection of HCV RNA by smiFISH in a patient with HCC. (**A**) Quantification of HCV RNA by RT-qPCR in non-tumoral and tumoral tissues of HCC patients. Patients #1 and #2 were negative for HCV and were used as controls. Means +/-SD of technical triplicates are shown. (**B**) smiFISH on patient#8 non tumoral and tumoral regions. Arrows indicate examples of single viral RNA molecules; arrowheads point to aggregates that may correspond to active viral replication sites.

Pan-HCV genotype 1b smiFISH probes were used on OCT-frozen liver tissue from patient #8, a 62-year-old male patient who had undergone HCC resection on cirrhotic liver with signs of chronic hepatitis C. Confirming the RT-qPCR analysis (Figure 2A), a clear HCV-specific signal was observed in both the tumoral and peri-tumoral tissue (Figure 2B). Many tumor cells gave a very strong HCV-specific signal, visualized as cytoplasmic aggregated dots (arrowheads), considerably larger than the size expected from a single RNA molecule detection (arrows). These may represent sites of intense RNA replication, further arguing for a strong productive infection of the tumoral tissue. As previously reported, the HCV-infected cells were mainly visible as clusters (see e.g., [19]), allowing laser microdissection and capture of HCV-positive and HCV-negative regions from the frozen liver specimen.

2.3. Gene Expression Profiling of HCV-Positive and HCV-Negative Cell Clusters

We used smiFISH-labelled tissue sections from patient #8 to delineate HCV-positive and negative regions. For this, we examined confocal tile scan images combining the smiFISH data for the tissue sections representing average areas of 2.5 cm^2, corresponding to circa 10,000 cells. Clusters of infected and HCV-free hepatocytes were identified by visual examination (Figure 3A). A total of 11 HCV-positive and 7 HCV-negative regions, each composed of 100–300 cells, were used as the dissection guide on serial tissue sections

surrounding these tile scan images for laser microdissection, RNA extraction, and RNAseq analyses. Indeed, since the smiFISH procedure is not compatible with good quality RNA preparation, the microdissection was performed on the adjacent cuts of serial sections used for the smiFISH imaging. We used 10-μm-thick sections. Because hepatocytes are large cells of about 40 μm diameter, we reasoned that adjacent serial sections will largely have the same pattern of HCV+ and HCV− cell clusters. Transcriptomic profiling confirmed the correct identification of HCV presence or absence in 70% of these samples. All of the discordant results were false positives, i.e., they concerned samples initially identified as HCV+; these were omitted from further analysis. The normalized HCV read values in the confirmed positive samples varied between 0.3–4.2 for the peritumoral and between 1.1 and 24.2 for the tumoral clusters (Figure 3B).

A total of eight samples from the tumor and four from the peri-tumoral tissues were thus subjected to further analysis by RNAseq. We identified 202 genes that were differentially expressed in peritumoral tissue as a function of viral presence (137 up and 65 down, $p < 0.05$, log2 fold change > 2) (Figure 3C). In the tumor, the total number of dysregulated genes was 257 (98 up, 159 down) (Figure 3D) (listed in Table S2). For the upregulated genes, GO analysis indicated strongest enrichment of the "Golgi membrane" category for the non-tumoral samples (fold enrichment = 3.1, $p = 1.5 \times 10^{-3}$) and "metal ion binding" and "DNA binding" for the tumoral part (fold enrichment = 2.2 for both, p-value = 1.1×10^{-3} and 6.2×10^{-3}, respectively) (Table S3).

Somewhat unexpectedly, we found very little overlap between genes that appeared dysregulated by HCV in the peritumoral and in the tumoral tissues. This negative result made us question the initial assumption that HCV-driven transcriptional changes would be apparent independently of the cellular context, in this case either the tumoral or the peritumoral tissue.

2.4. Expression of a Subset of HCV Dysregulated Genes Is Increased during Tumorigenesis

We reasoned that some alterations in gene expression caused by the virus are likely to play a role in tumor development [30,31] and their expression might be maintained in the tumor even if the initial stimulus, i.e., the virus, is no longer present. Of note, there is a precedent for such phenomena, both in the "hit-and-run" strategy of some oncogenic viruses and in the case of stable epigenetic changes following HCV eradication [14,32–34]. As a consequence, we sought to identify genes that are dysregulated in the HCV+ non-tumoral samples and whose expression remains dysregulated in the tumor, independently of the HCV status of the analyzed region. Twenty-five genes that were upregulated in the HCV+ areas and ten whose expression was decreased in the presence of the virus fell into this new category (Figure 3E). Our data reveal the presence of several cancer-related pathways in this small gene set, namely, ECM interactions and cytoskeleton dynamics (ARHGAP5, CADM1, CHI3L1, ITGA6, TMOD3, ATXN1L, MYO19), metabolism and oxidative stress (SUCLA2, ME1, OSER1, SLC35B1, TTC19, SLC5A9, HCCS), proliferation signaling (CCND2, STK38, RASSF4), circadian clock (METTL14, CLOCK, KDM8), and vesicle trafficking (SNX2, GOLT1B, AP4E1). We were particularly interested in this latter category since it is related to the most highly enriched gene set (Golgi membrane) detected by the GO analysis of peri-tumoral samples. In order to pinpoint the genes most likely to correspond to bona fide downstream transcriptional targets of HCV, we next concentrated on the upregulated genes and sought to correlate the level of expression of these putative targets with the level of HCV RNA present in the tissue. To do so, we performed RT-qPCR analysis on 10 candidates on peri-tumoral and corresponding tumoral regions in the 10 patients of the original cohort for whom we detected viral RNA (Figure 2A). For two out of 10 candidates, we could indeed detect a strong correlation between the viral presence and the gene's expression in the non-tumoral tissues (Figures 4A and S1). In contrast, in the tumors, the expression remained high, but was no longer correlated with the viral load. One such gene was CLOCK, a central component of the circadian clock, which regulates several steps in the HCV life cycle, including particle entry into hepatocytes and RNA genome

replication [35]. Another gene in this category was GOLT1B, an evolutionary conserved gene encoding a protein involved in vesicular Golgi trafficking [17,36]. Interestingly, analysis of an independent cohort of HCV-infected patients without liver tumors [37] revealed a correlation between CLOCK and GOLT1B expression as well as a correlation between both genes with ER stress and UPR gene sets (Figures 4B and S2).
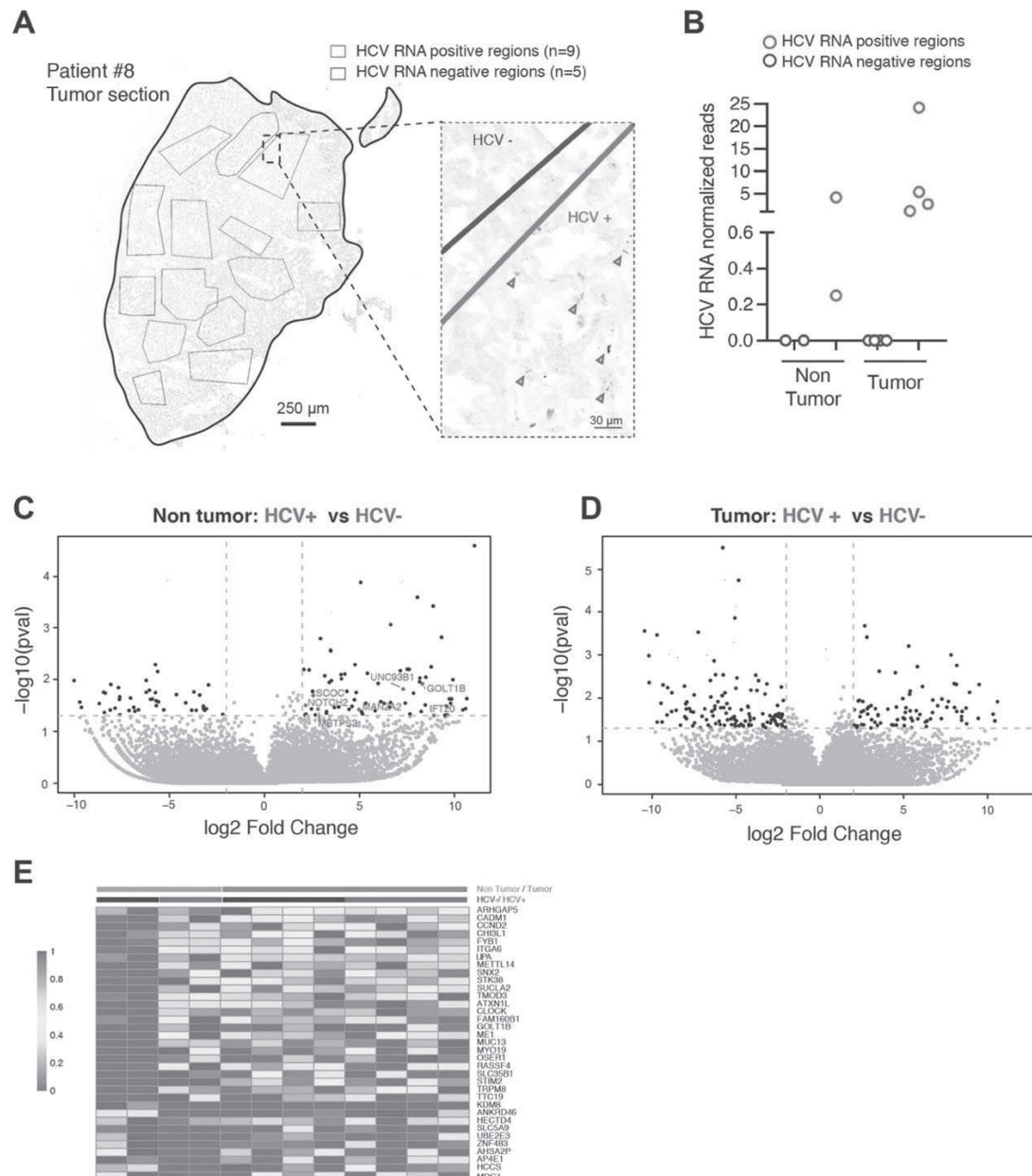

Figure 3. Transcriptomic analysis of HCV+ and HCV- clusters in tumoral and non-tumoral parts of a HCC patient. (**A**) An example of a tile scan image used to identify HCV-positive (red) and -negative (blue) regions detected by smiFISH that was used as guide for laser microdissection on serial tissue sections. Arrowheads on zoomed insert show examples of smiFISH-HCV positive cells. (**B**) HCV reads in RNAseq transcriptomes of HCV+ and HCV- regions used for further analysis. HCV reads were normalized to the total number of reads and number of cells in dissected regions. (**C**) Volcano plot presentation of genes deregulated in non-tumoral HCV+ vs. HCV- cells. (**D**) Volcano plot presentation of genes deregulated in tumoral HCV+ vs. HCV- cells. (**E**) Heatmap of genes deregulated in HCV+ non-tumoral samples that remained deregulated in the tumor.
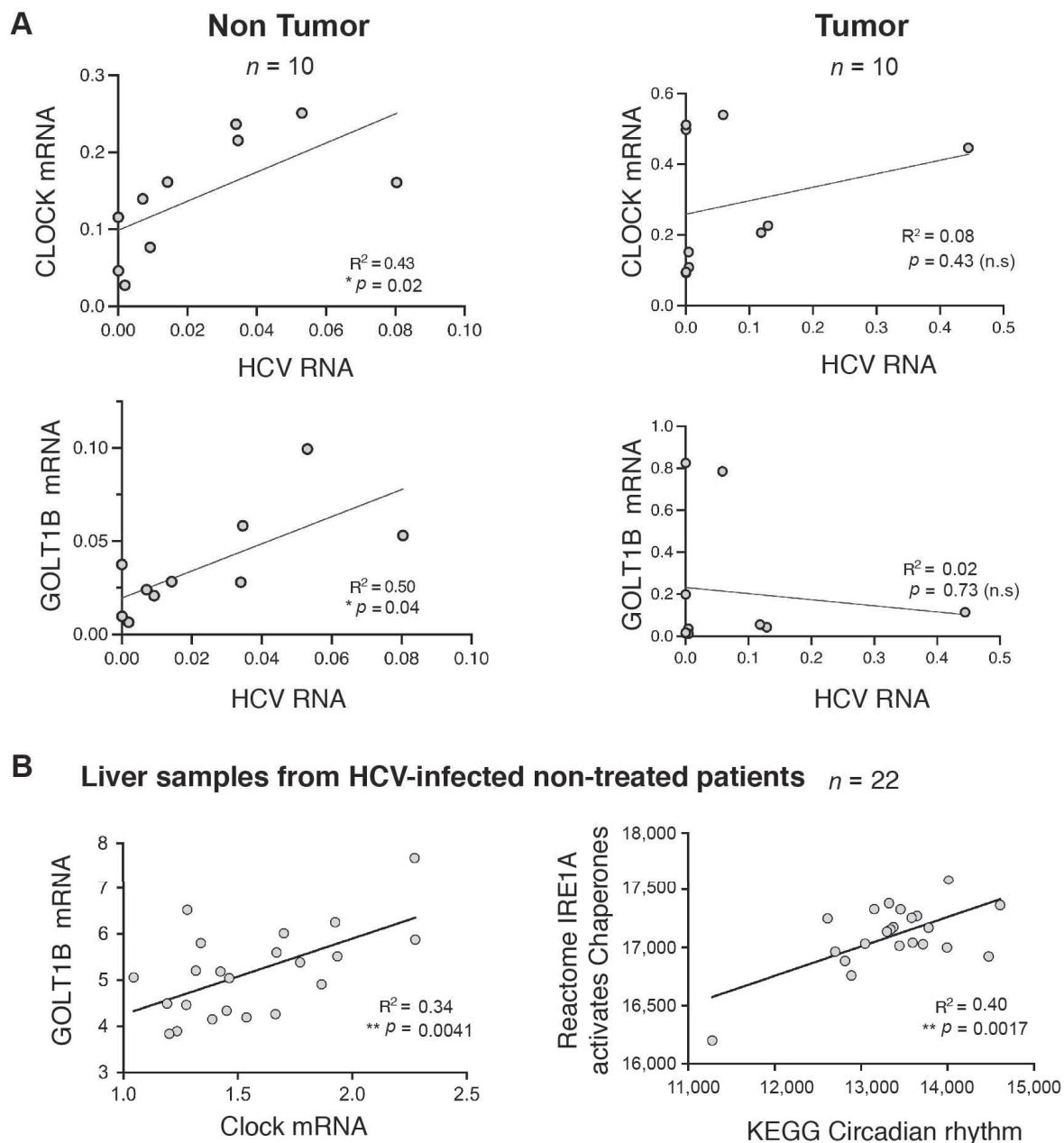
Figure 4. Correlation of CLOCK and GOLT1B expression with HCV RNA levels in non-tumoral liver in HCC patients. (**A**) mRNA levels of CLOCK/GOLT1B were quantified by qPCR in non-tumoral and tumoral samples from ten patients with HCC of HCV etiology. The R-squared and *p*-values from Pearson correlation tests (two-tailed) are indicated. (**B**) ER/UPR signaling pathways are associated with the circadian rhythm in the liver. The association of GOLT1B with CLOCK mRNA levels and the association of ER/UPR with the circadian rhythm signaling pathway were assessed in liver samples from HCV-infected non-treated patients (GSE84346, *n* = 22; [37]). Reactome and KEGG gene sets were obtained from the Molecular Signature Database (MSigDB) [38,39] are blotted as enrichment scores (ES); mRNA expression is plotted as normalized reads per kilo base per million mapped reads (RPKM). R-squared and *p*-values from Pearson correlation tests (two-tailed) are indicated. * *p* < 0.05, ** *p* < 0.01.

2.5. GOLT1B Is Required for Efficient HCV Replication

Because deficiencies in GOLT1B homologues in yeast and in rice (Got1p and Glup2 genes, respectively) disturb redistribution of proteins from the ER to the Golgi [17,18], we asked if GOLT1B might be associated with ER stress and a subsequent UPR in hepatocytes.

This proved to be the case, since pharmacological induction of ER stress and UPR by thapsigargin in Huh7.5.1 cells gave rise to a significant increase of GOLT1B expression (Figure 5A).
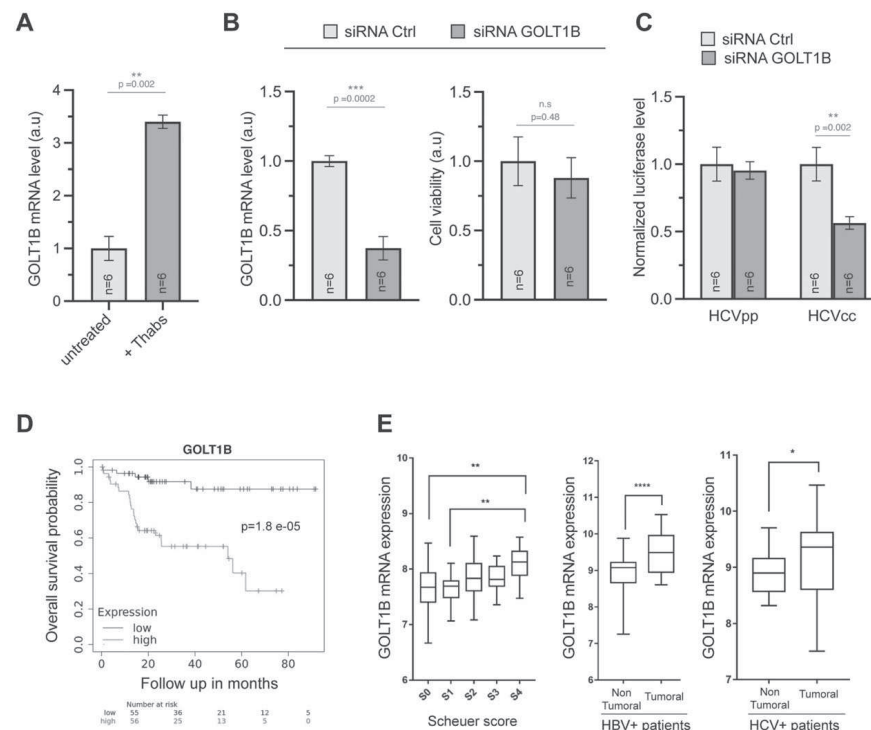
Figure 5. GOLT1B is an essential factor in HCV replication. (**A**) The Unfolded Protein Response (UPR) significantly increases GOLT1B expression in Huh7.5 cells treated for 8 h with 1 μM thapsigargin (Thabs). Results are displayed as average GOLT1B mRNA expression relative to GAPDH +/− SD (three biological replicates in technical duplicates). The *p*-value from the Mann–Whitney U-test test is indicated. (**B**) GOLT1B silencing efficacy in Huh7.5.1. (left panel). Measurement of cell viability in an HCVpp-infected cell using PrestoBlue (right panel). Means +/− SEM are shown. The Mann–Whitney U-test statistical significance is indicated. (**C**) Assessment of viral entry and replication via HCV pseudoparticles (HCVpp) and HCV viral construct (HCVcc). siGOLT1B impairs viral replication but not viral entry (mean +/− SEM, Mann–Whitney, U-test). (**D**) Kaplan–Meier analysis of TCGA data indicates that a high GOLT1B expression is correlated with poor overall survival probability in patients with HCC associated with viral hepatitis. Analysis conducted using Kaplan–Meier Plotter [40] (**E**) GOLT1B mRNA expression is significantly higher in HBV patients with severe fibrosis than in patients with mild fibrosis, according to the histological staging of fibrosis (Scheuer score "S"). In total, 124 liver biopsy samples were retrieved (GEO accession number: GSE84044) and used for the bioanalysis (S0 = 43 patients, S1 = 20 patients, S2 = 33 patients, S3 = 18 patients, S4 = 10 patients). For statistical analysis, the Kruskal–Wallis test (non-parametric ANOVA) was performed and GP *p*-values calculated: 0.0021 (**), GOLT1B expression in whole liver tissue was analyzed from 39 samples from HBV-associated HCC patients "Non tumor area (HBV)" and 81 samples from HBV-associated HCC patients "Tumor area (HBV)". The samples derived from 11 HBV-associated HCC patients who underwent liver transplantation for tumor (GEO accession number: GSE107170). GOLT1B expression in whole liver tissue was analyzed from 31 samples from HCV-associated HCC patients "Non tumor region (HCV)" and 44 samples from HCV-associated HCC patients "Tumor region (HCV)". The samples derived from 11 HCV-associated HCC patients who underwent liver transplantation for tumor (GEO accession number: GSE107170). For statistical analysis, the Mann–Whitney test was performed and GP *p*-values were calculated: >0.05 (not significant, n.s.), <0.05 (*), <0.01 (**), <0.001 (***), <0.0001 (****).

We next asked if increased GOLT1B expression in HCV-infected cells was a mere reflection of an overall ER stress or whether this protein had a functional importance in the viral life cycle. Efficient GOLT1B silencing by siRNA in Huh7.5.1 cells gave rise to only a minor effect on cell viability (Figure 5B). Control and GOLT1B KD cells were then infected with reporter viruses encoding Firefly luciferase. We tested viral entry by using HCV pseudoparticles (HCVpp) that display the HCV envelope glycoproteins on the backbone of a retroviral vector, as well as the entry and the replication of the bona fide HCV viral construct (HCVcc). While GOLT1B deficiency had no effect on viral entry, replication efficiency was significantly compromised in GOLT1B KD cells (Figure 5C). Thus, GOLT1B plays a role in the HCV life cycle.

We next questioned GOLT1B involvement in hepatic tumorigenesis. Kaplan–Meier analysis of TCGA data indicated that a high level of GOLT1B expression was associated with poor overall survival for virus-related HCC (Figure 5D) but had no significant impact on the survival of patients with many other tumor types, such as colorectal carcinoma, invasive breast cancer or glioblastoma. Moreover, GOLT1B hepatic expression was significantly higher in patients with severe fibrosis score compared to low fibrosis. Finally, in support of our conclusions regarding the role of GOLT1B in hepatic tumorigenesis, its expression was significantly higher in the tumor compared with non-tumoral tissues of both hepatitis B and hepatitis C patients (Figure 5E).

3. Discussion

Despite remarkable progress in prevention and treatment, chronic infections with HBV or HCV remain the major risk factors for HCC [41]. In addition to necro-inflammatory liver damage, characteristic of chronic viral hepatitis and responsible for creating a favorable environment for tumor development, direct effects of the virus on the host cell have been incriminated as HBV-induced pro-oncogenic events. These events include insertional mutagenesis, transcriptional deregulation or inactivation of the p53 tumor suppressor by a viral protein [42]. The case is less clear for HCV, although several transgenic mouse models expressing all or a subset of the HCV proteins are tumor-prone, strongly arguing for their oncogenic activity in the absence of any immune-mediated hepatic lesions [24,43]. Moreover, transcriptional and post-transcriptional activation of oncogenic signaling pathways, as well as inhibition of apoptosis, has been described for several viral proteins [9,10,44,45]. Importantly, both viruses trigger long-lasting alterations of the epigenome of their host cells [15], which may account for a proportion of HCC cases developing after the efficient clearance of HCV infection [44,45].

The lack of animal models recapitulating the events leading from chronic hepatitis C to tumorigenesis is a major hurdle for a full understanding of the underlying mechanisms. However, recent technological developments render a feasible in-depth analysis of surgical samples from liver resection of patients suffering from HCC that developed on the HCV infected liver. Here, we report such an effort that has led to the characterization of virus-mediated changes in the transcriptomic profile occurring in the true physiopathological context of naturally occurring hepatitis C infection.

There are two options for comparing the transcriptional profiles of patient-derived cells that either harbor the virus or are free of it. Single cell RNA sequencing is one of them. While extremely powerful, this technique has two major disadvantages: its high cost and the fact that it provides no positional information on the cells in the sample. In contrast, RNAseq analysis of microdissected regions, classified as infected or virus-free by single molecule FISH imaging, allows the study of infected cell clusters and their comparison with cells that are not in their immediate vicinity. This last point may be of importance because of the documented effects of infected cells on their non-infected counterparts, likely to primarily operate over short distance. Moreover, in situ hybridization and microdissection can be performed on frozen tissue samples allowing a retrospective study of appropriately preserved samples.

Driven by these considerations, we employed an improved version of smFISH: the single molecule inexpensive (smi)FISH [16]. Importantly, while smiFISH retains the high sensitivity of the classical smFISH, it is more versatile and considerably cheaper. This is due to the use of short unlabeled primary oligonucleotide probes that are tagged by a common sequence. Thus, a single fluorescent-labelled oligonucleotide, which is complementary to the common tag, is needed for in situ hybridization. An additional improvement came from designing a set of oligonucleotide probes that are expected to recognize all of the known sequences of the genotype 1b HCV. Of note, although we have not employed smiFISH for detection of any other low-abundance RNA viruses, there are no theoretical obstacles for doing so.

The RNAseq analysis of samples microdissected from clinical specimens remains technically challenging, and the quantity and quality of the recovered RNA did not allow high-depth analysis. It is therefore very likely that our dataset represents only a subset of alterations in gene expression triggered by the viral presence. Indeed, the number of genes dysregulated in the course of in vitro viral infection is 5 to10-fold higher than that detected in our work [10]. Noteworthy, however, our methodology is expected to strongly enrich the identification of cell-autonomously regulated genes and it has been performed on clinical samples from a chronic hepatitis C patient. Further analyses of additional patient samples will be required to better define the sets of genes commonly deregulated by HCV in a clinically relevant setting.

There are several common selective pressures that must be dealt with in a persistent viral infection and in a growing tumor, such as novel metabolic requirements and escape from elimination. This is presumably why some mechanisms used by viruses to pervert cellular functions constitute a risk factor for transformation [30,31]. Following this line of thought, we have discovered genes whose expression is dysregulated in infected, non-transformed cells as well as in tumor cells, independently of their infection status. These genes seem specifically related to an environment of advanced liver disease and HCC since they do not overlap with previous transcriptomics from HCV infection models [10]. This analysis led to the identification of GOLT1B, an ER protein involved in ER-Golgi protein trafficking [17,18]. GOLT1B has not previously been reported in the context of HCV infection, and we confirm that it is not directly induced by HCV, in accordance with previous omics studies on Huh7.5.1 cells and human liver chimeric mice [10]. We show, however, that the basal GOLT1B expression greatly facilitates HCV replication, presumably in relation to the major role played by ER in the viral life cycle. Indeed, HCV infection gives rise to major expansion and reorganization of the ER, leading to the creation of a membranous web, which is the site of viral replication and assembly (reviewed in [46]). This is achieved through the action of non-structural proteins NS4B and NS5A and is accompanied by the activation of the cellular response to stress, the unfolded protein response (UPR). The UPR is a common cellular adaptation to numerous stresses, originally described as a survival mechanism allowing cells to deal with an overload of protein processing in the ER (reviewed in [47]). Because viral or bacterial infection as well as cancer leads to an increased demand on protein synthesis and processing, these conditions are often associated with the UPR [48,49]. Interestingly, the UPR has also been described for other pathological conditions, including hepatic pathologies, such as NAFLD, fibrosis, and cirrhosis [50,51], which constitute independent risk factors for HCC.

Importantly, the UPR is not only a consequence of a strong protein synthetic activity in rapidly growing cells, it is also a necessary adaptation for cancer cell survival and growth [52]. Similar to many other cellular stress responses, overwhelming UPR can also trigger cell death, and novel anti-tumor therapies currently under investigation aim either at inhibiting or augmenting UPR processes.

While the general mechanism of the UPR is shared by many cell types and many types of stress, the response is fine-tuned by the preferential use of its three main sensors and many partners engaged in complex signaling networks [53]. It remains to be investigated if GOLT1B is involved in the generic UPR or rather remains specific to hepatic physiopathol-

ogy. Similarly, further work is necessary to establish whether the requirement for GOLT1B during HCV infection reflects a need for UPR-mediated ER reorganization or if this new HCV target gene plays a novel distinct role in the viral life cycle.

4. Material and Methods

4.1. Cells

Huh7 cells were cultured in DMEM supplemented with 10% fetal bovine serum, 100 µg/mL streptomycin, and 100 U/mL penicillin; 400 µg/mL of G418 was added to Huh7 Nneo/C-5B cells that harbored the full-length HCV genotype 1b replicon [23].

4.2. Mice

Three-month-old FL-N/35 males [24], transgenic for the entire HCV genotype 1b open reading frame and wild type controls, both in the C57Bl/6J genetic background, were used. Mice were housed and bred according to French Institutional guidelines. The protocols were approved (ID of approval for this study N° F 34-172-16) by the Languedoc-Roussillon ethics committee (CEEA-LR1013).

4.3. Patients

Freshly frozen tumoral and peritumoral tissue from 20 cases of HCC surgical resection on hepatitis C background were collected from Montpellier University Hospital. Two additional patients had HCC unrelated to HCV. The samples were anonymized and used after obtaining written informed consent from patients, in accordance with French legislation.

4.4. HCV Infection

Huh 7.5.1 cells [54] were infected with HCV-derived pseudoparticles (H77; genotype 1a) or cell culture-derived infectious HCV (HCVcc; strain Luc-jc1) harboring a luciferase reporter gene, as previously described [55]. Virus entry and infection were assessed two days after infection by measuring reporter gene luciferase activity in cell lysates using the Bright Glo Luciferase assay system (Promega, Charbonnières-les-Bains, France) and a Mithras LB 940 luminometer (Berthold Technologies, Bad Wildbad, Germany).

4.5. HCV Pan-Genotype 1b smiFISH Probe Design

More than 20,000 HCV genotype 1b sequences >9.5 Kb in length from the NCBI database were aligned using Galaxy [56]. A consensus HCV genotype 1b sequence was generated in Aliview (the Open Source Software License 'GNU General Public License, version 3.0 (GPLv3)). In the event of missing sequence information, the non-identified nucleotides were arbitrarily designated as C. Probes were designed by R-script Oligostan [16], which automatically eliminates C-stacks and thus excludes any artificially created C-blocks. Sixty-one primary probes corresponding to the HCV genotype 1b consensus sequence were designed. The same software was used to design primary probe sets for negative controls, the Firefly luciferase and Hygromycin resistance genes, as well as the positive controls, i.e., mouse and human GAPDH.

4.6. smiFISH Applied to Fixed Cells

The reagents used, primary probe preparations, FLAP probe sequence, and preparations, as well as the smiFISH protocol were carried out according to the detailed description found in [16]. Briefly, a set of target-specific probes was synthesized, each carrying an additional 28-nt long sequence ("FLAP"), which is not represented in either mouse or human genomes. Cy-3 labelled fluorescent probe complementary to FLAP was also synthesized and pre-annealed to primary probes prior to in situ hybridization.

4.7. smiFISH Applied to Frozen Tissue

Freshly dissected human or mouse liver tissue fragments were frozen in OCT in liquid nitrogen-cooled isopentane and stored at −80 °C. Ten-µM-thick tissue sections were

mounted on Superfrost^{TM} Plus Gold slides (Thermo Fisher Scientific, Waltham, MA, USA), fixed in 4% paraformaldehyde pH 9 for 30 min at room temperature, and permeabilized for 30 min in 1% Triton/PBS at room temperature. SmiFISH was performed as above using RNAse-free glass coverslips. Nuclei were counterstained by ProlongGold DAPI (Thermo Fisher Scientific, Waltham, MA, USA).

4.8. RNA Extraction and RT-qPCR

RNA was extracted from frozen samples of either tumoral or matched non-tumoral parts of HCC patient biopsies using the RNeasy mini kit (Qiagen, Hilden, Germany); 0.25 µg of total RNA was reverse-transcribed with the QuantiTect Reverse Transcription kit (Qiagen), and cDNA was quantified using LC Fast start DNA Master SYBR Green I Mix (Roche, Basel, Switzerland). Primers for RT-qPCR were designed using LightCycler probe design 2 software (Roche), targeting the pan-genotype small (i.e., 300 bp) conserved region in the 5′UTR of HCV viral sequence: forward: 5′- CAGGAGATGGGCG-GAAAC -3′, reverse: 5′- GCCGCAATGGATATTTCATTCTCA-3′. Results were normalized to SRSF4 housekeeping gene expression; forward: 5′- CGGAGTCCTAGCAGGCATA-3′, reverse: 5′-TTCCTGCCCTTCCTCTTGT-3′. GOLT1B expression was quantified using qPCR primers GOLT1B-fw (5′-CGGCTTCATTTCTCCCGACT-3′) and GOLT1B-rv (5′-TCCAATTTTCTGCGTGTCCG-3′) using the SYBR green method using a CFX96 Touch Thermal Cycler (Bio-Rad, Hercules, CA, USA). PCR products were validated by melting curve analysis and Sanger sequencing.

4.9. Microscopy and Imaging

In vitro smiFISH microscopy was performed using a widefield Zeiss Axioimager Z1 to capture Z-stacks using an X63 1.4 NA oil objective equipped with a CCD camera (Axiocam) and controlled with Metamorph (Molecular Devices). Tissue smiFISH imaging was performed using an inverse SP8-UV confocal microscope (Leica) for Z-stack capturing with a X63 1.4 NA oil objective and HyD detectors. Z-stacks and confocal tile scans were compiled using LAS software (Leica Application Suite). Signals were detected after adjustment of the negative control probe to zero (considered background noise). The remaining non-specific signal, likely due to spectral bleed-through, was calculated by image overlying upon excitement of the Cy3 and GFP channels. For the HCV signal the non-specific component was estimated to be 1.8% of the total signal and was therefore considered negligible. Estimation of the fraction of HCV-positive cells in tissue sections was performed by manual counting of HCV-positive cells relative to the total number of detected DAPI-stained nuclei in five randomly chosen fields (>100 cells/field).

4.10. Laser Microdissection and RNA Extraction

Serial 10 µM sections of human liver tissue were mounted on PET-membrane steel frame slides (Leica) and stored at −80 °C until use. For laser dissection, slides were fixed for 20 min in 70% ethanol at +4 °C followed by dissection using a PALM MicroBeam laser UV dissector (Zeiss, Oberkochen, Germany). Serial section alignment of the smiFISH and unstained slides was carried out at the Laser Microdissection platform at Bordeaux Neurocampus using an automated serial section alignment and microdissection (PALM 4 technology, Zeiss). RNA was extracted and purified from each sample (*circa* 100–200 cells) using the RNeasy® Micro kit (Qiagen, Hilden, Germany) with on-column DNAse I digestion. RIN values were calculated using the Agilent Bioanalyzer with RNA pico chips.

4.11. RNAseq

RNA samples containing a minimum of 1 ng RNA and a RIN > 4 were used for further RNAseq analysis. First, RNA was amplified using the Ovation® SoLo RNA-seq library preparation kit (NuGEN) used for low input or single-cell sequencing according to manufacturer's instructions, including a ribosomal RNA depletion step (Insert Dependent Adaptor Cleavage). Libraries were then monitored for concentration and fragment sizes

using a Fragment Analyzer (kit Standard Sensitivity NGS) and by qPCR (ROCHE Light Cycler 480) prior sequencing on Illumina HiSeq2000 platform (single end 50 bp length).

4.12. Single Sample Gene Set Enrichment Analysis

Transcriptomic data from HCV-infected non-treated human liver samples (GSE84346, *n* = 22) were preprocessed with the CollapseDataset tool available at GenePattern. Single sample gene set enrichment analysis (ssGSEA) was performed using gene sets belonging to the Molecular Signatures Database (MSigDB) version 7.4 [38,39]. The correlations of GOLT1B with CLOCK mRNA levels and ER/UPR with circadian rhythm signaling pathway scores were assessed with Pearson's correlation coefficient.

4.13. Statistical Analysis

- Read quality assessment

The first five bases of each read were trimmed using cutadapt v. 1.13 (option -u 5). The trimmed reads were aligned to the human genome (Hg38) using TopHat2 v2.1.1 [57]. The read quality was controlled using FastQC and FastQ Screen tools.

- Identification of viral reads

In order to confirm the presence of the hepatitis C virus in HCV-positive samples, we aligned the RNA reads on the HCV sequences using the BWA mapper tool (using BWA-backtrack algorithm). The results confirmed the presence of HCV in seven samples. Based on this result, we removed five HCV-positive samples for which no reads mapped on the HCV reference genome. We also removed one sample considered HCV-negative but that mapped reads to the HCV reference.

- Differential Gene Expression analysis

Differential Gene Expression analysis was performed using the DESeq package [58] from Bioconductor. First, the featureCounts program (version 1.6.2) was used to count the reads that mapped to gene annotations with the option '-s 1'. Then these counts were analyzed using the DESeq package to identify genes that were at least 1-fold (log2) differentially expressed relative to the reference sample using a *p*-value threshold of 0.05.

Statistical analysis of GOLT1B experiments was performed using GraphPad prism software (GraphPad Software, San Diego, CA, USA).

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/pathogens11010046/s1, Figure S1: Correlations of HCV RNA and eight target gene levels in non-tumoral regions of HCC patients; Figure S2: HCV infection induces an unfolded protein response in Huh7.5.1 and human liver chimeric mice; Table S1: Sequences of probes for smiFISH detection of HCV RNA; Table S2: List of genes differentially expressed in peritumoral tissue and tumor as a function of viral presence; Table S3: Gene Ontology analysis of deregulated genes.

References

1. Thrift, A.P.; El-Serag, H.B.; Kanwal, F. Global epidemiology and burden of HCV infection and HCV-related disease. *Nat. Rev. Gastroenterol. Hepatol.* **2017**, *14*, 122–132. [CrossRef]
2. Axley, P.; Ahmed, Z.; Ravi, S.; Singal, A.K. Hepatitis C Virus and Hepatocellular Carcinoma: A Narrative Review. *J. Clin. Transl. Hepatol.* **2018**, *6*, 79–84. [CrossRef] [PubMed]
3. Goto, K.; Roca Suarez, A.A.; Wrensch, F.; Baumert, T.F.; Lupberger, J. Hepatitis C Virus and Hepatocellular Carcinoma: When the Host Loses Its Grip. *Int. J. Mol. Sci.* **2020**, *21*, 3057. [CrossRef] [PubMed]
4. Virzì, A.; Roca Suarez, A.A.; Baumert, T.F.; Lupberger, J. Rewiring Host Signaling: Hepatitis C Virus in Liver Pathogenesis. *Cold Spring Harb. Perspect. Med.* **2020**, *10*, a037366. [CrossRef]
5. Heim, M.H. Innate immunity and HCV. *J. Hepatol.* **2013**, *58*, 564–574. [CrossRef]
6. Battaglia, S.; Benzoubir, N.; Nobilet, S.; Charneau, P.; Samuel, D.; Zignego, A.L.; Atfi, A.; Bréchot, C.; Bourgeade, M.-F. Liver cancer-derived hepatitis C virus core proteins shift TGF-beta responses from tumor suppression to epithelial-mesenchymal transition. *PLoS ONE* **2009**, *4*, e4355. [CrossRef]
7. Wilson, G.K.; Brimacombe, C.L.; Rowe, I.A.; Reynolds, G.M.; Fletcher, N.F.; Stamataki, Z.; Bhogal, R.H.; Simões, M.L.; Ashcroft, M.; Afford, S.C.; et al. A dual role for hypoxia inducible factor-1α in the hepatitis C virus lifecycle and hepatoma migration. *J. Hepatol.* **2012**, *56*, 803–809. [CrossRef]
8. Moreau, M.; Rivière, B.; Vegna, S.; Aoun, M.; Gard, C.; Ramos, J.; Assenat, E.; Hibner, U. Hepatitis C viral proteins perturb metabolic liver zonation. *J. Hepatol.* **2015**, *62*, 278–285. [CrossRef]
9. Simonin, Y.; Vegna, S.; Akkari, L.; Gregoire, D.; Antoine, E.; Piette, J.; Floc'h, N.; Lassus, P.; Yu, G.Y.; Rosenberg, A.R.; et al. Lymphotoxin signaling is initiated by the viral polymerase in HCV-linked tumorigenesis. *PLoS Pathog.* **2013**, *9*, e1003234. [CrossRef]
10. Lupberger, J.; Croonenborghs, T.; Roca Suarez, A.A.; Van Renne, N.; Jühling, F.; Oudot, M.A.; Virzì, A.; Bandiera, S.; Jamey, C.; Meszaros, G.; et al. Combined Analysis of Metabolomes, Proteomes, and Transcriptomes of Hepatitis C Virus-Infected Cells and Liver to Identify Pathways Associated With Disease Development. *Gastroenterology* **2019**, *157*, 537–551.e9. [CrossRef]
11. Van Renne, N.; Roca Suarez, A.A.; Duong, F.H.T.; Gondeau, C.; Calabrese, D.; Fontaine, N.; Ababsa, A.; Bandiera, S.; Croonenborghs, T.; Pochet, N.; et al. miR-135a-5p-mediated downregulation of protein tyrosine phosphatase receptor delta is a candidate driver of HCV-associated hepatocarcinogenesis. *Gut* **2018**, *67*, 953–962. [CrossRef]
12. Tao, Y.; Wang, M.; Chen, E.; Tang, H. Liver Regeneration: Analysis of the Main Relevant Signaling Molecules. *Mediat. Inflamm.* **2017**, *2017*, 4256352. [CrossRef]

13. Fujiwara, N.; Friedman, S.L.; Goossens, N.; Hoshida, Y. Risk factors and prevention of hepatocellular carcinoma in the era of precision medicine. *J. Hepatol.* **2018**, *68*, 526–549. [CrossRef] [PubMed]

14. Hamdane, N.; Jühling, F.; Crouchet, E.; El Saghire, H.; Thumann, C.; Oudot, M.A.; Bandiera, S.; Saviano, A.; Ponsolles, C.; Roca Suarez, A.A.; et al. HCV-Induced Epigenetic Changes Associated With Liver Cancer Risk Persist After Sustained Virologic Response. *Gastroenterology* **2019**, *156*, 2313–2329.e7. [CrossRef]

15. Perez, S.; Kaspi, A.; Domovitz, T.; Davidovich, A.; Lavi-Itzkovitz, A.; Meirson, T.; Alison Holmes, J.; Dai, C.-Y.; Huang, C.-F.; Chung, R.T.; et al. Hepatitis C virus leaves an epigenetic signature post cure of infection by direct-acting antivirals. *PLoS Genet.* **2019**, *15*, e1008181. [CrossRef] [PubMed]

16. Tsanov, N.; Samacoits, A.; Chouaib, R.; Traboulsi, A.-M.; Gostan, T.; Weber, C.; Zimmer, C.; Zibara, K.; Walter, T.; Peter, M.; et al. smiFISH and FISH-quant—A flexible single RNA detection approach with super-resolution capability. *Nucleic Acids Res.* **2016**, *44*, e165. [CrossRef] [PubMed]

17. Conchon, S.; Cao, X.; Barlowe, C.; Pelham, H.R. Got1p and Sft2p: Membrane proteins involved in traffic to the Golgi complex. *EMBO J.* **1999**, *18*, 3934–3946. [CrossRef] [PubMed]

18. Fukuda, M.; Kawagoe, Y.; Murakami, T.; Washida, H.; Sugino, A.; Nagamine, A.; Okita, T.W.; Ogawa, M.; Kumamaru, T. The Dual Roles of the Golgi Transport 1 (GOT1B): RNA Localization to the Cortical Endoplasmic Reticulum and the Export of Proglutelin and α-Globulin from the Cortical ER to the Golgi. *Plant Cell Physiol.* **2016**, *57*, 2380–2391. [CrossRef]

19. Wieland, S.; Makowska, Z.; Campana, B.; Calabrese, D.; Dill, M.T.; Chung, J.; Chisari, F.V.; Heim, M.H. Simultaneous detection of hepatitis C virus and interferon stimulated gene expression in infected human liver. *Hepatology* **2014**, *59*, 2121–2130. [CrossRef]

20. Femino, A.M.; Fay, F.S.; Fogarty, K.; Singer, R.H. Visualization of Single RNA Transcripts in Situ. *Science* **1998**, *280*, 585–590. [CrossRef] [PubMed]

21. Simmonds, P. Genetic diversity and evolution of hepatitis C virus—15 years on. *J. Gen. Virol.* **2004**, *85*, 3173–3188. [CrossRef]

22. Pichon, X.; Lagha, M.; Mueller, F.; Bertrand, E. A Growing Toolbox to Image Gene Expression in Single Cells: Sensitive Approaches for Demanding Challenges. *Mol. Cell* **2018**, *71*, 468–480. [CrossRef]

23. Ikeda, M.; Yi, M.; Li, K.; Lemon, S.M. Selectable subgenomic and genome-length dicistronic RNAs derived from an infectious molecular clone of the HCV-N strain of hepatitis C virus replicate efficiently in cultured Huh7 cells. *J. Virol.* **2002**, *76*, 2997–3006. [CrossRef]

24. Lerat, H.; Honda, M.; Beard, M.R.; Loesch, K.; Sun, J.; Yang, Y.; Okuda, M.; Gosert, R.; Xiao, S.-Y.; Weinman, S.A.; et al. Steatosis and liver cancer in transgenic mice expressing the structural and nonstructural proteins of hepatitis C virus. *Gastroenterology* **2002**, *122*, 352–365. [CrossRef]

25. Liang, Y.; Shilagard, T.; Xiao, S.; Snyder, N.; Lau, D.; Cicalese, L.; Weiss, H.; Vargas, G.; Lemon, S.M. Visualizing Hepatitis C Virus Infections in Human Liver by Two-Photon Microscopy. *Gastroenterology* **2009**, *137*, 1448–1458. [CrossRef] [PubMed]

26. Harouaka, D.; Engle, R.E.; Wollenberg, K.; Diaz, G.; Tice, A.B.; Zamboni, F.; Govindarajan, S.; Alter, H.; Kleiner, D.E.; Farci, P. Diminished viral replication and compartmentalization of hepatitis C virus in hepatocellular carcinoma tissue. *Proc. Natl. Acad. Sci. USA* **2016**, *113*, 1375–1380. [CrossRef] [PubMed]

27. Sobesky, R.; Feray, C.; Rimlinger, F.; Derian, N.; Dos Santos, A.; Roque-Afonso, A.-M.; Samuel, D.; Bréchot, C.; Thiers, V. Distinct hepatitis C virus core and F protein quasispecies in tumoral and nontumoral hepatocytes isolated via microdissection. *Hepatology* **2007**, *46*, 1704–1712. [CrossRef]

28. Young, K.-C.; Lin, P.-W.; Hsiao, W.-C.; Chang, T.-T.; Chang, Y.-C.; Wu, H.-L. Variation of hepatitis C virus load, hypervariable region 1 quasispecies and CD81 hepatocyte expression in hepatocellular carcinoma and adjacent non-cancerous liver. *J. Med. Virol.* **2002**, *68*, 188–196. [CrossRef]

29. Bandiera, S.; Pfeffer, S.; Baumert, T.F.; Zeisel, M.B. miR-122—A key factor and therapeutic target in liver disease. *J. Hepatol.* **2015**, *62*, 448–457. [CrossRef]

30. Hibner, U.; Grégoire, D. Viruses in cancer cell plasticity: The role of hepatitis C virus in hepatocellular carcinoma. *Contemp. Oncol.* **2015**, *19*, A62–A67. [CrossRef]

31. Mesri, E.A.; Feitelson, M.A.; Munger, K. Human Viral Oncogenesis: A Cancer Hallmarks Analysis. *Cell Host Microbe* **2014**, *15*, 266–282. [CrossRef] [PubMed]

32. Galloway, D.A.; McDougall, J.K. The oncogenic potential of herpes simplex viruses: Evidence for a "hit-and-run" mechanism. *Nature* **1983**, *302*, 21–24. [CrossRef]

33. Nevels, M.; Täuber, B.; Spruss, T.; Wolf, H.; Dobner, T. "Hit-and-run" transformation by adenovirus oncogenes. *J. Virol.* **2001**, *75*, 3089–3094. [CrossRef]

34. Niller, H.H.; Wolf, H.; Minarovits, J. Viral hit and run-oncogenesis: Genetic and epigenetic scenarios. *Cancer Lett.* **2011**, *305*, 200–217. [CrossRef]

35. Zhuang, X.; Magri, A.; Hill, M.; Lai, A.G.; Kumar, A.; Rambhatla, S.B.; Donald, C.L.; Lopez-Clavijo, A.F.; Rudge, S.; Pinnick, K.; et al. The circadian clock components BMAL1 and REV-ERBα regulate flavivirus replication. *Nat. Commun.* **2019**, *10*, 377. [CrossRef] [PubMed]

36. Lisauskas, T.; Matula, P.; Claas, C.; Reusing, S.; Wiemann, S.; Erfle, H.; Lehmann, L.; Fischer, P.; Eils, R.; Rohr, K.; et al. Live-cell assays to identify regulators of ER-to-Golgi trafficking. *Traffic* **2012**, *13*, 416–432. [CrossRef] [PubMed]

37. Boldanova, T.; Suslov, A.; Heim, M.H.; Necsulea, A. Transcriptional response to hepatitis C virus infection and interferon-alpha treatment in the human liver. *EMBO Mol. Med.* **2017**, *9*, 816–834. [CrossRef]

38. Liberzon, A.; Subramanian, A.; Pinchback, R.; Thorvaldsdóttir, H.; Tamayo, P.; Mesirov, J.P. Molecular signatures database (MSigDB) 3.0. *Bioinformatics* **2011**, *27*, 1739–1740. [CrossRef] [PubMed]

39. Subramanian, A.; Tamayo, P.; Mootha, V.K.; Mukherjee, S.; Ebert, B.L.; Gillette, M.A.; Paulovich, A.; Pomeroy, S.L.; Golub, T.R.; Lander, E.S.; et al. Gene set enrichment analysis: A knowledge-based approach for interpreting genome-wide expression profiles. *Proc. Natl. Acad. Sci. USA* **2005**, *102*, 15545–15550. [CrossRef] [PubMed]

40. Menyhárt, O.; Nagy, Á.; Győrffy, B. Determining consistent prognostic biomarkers of overall survival and vascular invasion in hepatocellular carcinoma. *R. Soc. Open Sci.* **2018**, *5*, 181006. [CrossRef]

41. Llovet, J.M.; Kelley, R.K.; Villanueva, A.; Singal, A.G.; Pikarsky, E.; Roayaie, S.; Lencioni, R.; Koike, K.; Zucman-Rossi, J.; Finn, R.S. Hepatocellular carcinoma. *Nat. Rev. Dis. Prim.* **2021**, *7*, 6. [CrossRef]

42. Levrero, M.; Zucman-Rossi, J. Mechanisms of HBV-induced hepatocellular carcinoma. *J. Hepatol.* **2016**, *64*, S84–S101. [CrossRef] [PubMed]

43. Koike, K. Molecular basis of hepatitis C virus-associated hepatocarcinogenesis: Lessons from animal model studies. *Clin. Gastroenterol. Hepatol.* **2005**, *3*, S132–S135. [CrossRef]

44. Llovet, J.M.; Villanueva, A. Liver cancer: Effect of HCV clearance with direct-acting antiviral agents on HCC. *Nat. Rev. Gastroenterol. Hepatol.* **2016**, *13*, 561–562. [CrossRef]

45. Onodera, H.; Ukai, K.; Suzuki, M.; Minami, Y. Incidence of hepatocellular carcinoma after interferon therapy in patients with chronic hepatitis C. *Tohoku J. Exp. Med.* **1997**, *181*, 275–283. [CrossRef] [PubMed]

46. Romero-Brey, I.; Bartenschlager, R. Endoplasmic Reticulum: The Favorite Intracellular Niche for Viral Replication and Assembly. *Viruses* **2016**, *8*, 160. [CrossRef]

47. Araki, K.; Nagata, K. Protein folding and quality control in the ER. *Cold Spring Harb. Perspect. Biol.* **2011**, *3*, a007526. [CrossRef] [PubMed]

48. Avril, T.; Vauléon, E.; Chevet, E. Endoplasmic reticulum stress signaling and chemotherapy resistance in solid cancers. *Oncogenesis* **2017**, *6*, e373. [CrossRef]

49. Vasallo, C.; Gastaminza, P. Cellular stress responses in hepatitis C virus infection: Mastering a two-edged sword. *Virus Res.* **2015**, *209*, 100–117. [CrossRef]

50. Henkel, A.; Green, R.M. The unfolded protein response in fatty liver disease. *Semin. Liver Dis.* **2013**, *33*, 321–329.

51. Xia, S.-W.; Wang, Z.-M.; Sun, S.-M.; Su, Y.; Li, Z.-H.; Shao, J.-J.; Tan, S.-Z.; Chen, A.-P.; Wang, S.-J.; Zhang, Z.-L.; et al. Endoplasmic reticulum stress and protein degradation in chronic liver disease. *Pharmacol. Res.* **2020**, *161*, 105218. [CrossRef]

52. Liang, D.; Khoonkari, M.; Avril, T.; Chevet, E.; Kruyt, F.A.E. The unfolded protein response as regulator of cancer stemness and differentiation: Mechanisms and implications for cancer therapy. *Biochem. Pharmacol.* **2021**, *192*, 114737. [CrossRef]

53. Hetz, C.; Zhang, K.; Kaufman, R.J. Mechanisms, regulation and functions of the unfolded protein response. *Nat. Rev. Mol. Cell Biol.* **2020**, *21*, 421–438. [CrossRef] [PubMed]

54. Zhong, J.; Gastaminza, P.; Cheng, G.; Kapadia, S.; Kato, T.; Burton, D.R.; Wieland, S.F.; Uprichard, S.L.; Wakita, T.; Chisari, F.V. Robust hepatitis C virus infection in vitro. *Proc. Natl. Acad. Sci. USA* **2005**, *102*, 9294–9299. [CrossRef]

55. Lupberger, J.; Zeisel, M.B.; Xiao, F.; Thumann, C.; Fofana, I.; Zona, L.; Davis, C.; Mee, C.J.; Turek, M.; Gorke, S.; et al. EGFR and EphA2 are host factors for hepatitis C virus entry and possible targets for antiviral therapy. *Nat. Med.* **2011**, *17*, 589–595. [CrossRef] [PubMed]

56. Afgan, E.; Baker, D.; Batut, B.; van den Beek, M.; Bouvier, D.; Cech, M.; Chilton, J.; Clements, D.; Coraor, N.; Grüning, B.A.; et al. The Galaxy platform for accessible, reproducible and collaborative biomedical analyses: 2018 update. *Nucleic Acids Res.* **2018**, *46*, W537–W544. [CrossRef]

57. Kim, D.; Pertea, G.; Trapnell, C.; Pimentel, H.; Kelley, R.; Salzberg, S.L. TopHat2: Accurate alignment of transcriptomes in the presence of insertions, deletions and gene fusions. *Genome Biol.* **2013**, *14*, R36. [CrossRef]

58. Anders, S.; Huber, W. Differential expression analysis for sequence count data. *Genome Biol.* **2010**, *11*, R106. [CrossRef]

Annex III: Combined analysis of metabolome, proteomes, and transcriptomes of HCV- infected cells and liver to identify pathways associated with disease development

Research article

Lupberger J, Croonenborghs T, Roca Suarez AA, Van Renne N, Jühling F, Oudot MA, **Virzì A**, Bandiera S, Jamey C, Meszaros G, Brumaru D, Mukherji A, Durand SC, Heydmann L, Verrier ER, El Saghire H, Hamdane N, Bartenschlager R, Fereshetian S, Ramberger E, Sinha R, Nabian M, Everaert C, Jovanovic M, Mertins P, Carr SA, Chayama K, Dali-Youcef N, Ricci R, Bardeesy NM, Fujiwara N, Gevaert O, Zeisel MB, Hoshida Y, Pochet N, Baumert TF.

Starting from a combination of -omic technologies, this study revealed insights on different aspects related to HCV infection such as HCC and immune evasion (Lupberger et al., 2019; Virzi, Roca Suarez, Baumert, & Lupberger, 2020). HCV represented a valuable model to study signal deregulation which occurs in other liver disease etiologies, such as NAFLD and NASH. Interestingly, it was observed that peroxisomal functions were strongly impaired in HCV-infected hepatocytes, in the liver of HCV-infected patients but not in patients with CHB. Remarkably, IL-6/STAT3 was identified as signaling regulator of peroxisomal function. Indeed, the inhibition of STAT3 signaling by niclosamide, caused a rescue of the peroxisomal genes, such as albumin and catalase.

Using computational analysis, **my contribution** was to predict potential transcription factor binding sites potentially involved in peroxisomal regulation during HCV infection (Supplementary table S5 *online*). These findings provide a potential therapeutic strategy of using STAT3 inhibitors to restore impaired peroxisomal function. Moreover, the study further

Combined Analysis of Metabolome, Proteomes, and Transcriptomes of HCV-infected Cells and Liver to Identify Pathways Associated With Disease Development

Joachim Lupberger[1,2], Tom Croonenborghs[3,4,5], Armando Andres Roca Suarez[1,2], Nicolaas Van Renne[1,2], Frank Jühling[1,2], Marine A. Oudot[1,2], Alessia Virzì[1,2], Simonetta Bandiera[1,2], Carole Jamey[2,6], Gergö Meszaros[2,7,8,9], Daniel Brumaru[2,6], Atish Mukherji[1,2], Sarah C. Durand[1,2], Laura Heydmann[1,2], Eloi R. Verrier[1,2], Hussein El Saghire[1,2], Nourdine Hamdane[1,2], Ralf Bartenschlager[10,11], Shaunt Fereshetian[12], Evelyn Ramberger[13,14], Rileen Sinha[3,4,5], Mohsen Nabian[3,4,5], Celine Everaert[3,4,5], Marko Jovanovic[12,15], Philipp Mertins[12,13,14], Steven A. Carr[12], Kazuaki Chayama[16,17], Nassim Dali-Youcef[2,6,7,8,9], Romeo Ricci[2,7,8,9], Nabeel M. Bardeesy[18], Naoto Fujiwara[19], Olivier Gevaert[4,20], Mirjam B. Zeisel[1,2], Yujin Hoshida[19], Nathalie Pochet[3,4,5], Thomas F. Baumert[1,2,21]

[1]Institut National de la Santé et de la Recherche Médicale, U1110, Institut de Recherche sur les Maladies Virales et Hépatiques, Université de Strasbourg (IVH) Strasbourg F-67000, France,

[2]Université de Strasbourg F-67000, France,

[3]Department of Neurology, Harvard Medical School, Boston, MA 02115, USA,

[4]Cell Circuits Program, Broad Institute of MIT and Harvard, Cambridge, MA 02142, USA,

[5]Ann Romney Center for Neurologic Diseases, Brigham and Women's Hospital, Boston, MA 02115, USA,

[6]Laboratoire de Biochimie et de Biologie Moléculaire, Pôle de biologie, Hôpitaux Universitaires de Strasbourg, Strasbourg F-67091, France,

[7]Institut de Génétique et de Biologie Moléculaire et Cellulaire, Illkirch F-67404, France,

[8]Centre National de la Recherche Scientifique, UMR7104, Illkirch F-67404, France,

[9]Institut National de la Santé et de la Recherche Médicale, U964, Illkirch F-67404, France,

[10]Department of Infectious Diseases, Molecular Virology, Heidelberg University, D-69120 Heidelberg, Germany,

[11]Division Virus-Associated Carcinogenesis, German Cancer Research Center (DKFZ), D-69120 Heidelberg, Germany,

[12]The Broad Institute of Massachusetts Institute of Technology and Harvard, Cambridge, MA 02142, USA,

[13]Proteomics Platform, Max Delbrück Center for Molecular Medicine in the Helmholtz Society, 13125 Berlin, Germany,

[14]Berlin Institute of Health, 13125 Berlin, Germany,

[15]Department of Biological Sciences, Columbia University, NY, USA,

[16]Department of Gastroenterology and Metabolism, Applied Life Sciences, Institute of Biomedical & Health Sciences, Hiroshima University, Hiroshima, Japan,

[17]Liver Research Project Center, Hiroshima University, Hiroshima, Japan,

[18]Massachusetts General Hospital, Boston, MA 02114, USA,

[19]Liver Tumor Translational Research Program, Simmons Comprehensive Cancer Center, Division of Digestive and Liver Diseases, Department of Internal Medicine, University of Texas Southwestern Medical Center, Dallas, TX 75390 USA,

[20]Stanford Center for Biomedical Informatics Research (BMIR), Department of Medicine & Biomedical Data Science, Stanford University, CA 94305, USA,

[21]Pôle Hépato-digestif, Institut Hopitalo-Universitaire, Strasbourg F-67000, France.

Abstract

Background & Aims: The mechanisms of hepatitis C virus (HCV) infection, liver disease progression, and hepatocarcinogenesis are only partially understood. We performed genomic, proteomic, and metabolomic analyses of HCV-infected cells and chimeric mice to learn more about these processes.

Methods: Huh7.5.1$^{\text{dif}}$ (hepatocyte-like cells) were infected with culture-derived HCV and used in RNA-Seq, proteomic, metabolomic, and integrative genomic analyses. uPA/SCID mice were injected with serum from HCV-infected patients; 8 weeks later, liver tissues were collected and analyzed by RNA-seq and proteomics. Using differential expression, gene set enrichment analyses, and protein interaction mapping, we identified pathways that changed in response to HCV infection. We validated our findings in studies of liver tissues from 216 patients with HCV infection and early-stage cirrhosis and paired biopsies from 99 patients with hepatocellular carcinoma, including 17 patients with histologic features of steatohepatitis. Cirrhotic liver tissues from patients with HCV infection were classified into 2 groups based on relative peroxisome function; outcomes assessed included Child-Pugh class, development of hepatocellular carcinoma,

survival and steatohepatitis. Hepatocellular carcinomas were classified according to steatohepatitis; the outcome was relative peroxisomal function.

Results: We quantified 21,950 mRNAs and 8297 proteins in HCV-infected cells. Upon HCV infection of hepatocyte-like cells and chimeric mice, we observed significant changes in levels of mRNAs and proteins involved in metabolism and hepatocarcinogenesis. HCV infection of hepatocyte-like cells significantly increased levels of mRNAs, but not proteins, that regulate the innate immune response—we believe this was due to the inhibition of translation in these cells. HCV infection of hepatocyte-like cells increased glucose consumption and metabolism and the STAT3 signaling pathway and reduced peroxisome function. Peroxisomes mediate beta-oxidation of very long-chain fatty acids (VLCFAs); we found intracellular accumulation of VLCFAs in HCV-infected cells, which is also observed in patients with fatty liver disease. Cells in livers from HCV-infected mice had significant reductions in levels of mRNAs and proteins associated with peroxisome function, indication perturbation of peroxisomes. We associated defects in peroxisome function with outcomes and features of HCV-associated cirrhosis, fatty liver disease, and hepatocellular carcinoma in patients.

Conclusions: We performed combined transcriptome, proteome, and metabolome analyses of liver tissues from HCV-infected hepatocyte-like cells and HCV-infected mice. We found that HCV infection increases glucose metabolism and the STAT3 signaling pathway and thereby reduces peroxisome function; alterations in expression of peroxisome genes were associated with outcomes of patients with liver diseases. These findings provide insights into liver disease pathogenesis and might be used to identify new therapeutic targets.

INTRODUCTION

Viruses have developed sophisticated strategies to promote their life cycle, evade the antiviral defense systems and cause disease. As a result, some viruses can persist beyond the stage of acute infection and develop a state of co-existence with the host through either chronic or latent infection. Viral and cellular gene expression are adjusted over time to meet the requirements of persistence. Considering chronic infection as merely an enduring acute phase is thus fundamentally inaccurate. In fact, the modulations of host biology in the long run are profound and continually damaging the host cell and its microenvironment[1]. This contributes to pathogenic phenotypes including chronic inflammation, tissue injury and cancer. Viral reprogramming of host cells can be investigated by systematic genome-wide profiling of gene-products. Such analysis does not only increase our understanding of the rearrangements of cellular architecture and functions, but also provides mechanistic insight into disease development.

Chronic hepatitis C virus (HCV) infection is an intriguing prototype to study general mechanisms of immune evasion and disease pathogenesis because of the refined strategies to evade antiviral responses, and alteration of metabolic pathways as well as regulatory cell circuits including signaling pathways, translation machinery and RNAi[2–5]. This may have

profound impact on the host cell's proteome and transcriptome resembling patterns induced by other pathologies such as alcohol or obesity. HCV establishes acute and chronic infection of the liver, which is a leading cause of liver disease progressing from chronic inflammation and metabolic disease to fibrosis, cirrhosis and ultimately hepatocellular carcinoma (HCC). It is assumed that HCV contributes to liver disease directly by viral factors and indirectly through signaling. Indeed, tumor expression profiling of cirrhotic livers revealed similar genetic profiles between HCV-associated HCCs and other etiologies[6]. Novel direct antivirals achieve very high cure rates, but despite HCV elimination patients with advanced liver disease remain at high risk for HCC development[7]. Treatment is costly and is currently only available for a fraction of all HCV-infected patients. Furthermore, resolved infection does not provide protection against reinfection, emphasizing the need for increased understanding of immune evasion for effective vaccine development[8].

During recent years thematically focused studies have revealed that virus-host interactions and microbial immune evasion involve the manipulation of both host gene transcription and RNA translation[9, 10]. However, a global and integrated view on the multiple biological layers is required to understand the host antiviral response and mechanisms leading to disease. Using HCV infection as a model we assessed perturbations of the cellular homeostasis contributing to chronic inflammatory disease and virus-induced cancer. We combined a state-of-the art HCV infection model with cutting edge screening technologies including proteomics, RNA sequencing (RNA-Seq), metabolomics and mathematical modeling to gain a multilayered insight into virus-host interaction and its impact for liver disease biology at the systems level. This approach sheds new light on how viruses evade innate immune responses, reprogram host cell metabolism and trigger chronic inflammatory and metabolic disease as well as cancer.

MATERIALS AND METHODS

HCV infection of human hepatocyte chimeric mice.

Mouse uPA+/+/SCID+/+ (uPA/SCID) liver samples used including ethical approval and informed consent have been described[11]. Mice with human albumin levels >10mg/mL (~80–90 % human hepatocytes repopulation) were used for this study. Briefly, mice were intravenously inoculated with HCV+ patient serum (10^5 HCV particles, genotype 1b). Five viremic mice (>10^7 HCV copies/mL) and five control mice were sacrificed at week eight. RNA-seq data on a subset of these mice (three HCV-infected and three non-infected mice) have been described[11]. All liver samples were snap frozen and stored at −80 °C prior to analysis.

Immunofluorescence microscopy.

Cells were fixed with 4 % paraformaldehyde for 20 min prior permeabilization (15 min) with 0.1 % Triton X-I00, 0.5 % BSA. Antibody incubation was performed as recommended. Slides were mounted with Fluoroshield including DAPI (Sigma). Fluorescence dots (puncta) of 25 uninfected and 25 HCV-infected cells were quantified as described[12].

Differentiation and infection of liver cells.

Huh7.5.1[13] and HepG2-NTCP[14] cells have been described. For proliferation arrest and differentiation (Huh7.5.1^{dif} cells), Huh7.5.1 cells were cultured in DMEM containing 1 % DMSO[15] for 10 days before infection. 2.5×10^4 Huh7.5.1^{dif} cells (RNA-Seq and metabolomics) or 1.5×10^6 Huh7.5.1^{dif} cells (proteomics) were infected with a MOI of 8 using affinity purified HCVcc (TCID$_{50}$ 6.7×10^5 / mL) or mock-inoculated with FLAG-peptide elution buffer. After 6 h incubation, the inoculum was replaced by fresh medium supplemented with 1 % DMSO. This MOI is >10× higher than the minimal MOI (0.12) required to infect 100 % of Huh7.5.1^{dif} cells after 7 days (Supplementary Fig. S1C).

Proteome analysis.

Protein lysates were harvested (duplicates) at day 0, 3, 7 and 10 post-infection (pi) for both HCV-infected and mock-infected Huh7.5.1^{dif} cells. Cells were lysed for 15 min at room temperature in 8.0 M urea buffer containing 75 mM NaCl, 50 mM Tris pH 8, 1 mM EDTA, aprotinin 2 μg/mL (Sigma-Aldrich), leupeptin 10 μg/mL (Roche), PMSF 1 mM (Sigma-Aldrich), phosphatase inhibitor cocktail 2 and phosphatase inhibitor cocktail 3 (Sigma-Aldrich). Snap-frozen mouse liver tissues were crushed in liquid nitrogen and lysed, accordingly. Lysates were cleared at full speed in a bench top centrifuge for 5 min, and stored at −80 °C. Proteomic analyses of Huh7.5.1^{dif} lysates were performed at the Broad Institute of MIT and Harvard (Cambridge, MA, USA) and the tissue lysates were analyzed at the Max Delbruck Center for Molecular Medicine/Berlin Institute of Health (Berlin, Germany) using 10-plex tandem mass tag (TMT) labeling as described[16].

Transcriptome profiling.

RNA samples were collected daily between days 0 and 10 pi (triplicates). Cells were lysed in TCL buffer (Qiagen) supplemented with 1 % 2-mercaptoethanol. Cellular mRNA was isolated and analyzed as described[17]. RNA-Seq was performed at the Broad Institute of MIT and Harvard (Cambridge, MA, USA). Therefore, HCV RNA was co-amplified with cellular mRNA using a SMART-compatible primer (sequence: 5'-Biotin-AAG CAG TGG TAT CAA CGC AGA GTA CTC TGC GGA ACC GGT GAG TA-3')[18]. Cellular mRNA was isolated and analyzed according to the SmartSeq2 protocol as described[17, 19]. RNA sequencing paired-end reads were aligned to the human hg19 UCSC reference using TopHat software (v2.0.14). HCV RNA sequencing paired-end reads were aligned to the HCV Jc1E2^{FLAG} genome using Bowtie2 (v2.2.5) software. Gene expression levels for 21,950 human genes were estimated using Cufflinks (v2.2.1) with the *fragments per kilobase of transcript per million mapped reads* method.

Metabolomics and lipid analysis.

Analysis of polar metabolites was performed in Huh7.5.1^{dif} cells infected with HCV Jc1E2^{FLAG} (MOI=8). Intra/extra-cellular metabolites were analyzed by mass spectrometry as described[20] by the Barteesy lab at the Massachusetts General Hospital (Boston, MA, USA). Methyl derivatives of very long-chain fatty acids (VLCFA) were extracted in the presence of internal standards by the Ricci and Dali-Youcef labs and analyzed by gas

chromatography-mass spectrometry (GC-MS) at the Hôpitaux Universitaires de Strasbourg (France) as described[21].

Bioinformatical analyses—Proteins and transcripts were mapped despite possible many-to-many relationships (i.e. isoforms) by constructing "analysis groups"[22] Host responses to HCV infection were assessed over a time course on a temporal population level. Pre-ranked gene set enrichment analysis (GSEA)[23] was performed on a temporal population level, taking time points as continuous class label, and taking the control time course as day 0 samples. To compare the two different sources, an indirect comparison was applied. Each time point for both sources were mapped independently to a functional metaspace by performing GSEA, implemented in GenePattern genomic analysis toolkits[24] for each gene set in the collection. For each time point, we compared the HCV-infected samples with the mock samples using the Signal-To-Noise ratio and the default setting for GSEA. Correlation to time point for the HCV-infected samples was used to obtain a general enrichment over the entire time course. Next, we compared the significant enrichment scores (ES) (p-value <0.005) for the transcriptional and proteomic data sets in this metaspace. The following gene sets from the Molecular Signature Database[25] (MSigDB, ver.4.0) were included for the analysis: BIOCARTA, BIOPLEX, KEGG, NABA, PID, REACTOME, SA, SIG, ST, CORUM, HCVpro, HALLMARK, E1, E2, E3, DUB. "Leading-edge" genes were extracted from all previous GSEA analyses, followed by overlap analyses using the hypergeometric test. The data set was compared to HCVpro database signatures identifying significant overlaps with the current knowledge in HCV-host interactions. Raw reads of patient's samples with low ISG[26] were trimmed using cutadapt[27] and mapped to the human genome hg19 using HISAT2. Reads mapping to GENCODE v19 genes were counted with htseq-count. Differentially expressed genes were analyzed using DESeq2. Common transcription factors as potential regulators of HCV-impaired peroxisome expression were identified using Enrichr[28]. The top 30 transcription factors were retrieved from a combined score ranking (calculated by multiplying p-value (Fisher extract test) and z-score) of the protein-protein interaction (PPI) database and those profiled by ChIP-Seq in mammalian cells (ChEA). These were then successively compared to transcription factors retrieved manually using GeneCards (www.genecards.org), that potentially regulate more than 2 peroxisomal genes. Inference of the transcriptional regulatory networks underlying HCV infection using AMARETTO is described in supplementary information.

RESULTS

An integrated proteogenomic approach reveals the spatiotemporal map of HCV-hepatocyte interactions during infection

We used an hepatocyte-like cell culture model consisting of DMSO-differentiated Huh7-derived liver cells (Huh7.5.1[dif]) because it is suitable for robust, long-term culture and has been shown to have a similar phenotype than primary human hepatocytes in cell culture[15, 29]. Indeed, Huh7.5.1[dif] are quiescent and display enhanced hepatocyte-specific marker expression compared to undifferentiated Huh7.5.1 cells (Supplementary Fig. S1A–B). Huh7.5.1[dif] were infected with HCVcc (Jc1E2[FLAG]) prior sampling during a 10 days culture period (Fig. 1A). HCV protein expression was visualized by a HCV peptide time

course analysis (Fig. 1B) and quantified 59 peptides from 8 viral proteins (Fig. 1C). During infection all HCV peptides increased in abundance until day 7 pi (Fig. 1C) simultaneously ($p<0.0001$, Chi-Square Test), with indifferent expression levels or kinetics ($p>0.05$, U-Test). These results suggest comparable half-lives of the viral proteins indicating that the HCV polyprotein abundance defines the amount of individual cleavage products. We therefore focused our investigations on these first 7 days. In total we quantified 21,950 mRNAs and 8,297 proteins providing a multidimensional atlas of persistent HCV infection. The atlas reveals a time-resolved proteogenomic state of HCV-infected cells. 7,416 proteins (90.8 %) were uniquely mapped to overlapping mRNAs using integrative functional genomic analyses (Fig. 2A, Supplementary table S1). The replicates (n=2) demonstrated excellent sensitivity and technical quality of the approach superior to recent studies for other viral infections[22] (Supplementary Fig. S2).

Disparate dynamics between host cell mRNA and proteins upon HCV infection

We then analyzed 2,006 predefined gene sets representing specific host pathways, cellular functions (MSigDB database) and protein complexes (Bioplex) with our proteogenomic data set using GSEA[23]. This approach classifies differentially expressed genes or proteins according to their representation within a pre-defined gene set associated to a phenotype. 47.4 % (956 gene sets, transcriptomic data set) and 31 % (621 gene sets, proteomic data set) of these gene sets were significantly altered by HCV over 7 days pi (Fig. 2B). Most of these gene sets were generally downregulated (negatively enriched). In gene set collections with >50 gene sets (BIOCARTA, BIOPLEX, KEGG, PID, REACTOME, CORUM, HALLMARK) a median of 62 % and 79 % of RNA and protein, were negatively enriched, respectively. Moreover, 83 % of these downregulated gene sets on the RNA level were also significantly impaired on the protein level. In contrast, only 17 % of the upregulated pathways on the RNA level matched to the corresponding protein trends (Fig. 2C). This suggests that HCV infection shuts down most of the non-vital processes on the transcriptional and/or at the posttranscriptional level to divert the resources toward viral replication and persistence. Upregulated pathways include the antiviral response and inflammation as well as pro-viral signaling pathways as discussed in the supplementary information (Supplementary Fig. S3, Supplementary table S2).

Persistent HCV infection impairs peroxisome function, lipid metabolism, fatty acid and bile acid metabolism

A striking observation from our proteogenomic atlas of HCV infection is a strongly impaired peroxisomal function as suggested by the GSEA at the RNA and protein levels (Fig. 3A). Peroxisomes are involved in lipid synthesis, signaling, ß-oxidation of VLCFAs and the detoxification of hydrogen peroxide[30]. Accordingly, we observed an impaired expression of genes involved in peroxisomal biogenesis, bile acid metabolism, fatty acid metabolism and cholesterol biosynthesis (Fig. 3A). We confirmed these findings in a transcriptomic database comprising liver biopsies of 25 patients with chronic HCV infection and 6 non-infected individuals[26] and in livers of HCV-infected chimeric mice (n=3) (Fig. 3A). Comparing the GSEA results of HCV-infected patients with Huh7.5.1^{dif} we identified 46 leading-edges of the HALLMARK_PEROXISOME gene signature that are impaired in infected Huh7.5.1^{dif} cells and in liver tissue of HCV-infected patients. The expression of 11

of these leading-edge genes changed significantly (p<0.05, Wald test) in HCV-infected Huh7.5.1[dif] cells and in the livers of HCV-infected patients (Fig. 3B, Supplementary Fig. S4). We further validated this finding by demonstrating impaired catalase expression in infected cells (Fig. 3C, Supplementary Fig. S5), which is a peroxisome-specific enzyme. Consistently, quantification of catalase-stained peroxisomes revealed a significantly (p<0.005, T-Test) lower number of catalase puncta formed in HCV-infected cells compared to uninfected hepatocytes (Fig. 3C) suggesting an impaired metabolic function of these organelles and an accumulation of fatty acids in infected cells. Whether less peroxisomes are formed during HCV infection cannot be concluded for sure, however a positive correlation between catalase expression and peroxisome abundance has been recently suggested[31]. Moreover, we demonstrate that HCV infection of Huh7.5.1[dif] increases intracellular concentrations of very long-chain fatty acids with a chain length of 20–26 carbons, while shorter fatty acids (C16–C18) are less accumulated (Fig. 4A). This is consistent with the formation of intrahepatic lipid droplets during infection, which are important during viral assembly[32] and the accumulation of hepatic lipids during steatosis. At the same time, impaired peroxisomes will increase oxidative stress imposed by HCV infection increasing the oncogenic pressure on infected cells. In contrast to HCV, persistent HBV infection increased peroxisomal function and its associated metabolic processes (Fig. 3D), which was confirmed by GSEA of gene expression in HBV-infected primary human hepatocytes (data set GSE69590)[33]. This is consistent with the clinical pathology of chronic HBV infection where steatosis is much less common[34].

Impaired PPAR signaling is a regulator of peroxisomal function during HCV infection

We hypothesized that impaired peroxisomal function was the result of direct, virus-related and indirect effects linked to host cell metabolism. The nuclear receptor peroxisomal proliferator-activated receptor (PPAR) is associated with peroxisome function and highly expressed in the liver. VLCFAs activate PPAR-alpha[35] and high glucose levels impair its activity[36]. Indeed, we observed an impaired PPAR signaling expression signature in HCV-infected Huh7.5.1[dif] cells and in the liver of patients on both RNA and the protein level (Fig. 3A), suggesting a perturbed switch between fatty acid and glucose metabolism in HCV-infected hepatocytes. This is consistent with suppressed PPAR-alpha expression by HCV infection[37]. Therefore, we studied the metabolic status of HCV-infected Huh7.5.1[dif] cells using mass spectrometry-based metabolomic profiling[20]. Analysis of polar metabolites revealed elevated concentrations of the glucose metabolites succinate, pyruvate, 3-phosphoglycerate and citrate in HCV-infected Huh7.5.1[dif] cells (Fig. 4B, Supplementary table S4), consistent with the high glucose-dependence of the HCV replication[38]. Indeed, our analyses revealed an increased glucose consumption (Fig. 4C) and an accumulation of lactate and decrease of malate arguing for impaired gluconeogenesis in infected cells (Fig. 4B). In healthy individuals, lactate is produced and secreted by glycolysis-dependent tissues including skeletal muscle, bone marrow and hypoxic tissue. Lactate is then rapidly metabolized in the liver to glucose and energy. As shown in Supplementary Fig. S6 HCV infection induces hypoxia presumably contributing to lactate production. When measuring the hepatocellular lactate flux, a significant accumulation inside the cells was observed (Fig. 4D). Collectively, these data demonstrate that persistent HCV infection causes elevated glucose levels in infected cells contributing to impaired peroxisomal functions. Moreover,

infection creates a Warburg-like metabolic shift of the host metabolism which is a hallmark of cancer or cells undergoing carcinogensis[39]. Next, we aimed to identify common transcription factors of peroxisomal genes that are potentially interacting with HCV proteins. Thereto, we identified common transcription factor binding sites among the combined 85 leading edge genes of the HALLMARK_PEROXISOME gene set in livers of HCV-infected patients and Huh7.5.1[dif] cells (Supplementary table S5) by using Enrichr28. Furthermore, we added to the 11 leading edge genes described in Fig. 3B common transcription factors potentially binding to more than 2 leading edge genes. As a result, we predicted 15 transcription factors potentially involved in the transcription of peroxisomal genes and suppressed by HCV infection (Supplementary table S5). Among these HCV-sensitive regulators were PPAR-alpha (*PPARA*) and its functional partner Retinoid X Receptor Beta (*RXRB*). Moreover, a comparison of the identified transcription factors with the HCVpro protein interaction database of HCV-host interactions[40], revealed that HCV core protein associates to PPAR-alpha and RXR-beta. In line with these computational analyses, we observed that HCV infection strongly inhibits PPAR-alpha expression in Huh7.5.1[dif] (Fig. 5A). Finally, we analyzed the RNA-Seq profiles of the HCV infection and non-infected control time courses using the AMARETTO algorithm[41]. This regulatory network inference tool learns regulatory modules by connecting known regulatory driver genes with co-expressed target genes that they control. Interestingly, AMARETTO highlighted interleukin 6 receptor (IL-6R) as a driver candidate of a module comprising a significant (FDR<0.001) functional negative enrichment of pathways associated to peroxisome function (7 genes), fatty acid (12 genes) and lipid metabolism (20 genes) (Fig. S7, Supplementary Table S6–7). This suggests a functional role for IL-6/STAT3 signaling in the regulation of peroxisome function in HCV infection. Indeed, infection induces a STAT3 transcriptional signature[5] (Supplementary Fig. S6) and STAT3 activation cause a rapid inhibition of IL-6R transcripts (Fig. 5B) as part of a negative feedback regulation. Moreover, inhibition of STAT3 activity by niclosamide (Fig. 5C) rescued the virus-induced inhibition of peroxisomal genes (Fig. 5D) suggesting a regulatory link between STAT3 signaling and peroxisomes. Indeed, inflammatory pathways regulate PPAR family members[42].

Taken together, our data suggest that HCV infection suppresses peroxisomal function at different levels by interfering with PPAR-alpha expression and function. This impacts the metabolic switch between fatty acid and glucose metabolism and thus contributes to glucose-dependence and lipid accumulations in infected hepatocytes that is a hallmark of chronic liver disease and HCC development.

Impaired peroxisomal function is associated with HCV clinical liver disease including cirrhosis and HCC

As perturbed peroxisome function might be relevant for outcome of HCV-associated liver disease, we analyzed the gene expression in liver biopsies from 216 patients with HCV-related early-stage liver cirrhosis[43] as well as in paired liver biopsies of patients with HCV-associated HCC[44] (Supplementary table S8–9). Comparing the enrichment of the HALLMARK_PEROXISOME gene set in each individual sample to the clinical outcome of the patients, we revealed a significant association of peroxisomal gene expression with liver cirrhosis (p=0.001, log-rank test), HCC development (p=0.03, log-rank test) and patient

survival from HCC (p=0.006, log-rank test) (Fig. 6A). This suggests that HCV patients with low peroxisomal function have high risk for poor clinical outcomes. Moreover, peroxisomal gene expression significantly correlates with collagen, type I, alpha 1 expression (*COL1A1*) (R=−0.16, p=0.02, Spearman correlation test) and with α-smooth muscle actin (ACTA2) (R= −0.19, p=0.004, Spearman correlation test) (Fig. 6B), suggesting elevated extracellular matrix deposition and liver injury in patient livers with low peroxisomal activity[6]. This also reflects the observed negative association of the peroxisomal gene expression with the Child-Pugh liver disease score in Fig. 6A. Interestingly, peroxisomal gene expression was reduced in tumor (*T*) and adjacent tissue (*A*) of HCC patients with steatohepatitis (SH-HCC) (Fig. 6C) suggesting that an impaired peroxisome is not only associated to fatty liver development but also a hallmark of tumors reflecting their glucose dependence and the shutting down of beta-oxidation[45, 46]. We validated key findings in Fig. 6 using *CAT* expression (Supplementary Fig. S8) thus, confirming the reliability of *CAT* as functional peroxisomal marker in patients. Reactivation of peroxisomal function during liver disease may therefore reduce oxidative stress and the risk of disease progression toward liver cirrhosis and HCC.

DISCUSSION

Chronic liver disease generally progresses from steatosis, inflammation to fibrosis, cirrhosis and ultimately to HCC. The striking similarities in liver disease progression independent of the underlying etiology, suggests the presence of common drivers and deregulated pathways that promote liver pathogenesis. In this study, we have unraveled the temporal proteogenomic atlas of persistent HCV infection that sheds light to open questions in HCV-host interactions and uncovers virus-induced perturbations of host cell circuits driving viral pathogenesis and disease progression. Using a highly efficient and reproducible infection model, we mapped and quantified for the first time the entire viral proteome in the early and steady-state phase of infection. Surprisingly, infection of the quiescent hepatocyte-like cells did mount a robust interferon response on the RNA level including expression signatures from dsRNA sensors RIG-I, MDA5, TLR3 and their downstream effector IRF3. This is consistent with reports demonstrating MDA5 being more critical for HCV sensing than RIG-I[47, 48]. HCV replication intermediates are also partially recognized by the TLR3 protein, however not sufficient enough to mount a fully effective host response against HCV[49]. However, all these antiviral gene expression patterns do not translate into protein in Huh7.5.1[dif]. These findings support a model where HCV infection overcomes the antiviral defense in hepatocytes mainly by inhibiting the translation of ISG mRNAs via the induction of PKR activity as previously suggested[50]. Indeed, HCV-infection of our model strongly induced PKR (*EIF2AK2*) expression at the RNA level which led to a self-limiting expression of PKR protein that spiked at day 1 pi (Supplementary table S1). Why in HCV-infected chimeric mice this evasion strategy seems to be distinctly different from HCV-infected patients and Huh7.5.1[dif] is an interesting observation and may be useful to refine our understanding of viral immune evasion. This shows that the Huh7.5.1[dif] is a useful model to study and understand the innate immune responses to HCV infection and serves as an example of how this data set can serve as a resource file to understand temporal changes and putative discrepancies in the proteogenomic landscape of HCV-host interactions. Using

this atlas, we validated cancer relevant pro-oncogenic pathways and gene signatures in a thus far unparalleled depth and comprehension (Supplementary Fig. S6) and correlated it with metabolic features resembling cancer, i.e. a Warburg-like shift of the lactate flux in infected cells. Of note, the transcriptomic patterns identified in HCV-infected Huh7.5.1[dif] were similar to those in liver tissue of patients and HCV-infected human liver chimeric mice (Figs. 3A, Supplementary Figs. S3 and S6) further emphasizing the suitability of this atlas to identify previously unrecognized disease-relevant processes *in vivo*.

Peroxisomes are key organelles for VLCF metabolism and detoxification of membrane-permeable peroxides and oxidative stress. We observed a marked decrease of the peroxisomal function in HCV-infected hepatocytes, in the livers of HCV patients and in infected chimeric mice, which is distinctly different from patients with chronic hepatitis B where peroxisomal function is increased (Fig. 3). Consistently, HCV but rarely HBV infection is associated with fat accumulations in the liver leading to NAFLD and NASH[51]. Both HBV and HCV induce NF-κB signaling[52], contributing to chronic liver inflammation, injury and disease progression. Inflammation is a regulator of host metabolism[53]. However, only HCV but not HBV infection impairs peroxisomal function and predominantly induces fat inclusions in hepatocytes arguing for an additional HCV-specific molecular mechanism suppressing fatty acid metabolism. Complementary to previous lipidomic analysis that suggested an HCV-induced accumulation of phospholipids and sphingomyelins[38] our data revealed that HCV cause a phenotype of VLCFA accumulation, which corresponds to impaired peroxisomal beta-oxidation. Consequently, HCV infection of differentiated cells as used here mimics a phenotype resembling fatty liver disease and its complication NASH, which is characterized by fatty acid accumulations and chronic inflammation[54]. HCV infection requires lipid droplets for particle assembly[55]. The suppressed peroxisomal activity in HCV-infected cells is thus of potential advantage to the virus since it contributes to accumulation of long-chain fatty acids in infected hepatocytes with potential impact on liver pathogenesis. Indeed, HCV-infected cells display a higher abundance of phosphatidylcholines and triglycerides with longer chain fatty acids[56], which are preferentially metabolized in the peroxisome. This resembles the situation in NASH patients, where increased systemic phosphatidylcholine levels are observed[57] and suggests that HCV infection is a model to study the molecular mechanisms of fatty liver disease independent of the underlying etiology. Moreover, high levels of VLCFA in serum provoked hepatic steatosis, NASH, and HCC in an animal model[58]. Our data suggest that re-activation of HCV-impaired nuclear receptors like PPAR-alpha has a potential clinical relevance for HCC prevention. Furthermore, targeting of PPAR-alpha is in development for NASH, where treatment options are currently limited. Indeed, PPAR stimulation improves steatosis, inflammation and fibrosis in pre-clinical models of NAFLD[59, 60], albeit with limited clinical efficacy. Alternatively, our data highlight a potential new strategy for a restoration of peroxisomal function using clinical STAT3 inhibitors. Collectively, these data indicate that HCV and NASH share similar pathways driving the pathogenesis of liver disease, which are distinctly different from HBV-associated liver disease. The highly similar results obtained in livers of HCV-infected chimeric mice and livers and HCV patients suggest the hepatocyte-like HCV-Huh7.5.1[dif] as suitable model for the discovery of targets and compounds of metabolic liver disease. Our atlas validated previous transcriptomic studies demonstrating an

association of HCV with genes involved in lipid metabolism and reactive oxygen species[61, 62] in an unprecedented temporal resolution, which allowed the prediction of regulatory networks (Supplementary data S7). The multi-omics approach in this study integrated transcriptome, proteome, polar metabolites and lipid analysis revealed novel mechanistic insights in the regulation of peroxisomal function by an interplay of glucose levels and HCV-induced cytokine signaling.

Finally, the temporal proteogenomic atlas of HCV infection is a useful and unique resource data set for researchers to validate individual hypotheses in virus-host interactions and liver disease biology. Moreover, the convenient upscaling, the high reproducibility, and the high similarity with gene expression profiles in livers of HCV patients emphasizes the potential of this model for screening approaches targeting drivers of liver disease pathobiology and cancer-risk to identify therapeutics for liver disease in general.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENTS

Abbreviations:

ATCC	American Type Culture Collection
ChEA	DNA sequencing after chromatin immunoprecipitation in mammalian cells
ChIP-Seq	DNA sequencing after chromatin immunoprecipitation
CTRL	control

DAPI	4',6-diamidino-2-phenylindole
DMSO	dimethyl sulfoxide
dsRNA	double-stranded RNA
EDTA	ethylenediaminetetra-acetic acid
EMT	epithelial-mesenchymal transition
ES	enrichment score
GC-MS	gas chromatography-mass spectrometry
GEO	Gene expression Omnibus
GSEA	gene set enrichment analysis
HBsAg	hepatitis B virus surface antigen
HBV	hepatitis B virus
HCC	hepatocellular carcinoma
HCV	hepatitis C virus
HCVcc	cell culture-derived hepatitis C virus
IL-6	Interleukin 6
ISGs	interferon-stimulated genes
LC-MS/MS	liquid chromatography-tandem mass spectrometry
MAPK	mitogen-activated protein kinase
MOI	Multiplicity of Infection
MSigDB	The Molecular Signatures Database
NAFLD	non-alcoholic fatty liver disease
NASH	non-alcoholic steatohepatitis
NES	normalized enrichment score
NF-κB	Nuclear factor kappa B
pi	post-infection
PMSF	phenylmethylsulfonyl fluoride
PPARA	Peroxisome proliferator-activated receptor alpha
PPI	protein-protein interaction
RNAi	RNA interference

RNA-Seq	RNA sequencing
SD	standard deviation
SH	steatohepatitis
SH-HCC	steatohepatitis-associated hepatocellular carcinoma
SRA	Sequence Read Archive
STAT3	Signal transducer and activator of transcription 3
TCID$_{50}$	50 % tissue culture infective dose
TMT	tandem mass tag
uPA/SCID	urokinase-type plasminogen activator/severe combined immunodeficiency
VLCFA	very long-chain fatty acid.

REFERENCES

1. Pittman KJ, Aliota MT, Knoll LJ. Dual transcriptional profiling of mice and Toxoplasma gondii during acute and chronic infection. BMC Genomics 2014; 15:806. [PubMed: 25240600]

2. Lupberger J, Casanova C, Fischer B, et al. PI4K-beta and MKNK1 are regulators of hepatitis C virus IRES-dependent translation. Sci Rep 2015;5:13344. [PubMed: 26323588]

3. Lupberger J, Zeisel MB, Xiao F, et al. EGFR and EphA2 are host factors for hepatitis C virus entry and possible targets for antiviral therapy. Nat Med 2011;17:589–95. [PubMed: 21516087]

4. Mailly L, Xiao F, Lupberger J, et al. Clearance of persistent hepatitis C virus infection in humanized mice using a claudin-1-targeting monoclonal antibody. Nat Biotechnol 2015;33:549–554. [PubMed: 25798937]

5. Van Renne N, Roca Suarez AA, Duong FHT, et al. miR-135a-5p-mediated downregulation of protein tyrosine phosphatase receptor delta is a candidate driver of HCV-associated hepatocarcinogenesis. Gut 2018;67:953–962. [PubMed: 28159835]

6. Nakagawa S, Wei L, Song WM, et al. Molecular Liver Cancer Prevention in Cirrhosis by Organ Transcriptome Analysis and Lysophosphatidic Acid Pathway Inhibition. Cancer Cell 2016;30:879–890. [PubMed: 27960085]

7. van der Meer AJ, Feld JJ, Hofer H, et al. Risk of cirrhosis-related complications in patients with advanced fibrosis following hepatitis C virus eradication. J Hepatol 2017;66:485–493. [PubMed: 27780714]

8. Bartenschlager R, Baumert TF, Bukh J, et al. Critical challenges and emerging opportunities in hepatitis C virus research in an era of potent antiviral therapy: Considerations for scientists and funding agencies. Virus Res 2018;248:53–62. [PubMed: 29477639]

9. Arvey A, Tempera I, Tsai K, et al. An atlas of the Epstein-Barr virus transcriptome and epigenome reveals host-virus regulatory interactions. Cell Host Microbe 2012;12:233–45. [PubMed: 22901543]

10. Ramage HR, Kumar GR, Verschueren E, et al. A combined proteomics/genomics approach links hepatitis C virus infection with nonsense-mediated mRNA decay. Mol Cell 2015;57:329–40. [PubMed: 25616068]

11. Hamdane N, Jühling F, Crouchet E, et al. HCV induces persistent epigenetic reprogramming post sustained virological response associated with liver cancer risk. Gastroenterology 2019;in press.

12. Di Cara F, Sheshachalam A, Braverman NE, et al. Peroxisome-Mediated Metabolism Is Required for Immune Response to Microbial Infection. Immunity 2017;47:93–106 e7. [PubMed: 28723556]

13. Zhong J, Gastaminza P, Cheng G, et al. Robust hepatitis C virus infection in vitro. Proc Natl Acad Sci U S A 2005;102:9294–9. [PubMed: 15939869]

14. Verrier ER, Yim SA, Heydmann L, et al. Hepatitis B virus evasion from cGAS sensing in human hepatocytes. Hepatology 2018.

15. Bauhofer O, Ruggieri A, Schmid B, et al. Persistence of HCV in quiescent hepatic cells under conditions of an interferon-induced antiviral response. Gastroenterology 2012;143:429–38 e8. [PubMed: 22522091]

16. Svinkina T, Gu H, Silva JC, et al. Deep, Quantitative Coverage of the Lysine Acetylome Using Novel Anti-acetyl-lysine Antibodies and an Optimized Proteomic Workflow. Molecular & cellular proteomics : MCP 2015;14:2429–40. [PubMed: 25953088]

17. Trombetta JJ, Gennert D, Lu D, et al. Preparation of Single-Cell RNA-Seq Libraries for Next Generation Sequencing. Curr Protoc Mol Biol 2014;107:4 22 1–4 22 17. [PubMed: 24984854]

18. Xiao F, Fofana I, Heydmann L, et al. Hepatitis C virus cell-cell transmission and resistance to direct-acting antiviral agents. PLoS Pathog 2014;10:e1004128. [PubMed: 24830295]

19. Picelli S, Faridani OR, Bjorklund AK, et al. Full-length RNA-seq from single cells using Smart-seq2. Nature protocols 2014;9:171–81. [PubMed: 24385147]

20. Nicolay BN, Gameiro PA, Tschop K, et al. Loss of RBF1 changes glutamine catabolism. Genes & development 2013;27:182–96. [PubMed: 23322302]

21. Takemoto Y, Suzuki Y, Horibe R, et al. Gas chromatography/mass spectrometry analysis of very long chain fatty acids, docosahexaenoic acid, phytanic acid and plasmalogen for the screening of peroxisomal disorders. Brain Dev 2003;25:481–7. [PubMed: 13129591]

22. Jovanovic M, Rooney MS, Mertins P, et al. Immunogenetics. Dynamic profiling of the protein life cycle in response to pathogens. Science 2015;347:1259038. [PubMed: 25745177]

23. Subramanian A, Tamayo P, Mootha VK, et al. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. Proc Natl Acad Sci U S A 2005;102:15545–50. [PubMed: 16199517]

24. Reich M, Liefeld T, Gould J, et al. GenePattern 2.0. Nat Genet 2006;38:500–1. [PubMed: 16642009]

25. Liberzon A, Birger C, Thorvaldsdottir H, et al. The Molecular Signatures Database (MSigDB) hallmark gene set collection. Cell Syst 2015;1:417–425. [PubMed: 26771021]

26. Boldanova T, Suslov A, Heim MH, et al. Transcriptional response to hepatitis C virus infection and interferon-alpha treatment in the human liver. EMBO Mol Med 2017;9:816–834. [PubMed: 28360091]

27. Martin M Cutadapt removes adapter sequences from high-throughput sequencing reads. EMBnet.journal 2011;17:10–12.

28. Chen EY, Tan CM, Kou Y, et al. Enrichr: interactive and collaborative HTML5 gene list enrichment analysis tool. BMC Bioinformatics 2013;14:128. [PubMed: 23586463]

29. Sainz B Jr., Chisari FV. Production of infectious hepatitis C virus by well-differentiated, growth-arrested human hepatoma-derived cells. J Virol 2006;80:10253–7. [PubMed: 17005703]

30. Lodhi IJ, Semenkovich CF. Peroxisomes: a nexus for lipid metabolism and cellular signaling. Cell metabolism 2014;19:380–92. [PubMed: 24508507]

31. Lee JN, Dutta RK, Maharjan Y, et al. Catalase inhibition induces pexophagy through ROS accumulation. Biochem Biophys Res Commun 2018;501:696–702. [PubMed: 29753736]

32. Paul D, Madan V, Bartenschlager R. Hepatitis C virus RNA replication and assembly: living on the fat of the land. Cell Host Microbe 2014;16:569–79. [PubMed: 25525790]

33. Yoneda M, Hyun J, Jakubski S, et al. Hepatitis B Virus and DNA Stimulation Trigger a Rapid Innate Immune Response through NF-kappaB. J Immunol 2016;197:630–43. [PubMed: 27288535]

34. Fan JG, Farrell GC. Epidemiology of non-alcoholic fatty liver disease in China. J Hepatol 2009;50:204–10. [PubMed: 19014878]

35. Kersten S, Desvergne B, Wahli W. Roles of PPARs in health and disease. Nature 2000;405:421–4. [PubMed: 10839530]

36. Roduit R, Morin J, Masse F, et al. Glucose down-regulates the expression of the peroxisome proliferator-activated receptor-alpha gene in the pancreatic beta -cell. J Biol Chem 2000;275:35799–806. [PubMed: 10967113]

37. Dharancy S, Malapel M, Perlemuter G, et al. Impaired expression of the peroxisome proliferator-activated receptor alpha during hepatitis C virus infection. Gastroenterology 2005;128:334–42. [PubMed: 15685545]

38. Diamond DL, Syder AJ, Jacobs JM, et al. Temporal proteome and lipidome profiles reveal hepatitis C virus-associated reprogramming of hepatocellular metabolism and bioenergetics. PLoS Pathog 2010;6:e1000719. [PubMed: 20062526]

39. San-Millan I, Brooks GA. Reexamining cancer metabolism: lactate production for carcinogenesis could be the purpose and explanation of the Warburg Effect. Carcinogenesis 2017;38:119–133. [PubMed: 27993896]

40. Kwofie SK, Schaefer U, Sundararajan VS, et al. HCVpro: hepatitis C virus protein interaction database. Infect Genet Evol 2011;11:1971–7. [PubMed: 21930248]

41. Champion M, Brennan K, Croonenborghs T, et al. Module Analysis Captures Pancancer Genetically and Epigenetically Deregulated Cancer Driver Genes for Smoking and Antiviral Response. EBioMedicine 2018;27:156–166. [PubMed: 29331675]

42. Mukherji A, Kobiita A, Ye T, et al. Homeostasis in intestinal epithelium is orchestrated by the circadian clock and microbiota cues transduced by TLRs. Cell 2013;153:812–27. [PubMed: 23663780]

43. Hoshida Y, Villanueva A, Sangiovanni A, et al. Prognostic gene expression signature for patients with hepatitis C-related early-stage cirrhosis. Gastroenterology 2013;144:1024–30. [PubMed: 23333348]

44. Hoshida Y, Villanueva A, Kobayashi M, et al. Gene expression in fixed tissues and outcome in hepatocellular carcinoma. N Engl J Med 2008;359:1995–2004. [PubMed: 18923165]

45. Buzzai M, Bauer DE, Jones RG, et al. The glucose dependence of Akt-transformed cells can be reversed by pharmacologic activation of fatty acid β-oxidation. Oncogene 2005;24:4165. [PubMed: 15806154]

46. Fujiwara N, Nakagawa H, Enooku K, et al. CPT2 downregulation adapts HCC to lipid-rich environment and promotes carcinogenesis via acylcarnitine accumulation in obesity. Gut 2018;67:1493–1504. [PubMed: 29437870]

47. Cao X, Ding Q, Lu J, et al. MDA5 plays a critical role in interferon response during hepatitis C virus infection. J Hepatol 2015;62:771–8. [PubMed: 25463548]

48. Hiet MS, Bauhofer O, Zayas M, et al. Control of temporal activation of hepatitis C virus-induced interferon response by domain 2 of nonstructural protein 5A. J Hepatol 2015;63:829–37. [PubMed: 25908268]

49. Grunvogel O, Colasanti O, Lee JY, et al. Secretion of Hepatitis C Virus Replication Intermediates Reduces Activation of Toll-Like Receptor 3 in Hepatocytes. Gastroenterology 2018;154:2237–2251 e16. [PubMed: 29535029]

50. Garaigorta U, Chisari FV. Hepatitis C virus blocks interferon effector function by inducing protein kinase R phosphorylation. Cell Host Microbe 2009;6:513–22. [PubMed: 20006840]

51. Pais R, Rusu E, Zilisteanu D, et al. Prevalence of steatosis and insulin resistance in patients with chronic hepatitis B compared with chronic hepatitis C and non-alcoholic fatty liver disease. Eur J Intern Med 2015;26:30–6. [PubMed: 25553983]

52. Hiscott J, Kwon H, Genin P. Hostile takeovers: viral appropriation of the NF-kappaB pathway. J Clin Invest 2001;107:143–51. [PubMed: 11160127]

53. Konner AC, Bruning JC. Toll-like receptors: linking inflammation to metabolism. Trends Endocrinol Metab 2011;22:16–23. [PubMed: 20888253]

54. Farrell GC, Haczeyni F, Chitturi S. Pathogenesis of NASH: How Metabolic Complications of Overnutrition Favour Lipotoxicity and Pro-Inflammatory Fatty Liver Disease. Adv Exp Med Biol 2018;1061:19–44. [PubMed: 29956204]

55. Miyanari Y, Atsuzawa K, Usuda N, et al. The lipid droplet is an important organelle for hepatitis C virus production. Nat Cell Biol 2007;9:1089–97. [PubMed: 17721513]

56. Hofmann S, Krajewski M, Scherer C, et al. Complex lipid metabolic remodeling is required for efficient hepatitis C virus replication. Biochim Biophys Acta Mol Cell Biol Lipids 2018;1863:1041–1056. [PubMed: 29885363]

57. Anjani K, Lhomme M, Sokolovska N, et al. Circulating phospholipid profiling identifies portal contribution to NASH signature in obesity. J Hepatol 2015;62:905–12. [PubMed: 25450212]

58. Hardwick JP, Osei-Hyiaman D, Wiland H, et al. PPAR/RXR Regulation of Fatty Acid Metabolism and Fatty Acid omega-Hydroxylase (CYP4) Isozymes: Implications for Prevention of Lipotoxicity in Fatty Liver Disease. PPAR Res 2009;2009:952734. [PubMed: 20300478]

59. Pawlak M, Lefebvre P, Staels B. Molecular mechanism of PPARalpha action and its impact on lipid metabolism, inflammation and fibrosis in non-alcoholic fatty liver disease. J Hepatol 2015;62:720–33. [PubMed: 25450203]

60. Friedman SL, Neuschwander-Tetri BA, Rinella M, et al. Mechanisms of NAFLD development and therapeutic strategies. Nat Med 2018;24:908–922. [PubMed: 29967350]

61. Blackham S, Baillie A, Al-Hababi F, et al. Gene expression profiling indicates the roles of host oxidative stress, apoptosis, lipid metabolism, and intracellular transport genes in the replication of hepatitis C virus. J Virol 2010;84:5404–14. [PubMed: 20200238]

62. Deng Y, Wang J, Huang M, et al. Inhibition of miR-148a-3p resists hepatocellular carcinoma progress of hepatitis C virus infection through suppressing c-Jun and MAPK pathway. J Cell Mol Med 2019;23:1415–1426. [PubMed: 30565389]
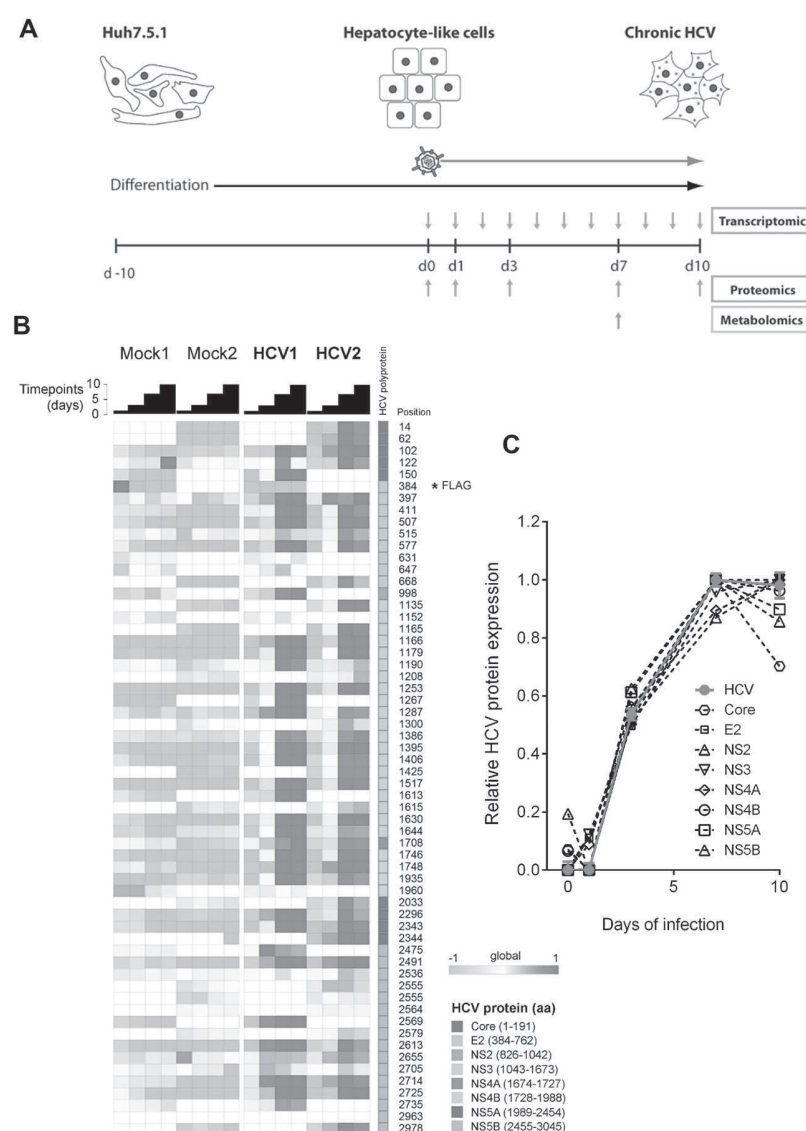
Fig. 1. Mapping of HCV protein expression by time-resolved proteogenomics.
(**A**) Hepatocyte-like Huh7.5.1^{dif} cells were infected with HCVcc (Jc1E2^{FLAG}) over 10 days. Sampling intervals for transcriptomics, metabolomics and proteomics are indicated. (**B**) Quantification of HCV-specific peptides over the infection time course relative to non-infected and mock-infected (Mock) cells. The data represent TMT ratios with the 131 channels in the denominator (internal reference = mix of all samples). All ratios were normalized for the median of all 120,000 distinct peptides for each time point. The FLAG peptide is indicated by asterisk. (**C**) Increase of HCV protein abundance between 3–7 days pi. Data are displayed as relative expression (mean) of all peptides corresponding to a given HCV protein over the time course. HCV replication is depicted in red as median of total HCV peptide abundance ± standard error of the median.
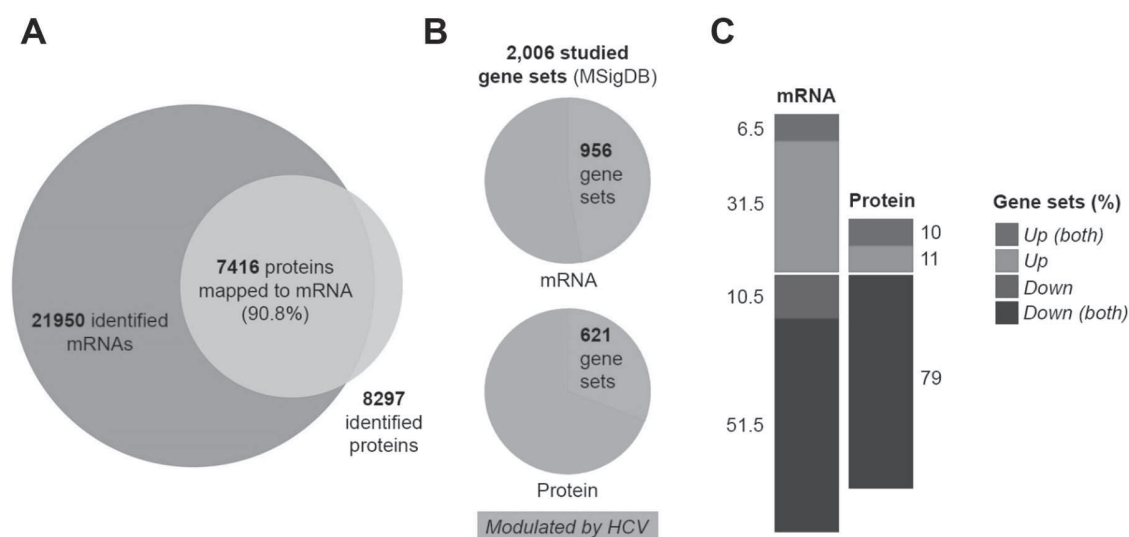
Fig. 2. Persistent HCV infection manipulates host pathways and triggers an attenuated innate immune response.

HCV-infected Huh7.5.1$^{\text{dif}}$ cells relative to mock-infected cells until 7 days pi. (**A**) Proteogenomic mapping of HCV infection. (**B**) Gene sets obtained from the MSigDB that are modulated by HCV infection on the RNA and protein level. (**C**) Stimulation (red) and suppression (blue) of host pathways (gene sets) by HCV infection.
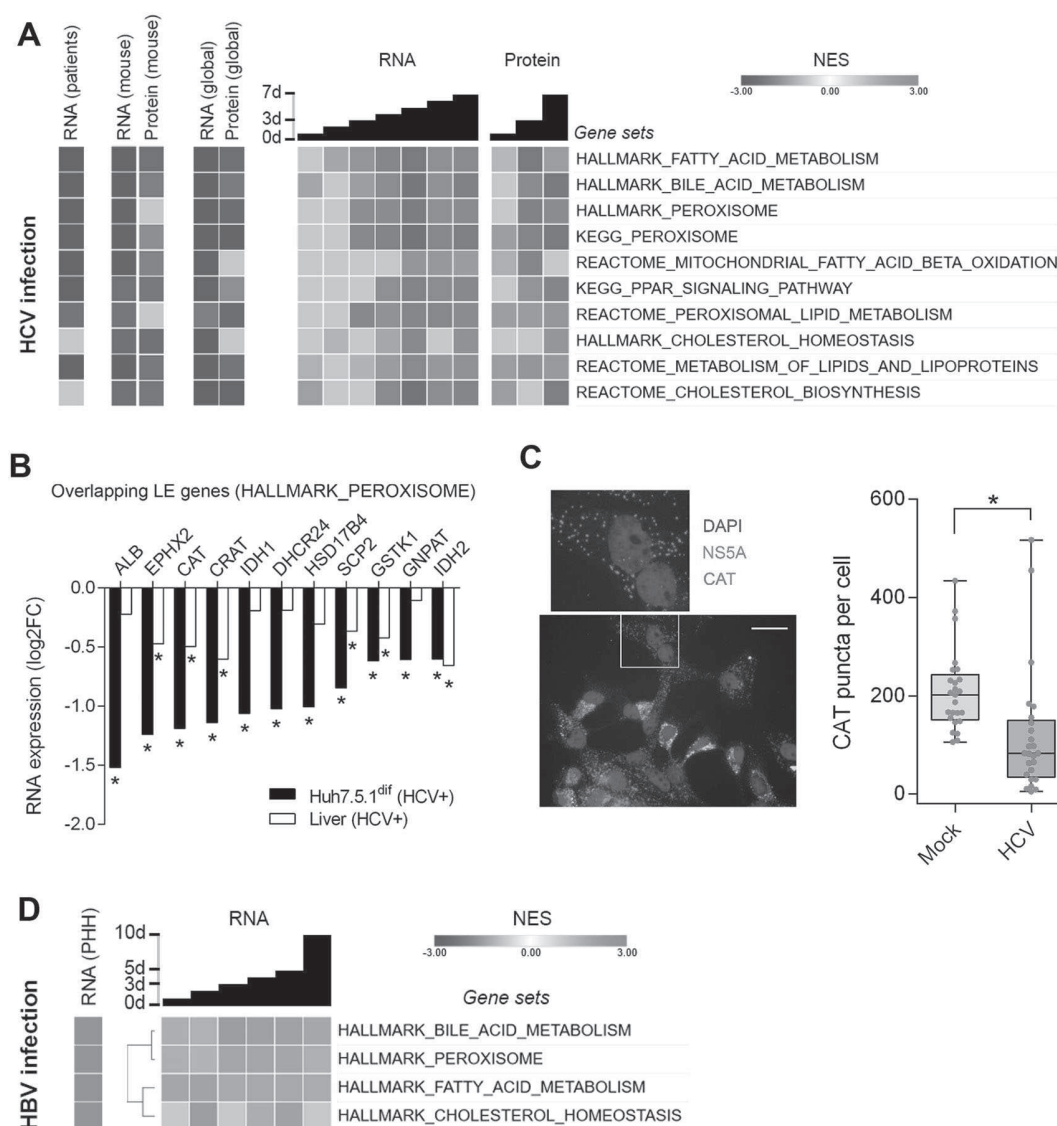
Fig. 3. HCV but not HBV infection impairs expression of peroxisomal genes in Huh7.5.1[dif] cells and liver tissue of patients.

(**A**) HCV infection impairs metabolic pathways associated to peroxisomal function and lipid homeostasis. GSEA of RNA-Seq data from liver tissue of 25 chronic HCV-infected patients vs. 6 non-infected individuals[26], and transcriptomics and proteomics of HCV-infection time-course of Huh7.5.1[dif] relative to mock-infected cells (n=2). (**B**) Expression of 11 peroxisomal genes is significantly (p<0.05, Wald test) suppressed by HCV in Huh7.5.1[dif] and in liver tissue of patients with chronic HCV infection[26]. Log fold change of leading-edge gene expression of the HALLMARK_PEROXISOME gene set (A). (**C**) Peroxisome marker expression is significantly (p=0.0048, T-Test) perturbed in HCV-infected hepatocyte-like cells. Immunofluorescence microscopy of Huh7.5.1[dif] cells infected for 3 days with HCVcc. The peroxisomal marker catalase (CAT) is stained in red, nuclear DNA (DAPI) in blue and HCV (NS5A) in green (see also supplementary Fig. S5). Quantification of catalase-stained peroxisomes in 25 random HCV-infected cells and 25 mock cells is shown in box/

whiskers; bar represents 200 μm. (**D**) HBV infection promotes peroxisome function. GSEA of transcriptomics of HBV-infection of HepG2-NTCP for 10 days (n=3), and primary human hepatocytes (n=3) infected for 40 days with HBV (genotype D) (GSE69590). NES are displayed in red (increased), blue (decreased), and gray (no significant change). Temporal analysis of infected Huh7.5.1$^{\text{dif}}$ are presented as global trend (global) and individual timepoints. Statistical cut-off for GSEA of liver tissues was FDR q<0.05 and for Huh7.5.1$^{\text{dif}}$ p<0.005.
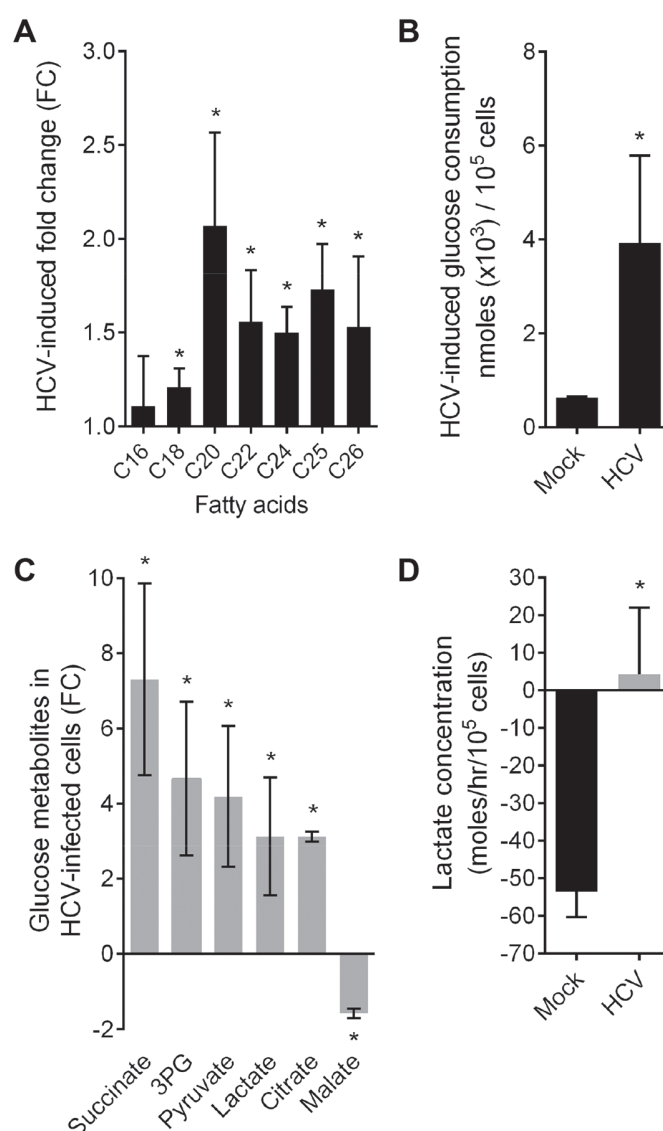
Fig. 4. Persistent HCV infection enhances metabolism of intracellular very long-chain fatty acids and glucose and creates a Warburg-like shift of the lactate flux.

(**A**) HCV infection induces intracellular accumulation of very long-chain fatty acids (VLCFA). Data expressed as mean fold change of intracellular C16 (palmitic acid), C18 (oleic acid), C20 (arachidic acid), C22 (behenic acid), C24 (lignoceric acid), C25 (pentacosanoic acid), C26 (cerotic acid) fatty acids relative to mock-infected cells ±SEM (2–3 independent experiments). (**B**) HCV-infection increases the concentration of glucose metabolites in infected Huh7.5.1^{dif} except for malate. Mean fold change of intracellular polar metabolites per 100,000 cells ±SD (4 independent experiments in triplicates). (**C**) HCV infection of Huh7.5.1^{dif} increases glucose consumption ~6 fold. Glucose consumption of HCV- and mock-infected (CTRL) cells was measured at day 7 pi in supernatants. Mean glucose consumption per 100,000 cells ±SD (2 independent experiments in triplicates). (**D**) HCV creates a Warburg-like shift in infected cells. Lactate uptake (negative value) from culture supernatant inverses to lactate secretion (positive value) in infected Huh7.5.1^{dif} cells.

Mean lactate flux ±SEM (mole/h/100,000 cells; 4 independent experiments in triplicates). * p<0.05, T-Test.
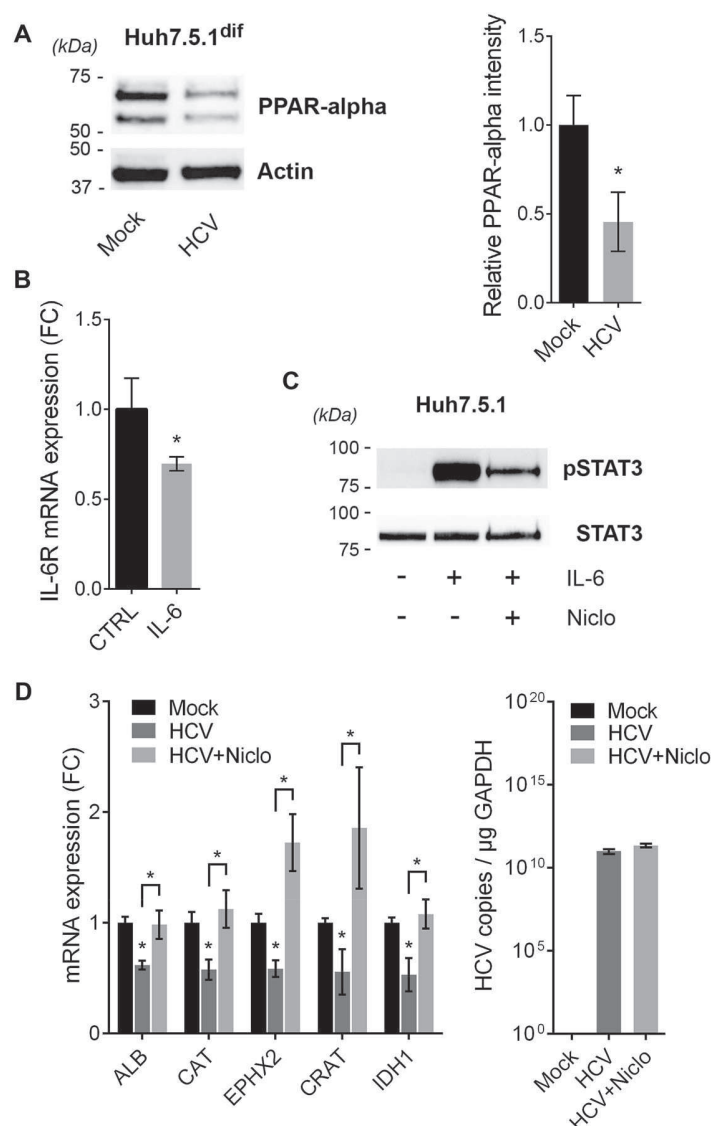
Fig. 5. HCV inhibits peroxisomal gene expression by suppressing PPAR-alpha function via STAT3 signaling.

(**A**) HCV infection of Huh7.5.1^{dif} significantly (p<0.05, U-Test) inhibits PPAR-alpha expression. Western blotting for PPAR-alpha and actin after 7 days of infection with HCVcc (Jc1). Quantification of band intensities of PPAR-alpha and actin. Mean relative PPAR-alpha intensity ±SD (2 independent experiments in triplicates). (**B**) Activation of the IL-6/STAT3 pathway rapidly downregulates IL-6 receptor (IL-6R). Huh7.5.1 cells were incubated 6 h with 10 ng/mL IL-6 prior RNA extraction and qPCR analysis. Mean fold change (FC) ±SEM triplicates. * p<0.05 (T-Test). (**C**) Niclosamide inhibits IL-6-induced STAT3 phosphorylation. Incubation of Huh7.5.1 cells with 2 μM niclosamide for 24 h and/or 10 ng IL-6 (30 min). Western blotting for pSTAT3 (Y705) and total STAT3. (**D**) HCV-induced inhibition of peroxisomal genes is rescued by the STAT3-inhibitor niclosamide. Huh7.5.1^{dif} cells were infected with HCVcc (Jc1) for 7 days. At day 6 pi cells were treated with solvent control or 2 μM niclosamide. Niclosamide reversed the HCV-induced inhibition of 5 top

leading-edge genes of the HALLMARK_PEROXISOME gene set (Fig. 3B, Supplementary Fig. S4), while it had no effect on HCV replication. Mean fold change of copy number normalized to GAPDH from triplicates ±SD. *p<0.05 T-Test.

225
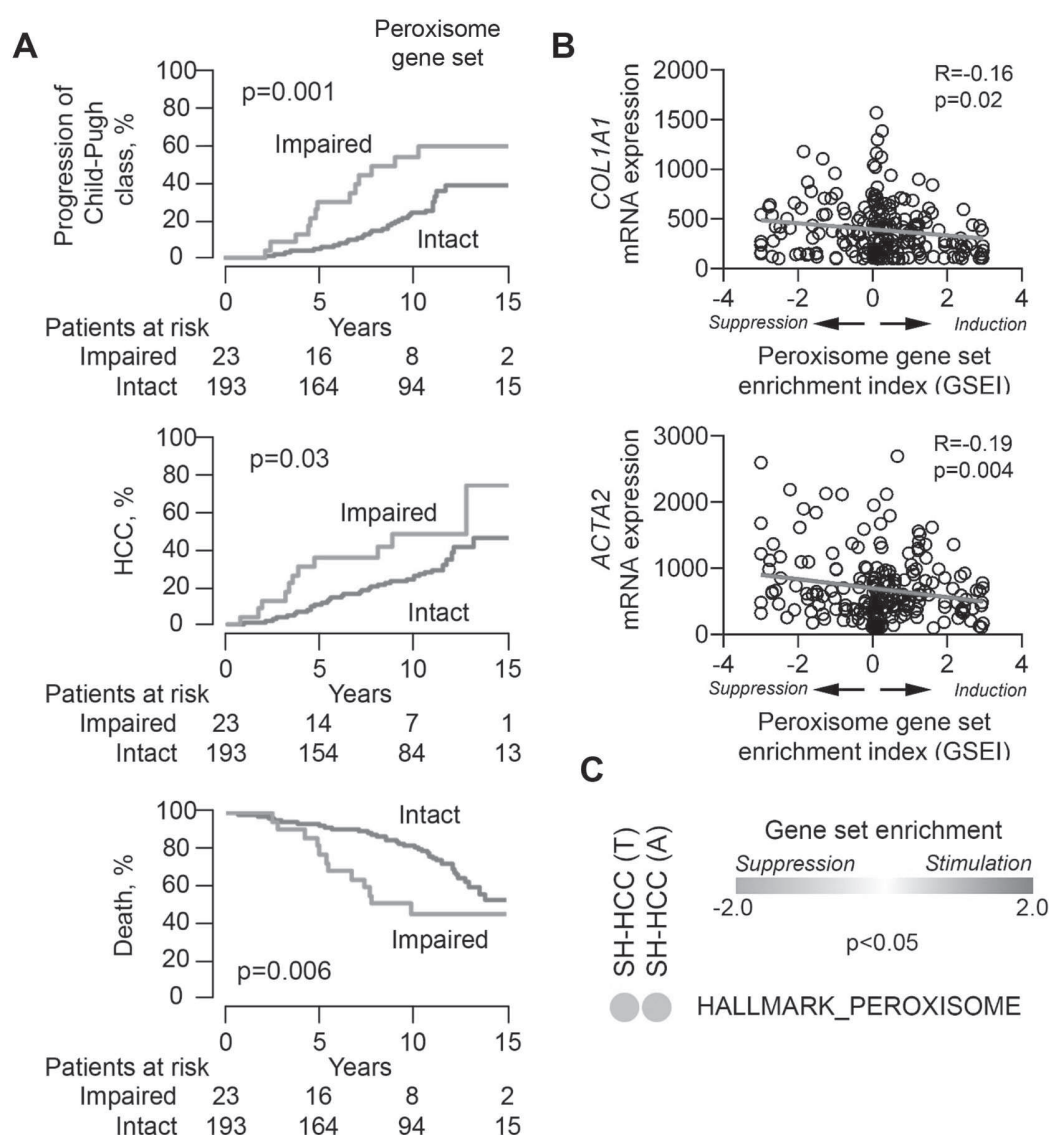
Fig. 6. Significant association of hepatic peroxisome expression with clinical outcomes and phenotypes in viral and metabolic liver disease.

(**A**) Patients with impaired peroxisomal function showed worse outcome compared to those with intact function (log-rank test). Early-stage HCV cirrhosis patients (n=216)[43] are classified into 2 groups based on relative peroxisomal function. Therefore, modulation of the HALLMARK_PEROXISOME gene set[25] in each individual sample was used to infer peroxisomal function in the liver tissues. Impaired peroxisomal function was defined by coordinated suppression of the gene set determined by modified gene set enrichment analysis with statistical significance (false discovery rate <0.10)[6]. (**B**) Expression of fibrosis-related genes, *COL1A1* and *ACTA2*, tend to be higher in the livers with a suppressed peroxisome pathway (Spearman correlation test). Induction or suppression of the HALLMARK_PEROXISOME gene set were measured by gene set enrichment index (GSEI)[6]. GSEI was calculated from gene set enrichment p value based on iterative random gene permutations (1,000 times). GSEI of +3 indicates induction at enrichment p= 0.001,

GSEI of −3 indicates suppression at enrichment p=0.001, and GSEI of 0 indicates no modulation at enrichment p=1.0. (**C**) The peroxisome is significantly (p<0.05) suppressed in histological steatohepatitis HCC (SH-HCC) (n=17) compared to other histological types (n=82) in tumor samples (*T*) (NES=−1.38) and in tissue adjacent to tumors (*A*) (NES=−1.43) of paired liver biopsies[44]. GSEA using the HALLMARK_PEROXISOME gene set.

proves the utility of HCV infection model for the investigation of targets related to metabolic

liver diseases.

Annex IV: Signaling Induced by Chronic Viral Hepatitis: Dependence and Consequences

Review

Boulahtouf Z, **Virzì A**, Baumert TF, Verrier ER, Lupberger J.

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My main contribution was in the preparation of the figures. I conceptualized and edited the writing together with the other authors.

Review

Signaling Induced by Chronic Viral Hepatitis: Dependence and Consequences

Zakaria Boulahtouf [1], Alessia Virzì [1], Thomas F. Baumert [1,2,3], Eloi R. Verrier [1] and Joachim Lupberger [1,*]

1 Institut de Recherche sur les Maladies Virales et Hepatiques UMR_S1110, Université de Strasbourg, Inserm, F-67000 Strasbourg, France; zakaria.boulahtouf@etu.unistra.fr (Z.B.); virzi@unistra.fr (A.V.); thomas.baumert@unistra.fr (T.F.B.); e.verrier@unistra.fr (E.R.V.)
2 Service d'Hépato-Gastroentérologie, Hôpitaux Universitaires de Strasbourg, F-67000 Strasbourg, France
3 Institut Universitaire de France (IUF), F-75005 Paris, France
* Correspondence: joachim.lupberger@unistra.fr

Abstract: Chronic viral hepatitis is a main cause of liver disease and hepatocellular carcinoma. There are striking similarities in the pathological impact of hepatitis B, C, and D, although these diseases are caused by very different viruses. Paired with the conventional study of protein–host interactions, the rapid technological development of -omics and bioinformatics has allowed highlighting the important role of signaling networks in viral pathogenesis. In this review, we provide an integrated look on the three major viruses associated with chronic viral hepatitis in patients, summarizing similarities and differences in virus-induced cellular signaling relevant to the viral life cycles and liver disease progression.

Keywords: HBV; HCV; HDV; liver; inflammation; oxidative stress; metabolic disease; fibrosis; cancer

1. Introduction

Viral hepatitis predominantly affects and damages the liver by commonly causing the progression from chronic inflammation to fibrosis, cirrhosis, and ultimately cancer. It is estimated that approximatively 350 million people worldwide are chronically infected with hepatitis viruses [1]. During a chronic infection of the liver, hepatic viruses persistently tweak and attenuate the host antiviral defenses and modulate cellular pathways that impact liver homeostasis and disease progression. Viral hepatitis is a major risk factor for liver fibrosis, cirrhosis, and hepatocellular carcinoma (HCC), which is the second leading and fastest rising cause of cancer death worldwide [2,3]. Although caused by very different viruses, virus-induced liver disease displays similar features, suggesting common molecular drivers. Moreover, chronic viral hepatitis may serve also as a model to understand the mechanism of non-viral etiologies like non-alcoholic fatty liver disease (NAFLD) and non-alcoholic steato-hepatitis (NASH).

Although replication strategies of hepatotropic viruses are very diverse, we can highlight common molecular mechanisms that occur during chronic viral hepatitis: (1) induction of intrahepatic oxidative stress damage by viral proteins, (2) dysregulation of cellular metabolic pathways, (3) persistence of liver inflammation, (4) activation of pro-fibrotic, pro-oncogenic processes that can lead to the accumulation of genetic alterations and genomic instability. Therefore, the common denominator of these events comprises a virus-induced dysregulation of signaling events that holds the potential for the identification of novel host-targeting and chemo-preventive strategies targeting the viral life cycle and/or liver disease progression. In this review, we summarize similarities and differences in virus-induced cellular signaling associated with the three major viruses that cause chronic viral hepatitis in patients.

2. Hepatitis B, C, D Viruses

The three hepatotropic viruses causing chronic liver infection are Hepatitis B virus (HBV), Hepatitis C virus (HCV), and Hepatitis Delta virus (HDV). HBV is a DNA virus of the *Hepadnaviridae* family whose partially double-stranded genome is translocated into the host nucleus. Here, a covalently closed circular DNA (cccDNA) is formed and serves as a template for transcription. cccDNA is highly persistent and epigenetically regulated as a host chromosome, a critical feature that makes it difficult to achieve a complete cure for HBV infection [4,5]. HBV is the only hepatotropic virus that causes integration of the viral DNA into the host genome. It thus contributes directly to an elevated liver cancer risk even in non-cirrhotic patients by cis-mediated insertional mutagenesis, chromosomal instability, and expression of aberrant viral proteins [6]. HDV is a single-stranded RNA virus and the only virus of the genus *Deltavirus*. It is a satellite virus of HBV, which requires the HBV surface antigen (HBsAg) for its lifecycle [7,8]. Importantly, HBV/HDV coinfection causes the most severe form of chronic viral hepatitis, with accelerated liver disease progression to cirrhosis and HCC and increased liver-related and overall mortality [9]. However, little is known about the HBV/HDV–host interactions driving these complications. HCV, a member of the *Flaviviridae* family, is a positive-sensed single-stranded RNA virus that depends on and interacts with hepatocyte lipid metabolism during its lifecycle [10]. HCV triggers phenotypic changes closely resembling metabolic liver disease, including hepatic steatosis and insulin resistance [11], and profoundly influences the proteogenomic landscape of the host cell [12].

Challenges in the treatment of these viruses differ very much. While an efficient HBV vaccine is available protecting from HBV and HDV, the development of an HCV vaccine is hampered by its lipoviral composition and its highly variable quasispecies that contribute to its shielding and escape from neutralizing antibodies. However, over the last decade, novel and highly efficient antivirals have been developed to cure HCV infection [13]. In contrast, chronic HBV infection can only be controlled by long-term antiviral strategies due to the persistence of cccDNA pools in patients' liver [14,15]. For HDV, interferon-based therapies had only limited success; however, novel antivirals such as entry inhibitors have shown encouraging results in clinical practice to control HDV infection and to improve liver function [16]. Nevertheless, even if a viral infection is controlled or cured, the risk of developing HCC may not be fully reversed, depending on the duration of chronic infection, liver disease stage, and type of virus [17,18]. Moreover, evidence points towards an epigenetic imprinting by hepatic viruses (HCV, HBV) and underlying liver fibrosis in the host genome which maintains a persistent transcriptomic environment in cured livers that acts in a pro-oncogenic manner [17,19,20].

3. Virus-Induced Oxidative Stress Signaling

In healthy cells, reactive oxygen species (ROS) are predominantly produced through mitochondria oxidative phosphorylation, protein folding in the endoplasmic reticulum (ER), and the catabolism of lipids and amino acids [21–23]. ROS are considered to be harmful for the cell, exerting damage-promoting, detrimental effects. However, ROS are also an essential signaling trigger regulating apoptosis and immune response against pathogens [24]. ROS are neutralized by the enzymatic and non-enzymatic cellular antioxidant system. The enzymatic antioxidant system includes various types of ROS-scavenging phase II enzymes such as glutathione peroxidase (GPx), superoxide dismutase (SOD), and catalase (CAT) catalyzing free radicals' neutralization. In contrast, the non-enzymatic antioxidants system is composed of low-molecular-weight compounds such as glutathione and vitamin C, scavenging ROS with a slow kinetics. Both systems are regulated by the expression of genes comprising antioxidant response elements (ARE), which are controlled by the transcription factor Nrf2 [25,26]. A persistent imbalance of ROS is an important driver of chronic liver disease, and the associated redox imbalance has been suggested to be highly relevant to NAFLD pathogenesis [27]. Moreover, oxidative stress has been associated with oncogenic transformation in patients with chronic viral hepatitis [28,29].

The mechanism of virus-induced oxidative stress by HBV, HCV, HDV can be summarized in four main categories: (1) alteration of mitochondrial function mediated by Ca^{2+} uptake; (2) triggering of ER stress and unfolded protein response (UPR); (3) virus-induced expression of ROS-producing enzymes; (4) dysregulation of antioxidative pathways (Figure 1). In the case of HCV, viral core proteins [30], E1/E2 [31], and NS4B [32,33] induce oxidative stress via calcium efflux through the induction of ER stress and UPR, which is a component of the ER adaptative system. In addition, the HCV core at the mitochondrial outer membrane [34] interacts with heat shock protein Hsp60 [35], triggering the release of Ca^{2+} from the ER and its accumulation in the mitochondria. Moreover, the HCV core stimulates the expression oxidoreductin 1α (ERO1α) in the ER. This promotes the formation of mitochondria-associated membranes and induces Ca^{2+} translocation from the ER to the mitochondria. Mitochondrial Ca^{2+} accumulation alters the respiratory chain and promotes ROS production [36,37]. For HBV, HBx protein expression reduces the activity of several respiratory chain complexes, causing the loss of mitochondrial membrane potential and therefore enhancing the production of ROS [38]. Moreover, HBx dysregulates mitochondrial functioning by interacting with two partners: voltage-dependent anion channel 3 (VDAC3), involved in calcium transport across the mitochondrial outer membrane [39], and cytochrome c oxidase subunit III (COX3) [40,41]. Other HBV proteins may also be involved in the induction of oxidative stress. HBsAg is generally secreted during the HBV lifecycle; however, secretion-deficient mutants can appear during infection and accumulate in the ER. This also occurs with the HBV core antigen (HBcAg). Both proteins induce ER stress and UPR signaling, leading to calcium release from the ER and subsequent ROS production [42,43]. Viral components also enhance oxidative stress by inducing the expression of ROS-producing enzymes. The HCV core proteins and NS5A enhance the expression of cytochrome P450 2E1 (CYP2E1) and NADPH oxidase 1 and 4 (NOX1 and 4), leading to elevated levels of ROS, including superoxide and hydrogen peroxide [37,44]. Similarly, the large HDV surface antigen (L-HDAg) induces oxidative stress by promoting NOX4 expression [45].

A virus-induced, but not always consistent, dysregulation of the antioxidant system is observed during HCV and HBV infection. For HCV, the expression of nonstructural proteins downregulates SOD1 and SOD2 and induce catalase, whereas HCV core alone enhances the expression of SOD2 [46]. The expression of full-length HCV or the nonstructural proteins and core leads to impaired Nrf2/ARE activity [47], whereas a full HCV infection activates the Nrf2/ARE axis [48–50]. Overexpression studies in Huh7 cells point towards Nrf2-activating phosphorylation during HCV infection by protein kinase C in response to ROS or by casein kinase 2 and phosphoinositide 3-kinase (PI3K) in a ROS-independent manner [48]. Moreover, in an HCV infection model, Nrf2/ARE activation was promoted by the inhibitory phosphorylation of glycogen synthase kinase 3β (GSK3β) [49]. While in non-transformed hepatocytes several Nrf2-dependent genes are induced by HCV [51], downregulation of a wide spectrum of antioxidant defense proteins can be observed in hepatoma cell line-based models [52,53]. Several theories have been proposed to explain this discrepancy, one of which is the bi-phasic nature of oxidative stress, which at low and moderate ROS levels activates antioxidants, whereas at high ROS level induces damage and inhibits the expression of antioxidant genes. The used study model and the readout have thus a significant impact on the results. HCV-induced gene expression is often not translated into a protein response to blunt the antiviral response of the host cells [12]. For HBV, the Nrf2/ARE pathway is activated by the virus in both infected cells and liver tissues of chronic HBV carriers in a genotype-dependent manner via HBx and the large surface antigen (LHBs) [54]. HBx sequesters the Nrf2 partner protein, Keap1, forming a HBx–p62–Keap1 triple complex in a ROS-independent manner [55]. However, the activation of the antioxidant system by HBV is challenged by several studies in HBV-infected cells and in HBV patients [56–58]. Indeed, some Nrf2-dependent genes such as *GSTM3* [59] and *GSTP1* [60] are epigenetically silenced by HBx expression or HBV infection. HBx also alters type II enzyme expression by interfering with the expression of other regulatory ele-

ments/factors of the Nrf2/ARE signaling pathway [61]. Furthermore, HBV suppresses the expression of proteins indirectly implicated in the antioxidant system such as selenoprotein P (SeP) and selenium-binding protein 2 (Selenbp2) [62,63]. Beside the observed effects of HBV on ROS, also HDV promotes oxidative stress in the ER through the interaction between L-HDAg and NOX4. The activation of the NOX4 pathway induces the release of ROS from the ER, activating the signal transducer and activator of transcription-3 (STAT3) and nuclear factor kappa B (NF-κB) signaling [45]. Moreover, the small hepatitis delta antigen (S-HDAg) can directly bind to glutathione S-transferase P1 mRNA causing the downregulation of its expression, therefore increasing ROS and promoting apoptosis [64].
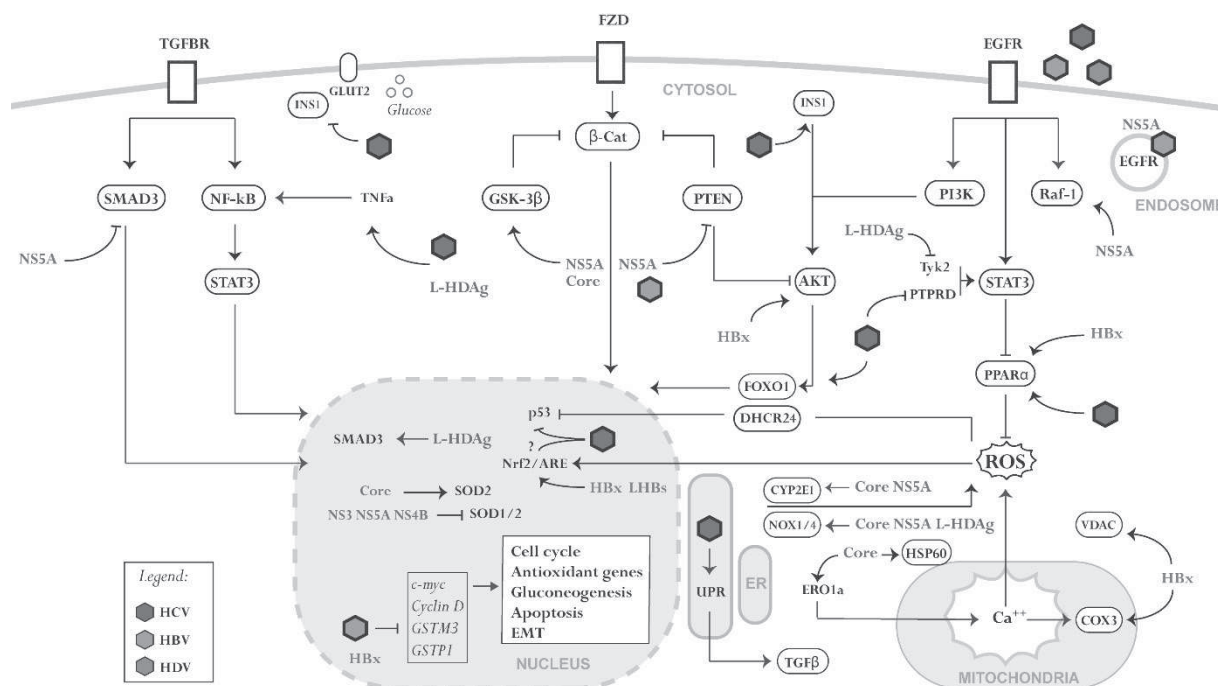
Figure 1. Signaling pathways perturbed by hepatotropic viral proteins. HCV, HBV, and HDV alter liver homeostasis by disrupting several signaling processes associated with (1) the generation of oxidative stress and the dysregulation of the antioxidant system, (2) the alteration of a pro-inflammatory signaling, (3) the hijacking of glucose and lipid metabolism, (4) the dysregulation of host genome expression. NS3, NS4B, NS5A, (non-structural protein 3/4B/5A); HBx, (hepatitis b X antigen); LHBs, (large HBV surface antigen); L-HDAg, (large HDV antigen).

Oxidative stress, ER stress, and UPR trigger a cascade of signaling events that may protect but also damage the liver, depending on the duration of the insult. During HCV infection, elevated ROS levels induce the production of pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF-α) and IL-8 [65]. The underlying NF-κB pathway regulating their production is very sensitive to oxidative stimuli. HCV core proteins, NS4B, and NS5A activate NF-κB as well as STAT3 expression through the elevation of ROS and the disruption of calcium homeostasis [66,67]. Interestingly, the induction of oxidative stress during HCV infection is positively associated with the progression of liver fibrosis, which is characterized by the overproduction of extracellular matrix. A key mechanism of fibrosis is the activation of hepatic stellate cells (HSCs) by pro-inflammatory cytokines, leading to collagen deposition [68]. HCV core proteins, NS3A/4A, NS4B, and NS5A activate transforming growth factor beta 1 (TGF-β1) secretion through ROS and calcium-dependent mechanisms [69]. Viruses also benefit from manipulating ROS levels. Studies revealed that HCV activates the Nrf2/ARE axis, promoting ROS scavenging and preventing ROS accumulation to levels with antiviral and/or lethal effects in the host cell [48,70]. Moreover, under conditions of oxidative stress, viruses promote cell survival and proliferation via associated signaling. HCV activates β-catenin that induces c-Myc and cyclin D1 expres-

sion, thus promoting cell cycle progression [71]. Moreover, ROS disrupt p53 binding to Mdm2 via the upregulation of DHCR24 expression, thus attenuating apoptosis [72]. HCV further prevents apoptosis by activating the peroxisome proliferator-activated receptor alpha (PPARα) and suppressing the voltage-gated K$^+$ channel Kv2.1 through NS5A and ROS [73,74]. ROS also suppress the expression of p14, which is implicated in the induction of the pro-apoptotic p53/Mdm2 pathway [75]. ROS upregulates p21, a cyclin-dependent kinase inhibitor activating Nrf2 [76]. While in low stress conditions p21 induces cell cycle arrest, in the presence of high oxidative stress levels, it induces apoptosis [77,78]. HCV core proteins and NS5A inhibit p21 and therefore render Nrf2 less sensitive to ROS. This hampers the induction of apoptosis and stimulates the proliferation of damaged hepatocytes [79,80].

4. Virus-Induced Pro-Inflammatory Signaling

A common consequence of chronic viral hepatis is the induction of liver inflammation (hepatitis). Upon viral sensing, infected hepatocytes trigger the activation of innate immune receptors and sensors that are referred to as the inflammasome and are large protein complexes. The inflammasome serves as a signaling hub triggering type I interferons and the processing and release of proinflammatory cytokines. It is activated by pattern recognition receptors (PRRs) triggered by pathogen-related molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs) (reviewed in more detail in [81]). The host inflammasome represents an important line of defense and is a decision maker with regard to fight (antiviral response) and containment (apoptosis). It is therefore not surprising that hepatic viruses evade the host innate immune response and twist pro-inflammatory signaling to their own benefit to persist and prevent apoptosis of the infected cells. The induction of the inflammasome by viral hepatitis was already evident in the previous section highlighting the common mechanism of virus-induced ROS and ER stress activating STAT3 and NF-κB signaling. Presumably, ROS-independent or -related mechanisms comprise the induction of NF-κB signaling by HDV. Indeed, L-HDAg renders NF-κB signaling more susceptible to TNF-α [82] and induces STAT3 [83]. NF-κB-independent induction of inflammation has been observed for HCV, which involves NS5A induction of cyclooxygenase-2 (COX-2) and, consequently, second messenger signaling and prostaglandin production [84]. The JAK/STAT signaling pathway is an important mediator of the host innate immune response as well as of cellular apoptosis and survival. Viral sensing triggers type I and II interferon responses via STAT1 and STAT2, promoting the expression of antiviral interferon response genes and apoptosis if the pathogen is not cleared [85]. Viruses causing chronic hepatitis have developed elaborated strategies to evade the innate response (reviewed in [86–89]). An important aspect in this evasion strategy is the pro-viral role of STAT3 signaling. In the liver, STAT3 signaling is a mediator of liver regeneration and balances the pro-apoptotic role of STAT1 by heterodimerization in response to IL-6 and proliferative signaling by HGF and EGF [90]. Attenuation of STAT3 signaling in functional studies impaired the replication of HCV and HBV, suggesting a pro-viral role of STAT3 signaling [90–92]. Proliferative signaling via the epidermal growth factor receptor (EGFR) is required for HBV, HCV, and HDV infection [93,94]. Interestingly, EGFR further promotes STAT3 activity by repressing a negative regulator of STAT3, i.e., suppressor of cytokine signaling 3 (SOCS3). Consequently, this tempers the pro-apoptotic, antiviral effect of type I interferon signaling by promoting STAT1/STAT3 heterodimerization over STAT1 homodimerization [95]. STAT3 signaling is further maintained by HCV-induced downregulation of another negative regulator of STAT3, protein tyrosine phosphatase delta (PTPRD) [96]. A potential similar effect may be triggered by HDV infection, which attenuates STAT1/STAT2 signaling via the suppression of Tyk2 [89], which is another negative regulator of STAT3 transcriptional activity [97]. Whether this may promote the pro-viral effect of STAT3 signaling in HBV/HDV-infected hepatocytes remains to be demonstrated.

5. Deregulation of Cellular Metabolism Pathways

The liver is an essential hub for metabolic processes and energy storage. Chronic viral hepatitis has an important impact on metabolic processes in the liver with distinct virus-specific manifestations. While chronic HCV infection strongly resembles clinical manifestations caused by NAFLD and NASH [98], the role of HBV in metabolic disease is controversial, and it has been suggested that HBV may potentially exhibit protective effects towards NAFLD development [99,100]. However, metabolic disease and obesity in HBV- or HCV-infected patients are considered important co-morbidities promoting liver disease progression and increasing cancer risk [101]. The accumulation of free fatty acids induces mitochondrial and ER oxidative stress. Moreover, the accumulation of ROS stimulates lipid peroxidation and inflammatory cascades such as those associated with TNF-α and IL-6, leading to the development of hepatic steatosis and insulin resistance [102].

HCV lifecycle is tightly linked to human lipid metabolism also because HCV requires lipid droplets to replicate and circulates as lipoviral particles to evade the host immune response [103]. HCV proteins directly interact with or regulate the expression of key effector molecules of the lipid metabolism, including apolipoproteins and diacylglycerol acyltransferase-1 [104–106]. HCV also activates IKK, which induces the expression of lipogenic genes and promotes lipid droplet formation [107]. Indeed, recent proteogenomic analysis revealed a massive suppression of pathways required for fatty acid metabolism, perturbing the capacity of infected hepatocytes to use fat as an energy source. This coincided with a shift towards a highly glutamine-/glucose-dependent metabolism, which promoted HCV replication [12,108] and resembled the high energy dependence of tumor cells. HCV also tweaks host signal transduction, promoting a favorable metabolic environment for its replication and persistence.

A central role in metabolic liver disease is a chronically dysregulated STAT3 and NF-κB signaling [109,110]. As reviewed above, both pathways are induced by chronic viral hepatitis and associated oxidative stress and are associated with liver disease progression and increased HCC risk [12,90,111–114]. However, while during HCV infection STAT3 signaling contributes to the accumulation of free fatty acids by suppressing peroxisomal beta-oxidation via inhibiting PPARα [12], HBV-induced STAT3 signaling does not produce the same phenotype. In contrast, HBV replication requires PPARα [115,116] and therefore prevents PPARα inhibition by inducing STAT3 activity. Indeed, HBV infection of primary human hepatocytes induces peroxisomal function, which may be a consequence of a direct rescue of PPARα activity by an HBV protein [12], as observed for PPARγ activation by the protein HBx [117]. HBx maintains fatty acid oxidation [118] and has been observed to bind PPARα in cell culture [119]. This matches observations that PPARα stimulation improves NAFLD in pre-clinical models [120,121]. However, evidence points also to a pro-steatotic impact of HBV infection, even though this is more rarely observed in patients compared to patients infected with HCV [122]. HBV infection does promote the biosynthesis of fatty acids in HBV transgenic (HBV-Tg) mice by the upregulation of fatty acid-binding protein 5 (FABBP5) and acyl-CoA-binding protein (ACBP) [123]. Moreover, HBV-Tg mice display the upregulation of lipid biosynthetic pathways such as those involving retinol-binding protein 1 (RBP1), sterol regulatory element-binding protein 2 (SREBP2), ATP citrate lyase, and fatty acid synthase (FAS) [124]. Additionally, other factors participating in fatty acid transport and biogenesis are dysregulated by HCV, including fatty acid-binding protein 1 (FABP1), responsible for the uptake and transport of long-chain fatty acids (LFA) [125,126], SREBP1, and PPARγ, which induces the expression of hepatic lipogenic and adipogenic genes, accompanied by the accumulation of lipid droplets [127–130]. HDV infection also impacts metabolic pathways, since HDV decreases the availability of triosephosphate isomerase and pyruvate carboxylase, leading to an abnormal retention of lipids. This effect may also be responsible for microvesicular steatosis during HDV infection [131].

A particular feature of HCV infection is its association with insulin resistance (IR) in patients, which is less frequently observed in HBV- or HBV/HDV-infected patients [132], although a recent genetic screen highlighted the importance of metabolic pathways in

HDV lifecycle, including insulin resistance-related genes [133]. This may be due to the different dependency of the hepatic viruses on intracellular glucose levels. Insulin is a central regulator of glucose levels in the blood and of gluconeogenesis in the liver. It therefore also impacts the glucose levels within the hepatocyte. During IR, insulin fails to suppress gluconeogenesis in the hepatocytes [134]. While HCV is a highly glucose-dependent virus [12], HBV replication favors low glucose levels, and thus glucose-induced mTOR signaling hampers HBV replication [135]. HCV proteins directly promote IR by interacting with insulin pathways' components, including insulin receptor substrate 1 (IRS-1) [136–138]. It also alters Akt-induced forkhead box O1 (FOXO1) phosphorylation and its nuclear exclusion, which is required for the transcription of the gluconeogenic gene phosphoenolpyruvate carboxykinase 1 (PCK1) in hepatocytes [139]. Moreover, HCV suppresses glucose transporter 2 (GLUT2) and IRS-2, contributing to higher endogenous glucose levels. Indirect mechanisms of IR involve HCV-induced oxidative stress, steatosis, and pro-inflammatory cytokines, e.g., TNF-α. These indirect effects induce the expression of gluconeogenic genes, such as glucose 6 phosphatase (G6P) and PCK2 [140,141]. Moreover, HCV mediates oxidative stress, leading to hypoxia. This activates H1Fα via c-Myc and Nrf2, controlling the expression of key enzymes in glycolysis [108]. One may speculate that HBV and HDV infection rather indirectly contribute to insulin resistance in patients via virus-induced inflammation and oxidative stress, which is further promoted by comorbidities such as overweight. Direct effects are observed, however, in HBx-Tg mice, which develop hyperglycemia and impaired glucose tolerance [142], and in the HBV-expressing cell line HepG2.2.15, with stimulated TCA cycle and glycolysis [143].

6. Virus-Induced Pro-Fibrotic/Pro-Oncogenic Signaling

Many of the above-mentioned dysregulated signaling pathways promote viral replication and persistence mostly by diverting the host antiviral response to prevent apoptosis and ensure the survival of the infected cell. Strikingly, many of these survival signals are also involved in regenerative processes during liver injury and orchestrate a delicate balance between pro-inflammatory and proliferative signals [144]. As mentioned earlier in this review, all three hepatis viruses chronically infecting the liver engage EGFR signaling to maintain their life cycle [93,94]. EGFR orchestrates the entry of HBV [93] and HCV [94]. EGFR signaling is active during ligand-induced receptor dimerization and internalization and is regulated by phosphatases and endosomal recycling/degradation [145]. HCV has developed strategies to maintain EGFR signaling to its own benefit. HCV infection induces EGFR signaling [146,147] and prolongs EGFR signaling by retaining EGFR in the early endosome via NS5A. This prevents EGFR degradation and leads to EGFR accumulation in infected cells [148,149]. HCV also alters the expression of other ErbB receptors in favor of EGFR [150]. In contrast to HCV, HBV internalization requires EGFR transport to the late endosome, which is critical for efficient HBV infection [151]. Consistently, the inhibition of EGFR degradation abrogated the internalization of HBV via its receptor sodium/taurocholate cotransporter (NTCP) and prevented viral infection [151]. Downstream of EGFR signaling, several viral proteins interact with the MAPK signaling pathways and stimulate cell proliferation [152–155]. For HCV, NS5A associates with Raf-1 kinase, promoting HCV replication [152]. Consistently, inhibiting Raf kinases with sorafenib blocked the infection, while a further downstream inhibition of MEK1/2 and Erk1/2 showed only marginal effects. This suggests a direct virus–host dependency independent of pathway-associated transcriptional changes. The same holds true for HBV, for which the inhibition of EGFR-associated MAPK or PI3K signaling during infection seems only to have marginal effects [151]. Although no studies have so far demonstrated a role of EGFR during HDV infection, it can be assumed that EGFR may also be required for HDV internalization, since this virus uses the HBV envelope to enter the cell and shares the same entry pathway through HSPG and NTCP.

PI3K/Akt signaling regulates glucose metabolism, cell growth, and survival [156] and it is tightly regulated by phosphatase and tensin homolog deleted on chromosome 10

(PTEN) [157,158]. Independently of its role in insulin signaling, HCV NS5A downregulates PTEN expression through a cooperation of ROS-dependent and -independent pathways that subsequently drives a PTEN–PI3K/Akt feedback loop supporting cell survival [159]. For HBV, the role of PI3K/Akt signaling is more diverse. HBx activates Akt in hepatocytes thereby self-limiting HBV replication [160]. This is consistent with a decreased HBV replication upon PI3K/Akt pathway inhibition using a small-molecule inhibitor in cell culture [161]. However, despite the self-limiting effect on HBV replication, HBx inhibits hepatocyte apoptosis via Akt stimulation and potentially facilitates the persistent, noncytopathic HBV replication [160]. As observed for HCV, HBV impairs PTEN expression, promoting β-catenin/c-Myc signaling and PD-L1 expression [162]. The authors found that PTEN rescue in hepatocytes inhibited β-catenin/PD-L1 signaling and promoted HBV clearance.

Wnt/ß-catenin signaling is essentially involved in the regulation of cell fate during embryogenesis and hepatobiliary development, as well as in liver homeostasis, epithelial–mesenchymal transition (EMT), and tissue regeneration during adulthood. If dysregulated, it promotes liver disease and cancer [163]. The Wnt/β-catenin signaling pathway is activated by hepatic viruses via direct engagement of viral proteins. β-catenin signaling is stimulated by HCV infection via NS3 and NS5A [164] or the phospho-inactivation of GSK-3β by NS5A and core proteins [165,166]. Strikingly, despite a highly genetic heterogeneity, a relative higher frequency of mutations in the β-catenin gene *CTNNB1* can be observed in HCC associated with HCV than with HBV [164]. Wnt/ß-catenin signaling is involved in EMT, which is a hallmark of wound healing and liver fibrosis [167]. During chronic infection, dysregulated wound healing processes cause an excessive deposition of extracellular matrix in the liver, leading to liver fibrosis and cirrhosis, which involve not only hepatocytes but also non-parenchymal cells like HSCs and liver macrophages [167]. The Wnt/ß-catenin cascade has a central role in regulating profibrotic pathways in hepatocytes, which involve oxidative stress signaling and transforming growth factor beta (TGF-β)/SMAD signaling [167]. HCV induces TGF-β signaling indirectly via UPR [168]. Interestingly, TGF-β signaling seems to limit HCV infection in hepatocytes [169]. The virus counteracts this activation by an NS5A-mediated inhibition of the phosphorylation and transcriptional activity of SMAD2 and SMAD3/4 heterodimers [170]. HBV infection is also restricted by TGF-β [171], while HDV seems to stimulate TGF-β in luciferase reporter gene assays [172]. This is consistent with a reported activation of TGF-β expression by HDV via an L-HDAg-mediated activation of the Twist promoter through binding to SMAD3 on Smad-binding elements (SBEs) [173]. This is an interesting finding that may help to understand the aggravation of HBV liver disease and the rapid fibrosis progression in HDV/HBV-infected patients [174].

7. Discussion

Chronic liver disease and associated complications including cancer constitute an important burden for public health, with a long-lasting impact on affected individuals even after viral infection cure. The comparison of virus-induced signaling during chronic infection with HBV, HCV, and HDV outlined common pathogenic mechanisms that predominantly result in the failure of the antiviral response to clear infection and in a diversion of the final antiviral safeguard apoptosis towards cell survival. It is evident that the involved signaling pathways that are thereto manipulated largely overlap for different viruses (Table 1), although the detailed strategies differ (Figure 2). Also hepatitis E virus (HEV) seem to dysregulate similar pro-oncogenic signaling pathways linked to oxidative stress, inflammation, apoptosis, and cell proliferation, as reviewed elsewhere [175]. However, chronic infections are relatively rare, and only a fraction of patients progress to fibrosis and HCC [176]. The majority of studies reviewed here were based on cell culture models and performed a limited analysis of canonical pathways. Given these limitations, the currently available literature for some viruses is biased by functional studies of individual proteins (e.g., HBx for HBV) and does not consider protein dynamics or synergic effects of the virus

interactome. However, the fast-moving technological development in the recent years and the diffusion of -omics studies in the scientific routine are allowing a more profound study of virus-induced signaling. This should be combined with the use of better infection models representing the three-dimensional architecture of the liver, the heterogeneity of its cell populations, and the contribution of immune cells. Signaling pathways are established targets in cancer therapy [177] and have previously drawn attention as targets for cancer prevention attenuating liver disease progression [178,179]. Host signaling-targeting approaches to battle chronic infection have been discussed [133,180,181] as they hold the potential to lower the genetic barrier of resistance to direct-acting antivirals. However, currently, only interferons are in clinical use targeting chronic viral hepatitis. Thus, a better understanding of virus-induced signaling could promote the development of common therapeutic strategies to help not only patients with chronic infection but also patients suffering from non-viral disease etiologies that display a similar course of liver disease and fibrosis-associated carcinogenesis.

Table 1. Virus-perturbed signaling pathways during chronic viral hepatitis.

Perturbed Signaling Pathway	Virus	References
IL-6/JAK/STAT3	HBV, HCV, HDV	[45,83,89–92,96]
EGFR	HBV, HCV	[93,94,146–151]
TNF-α/NF-κB	HCV, HDV	[45,65,66,82]
Nrf2/ARE	HBV, HCV	[47–50,54,55,59–61]
PI3K/Akt	HBV, HCV	[48,160]
Ras/Raf	HCV	[152]
TGF-β/SMAD	HBV, HCV, HDV	[66,67,69,168,172,173]
Wnt/β-catenin	HBV, HCV	[71,162,164–166]
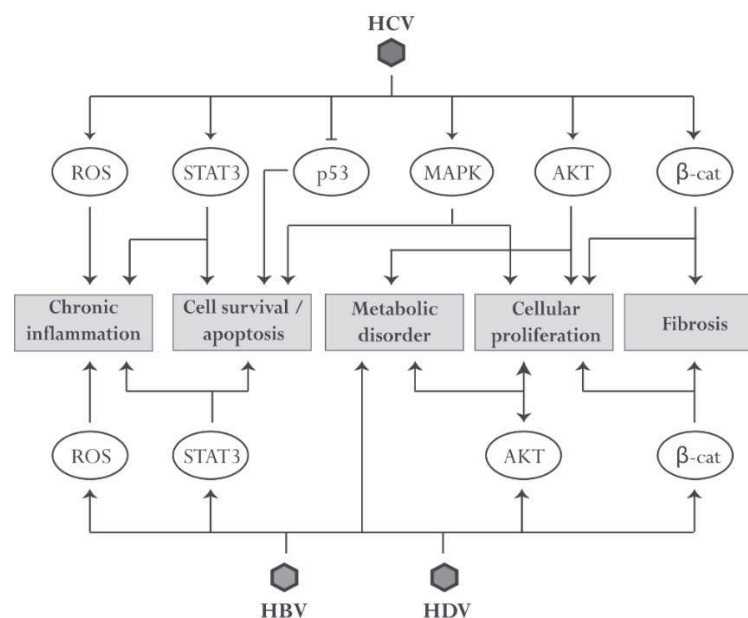
Figure 2. Common pathways associated with virus-induced liver disease progression. Several perturbations are mediated by HBV, HCV, and HDV infection. Reactive oxygen species (ROS) production and activation of STAT3 contribute to the establishment of chronic liver inflammation. Upregulation of mitogen-activated protein kinase (MAPK) and STAT3 signaling as well as downregulation of p53 reduce apoptosis and promote cell survival. Similarly, activation of AKT, MAPK, and β-catenin induces cell proliferation. AKT upregulation contributes to the development of metabolic disorders, while β-catenin is involved in the progression of liver fibrosis.

References

1. WHO Team Global HIV, H. a. S. T. I. P. *Global Progress Report on HIV, Viral Hepatitis and Sexually Transmitted Infections, 2021*; World Health Organization: Geneva, Switzerland, 2021; p. 108.
2. Villanueva, A. Hepatocellular Carcinoma. *N. Engl. J. Med.* **2019**, *380*, 1450–1462. [CrossRef] [PubMed]
3. Ward, E.M.; Sherman, R.L.; Henley, S.J.; Jemal, A.; Siegel, D.A.; Feuer, E.J.; Firth, A.U.; Kohler, B.A.; Scott, S.; Ma, J.; et al. Annual Report to the Nation on the Status of Cancer, Featuring Cancer in Men and Women Age 20–49 Years. *J. Natl. Cancer Inst.* **2019**, *111*, 1279–1297. [CrossRef] [PubMed]
4. Yuen, M.F.; Chen, D.S.; Dusheiko, G.M.; Janssen, H.L.A.; Lau, D.T.Y.; Locarnini, S.A.; Peters, M.G.; Lai, C.L. Hepatitis B virus infection. *Nat. Rev. Dis. Primers* **2018**, *4*, 18035. [CrossRef] [PubMed]
5. Lucifora, J.; Protzer, U. Attacking hepatitis B virus cccDNA—The holy grail to hepatitis B cure. *J. Hepatol* **2016**, *64*, S41–S48. [CrossRef] [PubMed]
6. Tu, T.; Budzinska, M.A.; Shackel, N.A.; Urban, S. HBV DNA Integration: Molecular Mechanisms and Clinical Implications. *Viruses* **2017**, *9*, 75. [CrossRef]
7. Mentha, N.; Clement, S.; Negro, F.; Alfaiate, D. A review on hepatitis D: From virology to new therapies. *J. Adv. Res.* **2019**, *17*, 3–15. [CrossRef]
8. Turon-Lagot, V.; Saviano, A.; Schuster, C.; Baumert, T.F.; Verrier, E.R. Targeting the Host for New Therapeutic Perspectives in Hepatitis D. *J. Clin. Med.* **2020**, *9*, 222. [CrossRef]
9. Wedemeyer, H.; Negro, F. Devil hepatitis D: An orphan disease or largely underdiagnosed? *Gut* **2019**, *68*, 381–382. [CrossRef]
10. Dubuisson, J.; Cosset, F.L. Virology and cell biology of the hepatitis C virus life cycle: An update. *J. Hepatol.* **2014**, *61*, S3–S13. [CrossRef]
11. Comparcola, D.; Alisi, A.; Nobili, V. Hepatitis C virus and nonalcoholic Fatty liver disease: Similar risk factors for necroinflammation, fibrosis, and cirrhosis. *Clin. Gastroenterol. Hepatol.* **2010**, *8*, 97. [CrossRef]
12. Lupberger, J.; Croonenborghs, T.; Roca Suarez, A.A.; Van Renne, N.; Juhling, F.; Oudot, M.A.; Virzi, A.; Bandiera, S.; Jamey, C.; Meszaros, G.; et al. Combined Analysis of Metabolomes, Proteomes, and Transcriptomes of Hepatitis C Virus-Infected Cells and Liver to Identify Pathways Associated with Disease Development. *Gastroenterology* **2019**, *157*, 537–551.e9. [CrossRef] [PubMed]
13. Pawlotsky, J.M.; Negro, F.; Aghemo, A.; Berenguer, M.; Dalgard, O.; Dusheiko, G.; Marra, F.; Puoti, M.; Wedemeyer, H.; European Association for the Study of the Liver. EASL recommendations on treatment of hepatitis C: Final update of the series. *J. Hepatol.* **2020**, *73*, 1170–1218. [CrossRef] [PubMed]
14. Martinez, M.G.; Boyd, A.; Combe, E.; Testoni, B.; Zoulim, F. Covalently closed circular DNA: The ultimate therapeutic target for curing HBV infections. *J. Hepatol.* **2021**, *75*, 706–717. [CrossRef]
15. Roca Suarez, A.A.; Testoni, B.; Zoulim, F. HBV 2021: New therapeutic strategies against an old foe. *Liver Int.* **2021**, *41* (Suppl. S1), 15–23. [CrossRef]

16. Asselah, T.; Loureiro, D.; Le Gal, F.; Narguet, S.; Brichler, S.; Bouton, V.; Abazid, M.; Boyer, N.; Giuly, N.; Gerber, A.; et al. Early virological response in six patients with hepatitis D virus infection and compensated cirrhosis treated with Bulevirtide in real-life. *Liver Int.* **2021**, *41*, 1509–1517. [CrossRef]

17. Hamdane, N.; Juhling, F.; Crouchet, E.; El Saghire, H.; Thumann, C.; Oudot, M.A.; Bandiera, S.; Saviano, A.; Ponsolles, C.; Roca Suarez, A.A.; et al. HCV-Induced Epigenetic Changes Associated with Liver Cancer Risk Persist after Sustained Virologic Response. *Gastroenterology* **2019**, *156*, 2313–2329.e7. [CrossRef]

18. Kim, S.U.; Seo, Y.S.; Lee, H.A.; Kim, M.N.; Lee, E.J.; Shin, H.J.; Lee, Y.R.; Lee, H.W.; Park, J.Y.; Kim, D.Y.; et al. Hepatocellular Carcinoma Risk Steadily Persists over Time Despite Long-Term Antiviral Therapy for Hepatitis B: A Multicenter Study. *Cancer Epidemiol. Biomark. Prev.* **2020**, *29*, 832–837. [CrossRef]

19. Juhling, F.; Hamdane, N.; Crouchet, E.; Li, S.; El Saghire, H.; Mukherji, A.; Fujiwara, N.; Oudot, M.A.; Thumann, C.; Saviano, A.; et al. Targeting clinical epigenetic reprogramming for chemoprevention of metabolic and viral hepatocellular carcinoma. *Gut* **2021**, *70*, 157–169. [CrossRef] [PubMed]

20. Andrisani, O. Epigenetic mechanisms in hepatitis B virus-associated hepatocellular carcinoma. *Hepatoma Res.* **2021**, *2021*, 7. [CrossRef] [PubMed]

21. Phaniendra, A.; Jestadi, D.B.; Periyasamy, L. Free radicals: Properties, sources, targets, and their implication in various diseases. *Indian J. Clin. Biochem.* **2015**, *30*, 11–26. [CrossRef]

22. Santos, C.X.; Tanaka, L.Y.; Wosniak, J.; Laurindo, F.R. Mechanisms and implications of reactive oxygen species generation during the unfolded protein response: Roles of endoplasmic reticulum oxidoreductases, mitochondrial electron transport, and NADPH oxidase. *Antioxid. Redox Signal.* **2009**, *11*, 2409–2427. [CrossRef] [PubMed]

23. Scialo, F.; Fernandez-Ayala, D.J.; Sanz, A. Role of Mitochondrial Reverse Electron Transport in ROS Signaling: Potential Roles in Health and Disease. *Front. Physiol.* **2017**, *8*, 428. [CrossRef]

24. Chen, Y.; Zhou, Z.; Min, W. Mitochondria, Oxidative Stress and Innate Immunity. *Front. Physiol.* **2018**, *9*, 1487. [CrossRef] [PubMed]

25. Xu, D.; Xu, M.; Jeong, S.; Qian, Y.; Wu, H.; Xia, Q.; Kong, X. The Role of Nrf2 in Liver Disease: Novel Molecular Mechanisms and Therapeutic Approaches. *Front. Pharmacol.* **2018**, *9*, 1428. [CrossRef] [PubMed]

26. Aleksunes, L.M.; Manautou, J.E. Emerging role of Nrf2 in protecting against hepatic and gastrointestinal disease. *Toxicol. Pathol.* **2007**, *35*, 459–473. [CrossRef] [PubMed]

27. Spahis, S.; Delvin, E.; Borys, J.M.; Levy, E. Oxidative Stress as a Critical Factor in Nonalcoholic Fatty Liver Disease Pathogenesis. *Antioxid. Redox Signal.* **2017**, *26*, 519–541. [CrossRef] [PubMed]

28. Ivanov, A.V.; Valuev-Elliston, V.T.; Tyurina, D.A.; Ivanova, O.N.; Kochetkov, S.N.; Bartosch, B.; Isaguliants, M.G. Oxidative stress, a trigger of hepatitis C and B virus-induced liver carcinogenesis. *Oncotarget* **2017**, *8*, 3895–3932. [CrossRef]

29. Block, T.M.; Mehta, A.S.; Fimmel, C.J.; Jordan, R. Molecular viral oncology of hepatocellular carcinoma. *Oncogene* **2003**, *22*, 5093–5107. [CrossRef]

30. Benali-Furet, N.L.; Chami, M.; Houel, L.; De Giorgi, F.; Vernejoul, F.; Lagorce, D.; Buscail, L.; Bartenschlager, R.; Ichas, F.; Rizzuto, R.; et al. Hepatitis C virus core triggers apoptosis in liver cells by inducing ER stress and ER calcium depletion. *Oncogene* **2005**, *24*, 4921–4933. [CrossRef]

31. Chan, S.W.; Egan, P.A. Hepatitis C virus envelope proteins regulate CHOP via induction of the unfolded protein response. *FASEB J.* **2005**, *19*, 1510–1512. [CrossRef]

32. Li, S.; Ye, L.; Yu, X.; Xu, B.; Li, K.; Zhu, X.; Liu, H.; Wu, X.; Kong, L. Hepatitis C virus NS4B induces unfolded protein response and endoplasmic reticulum overload response-dependent NF-kappaB activation. *Virology* **2009**, *391*, 257–264. [CrossRef]

33. Zheng, Y.; Gao, B.; Ye, L.; Kong, L.; Jing, W.; Yang, X.; Wu, Z.; Ye, L. Hepatitis C virus non-structural protein NS4B can modulate an unfolded protein response. *J. Microbiol.* **2005**, *43*, 529–536.

34. Wang, T.; Campbell, R.V.; Yi, M.K.; Lemon, S.M.; Weinman, S.A. Role of Hepatitis C virus core protein in viral-induced mitochondrial dysfunction. *J. Viral Hepat.* **2010**, *17*, 784–793. [CrossRef] [PubMed]

35. Kang, S.M.; Kim, S.J.; Kim, J.H.; Lee, W.; Kim, G.W.; Lee, K.H.; Choi, K.Y.; Oh, J.W. Interaction of hepatitis C virus core protein with Hsp60 triggers the production of reactive oxygen species and enhances TNF-alpha-mediated apoptosis. *Cancer Lett.* **2009**, *279*, 230–237. [CrossRef] [PubMed]

36. Anelli, T.; Bergamelli, L.; Margittai, E.; Rimessi, A.; Fagioli, C.; Malgaroli, A.; Pinton, P.; Ripamonti, M.; Rizzuto, R.; Sitia, R. Ero1alpha regulates Ca(2+) fluxes at the endoplasmic reticulum-mitochondria interface (MAM). *Antioxid. Redox Signal.* **2012**, *16*, 1077–1087. [CrossRef]

37. Ivanov, A.V.; Smirnova, O.A.; Petrushanko, I.Y.; Ivanova, O.N.; Karpenko, I.L.; Alekseeva, E.; Sominskaya, I.; Makarov, A.A.; Bartosch, B.; Kochetkov, S.N.; et al. HCV core protein uses multiple mechanisms to induce oxidative stress in human hepatoma Huh7 cells. *Viruses* **2015**, *7*, 2745–2770. [CrossRef] [PubMed]

38. Lee, Y.I.; Hwang, J.M.; Im, J.H.; Lee, Y.I.; Kim, N.S.; Kim, D.G.; Yu, D.Y.; Moon, H.B.; Park, S.K. Human hepatitis B virus-X protein alters mitochondrial function and physiology in human liver cells. *J. Biol. Chem.* **2004**, *279*, 15460–15471. [CrossRef]

39. Rahmani, Z.; Huh, K.W.; Lasher, R.; Siddiqui, A. Hepatitis B virus X protein colocalizes to mitochondria with a human voltage-dependent anion channel, HVDAC3, and alters its transmembrane potential. *J. Virol.* **2000**, *74*, 2840–2846. [CrossRef] [PubMed]

40. Zou, L.Y.; Zheng, B.Y.; Fang, X.F.; Li, D.; Huang, Y.H.; Chen, Z.X.; Zhou, L.Y.; Wang, X.Z. HBx co-localizes with COXIII in HL-7702 cells to upregulate mitochondrial function and ROS generation. *Oncol. Rep.* **2015**, *33*, 2461–2467. [CrossRef]

41. Li, D.; Ding, J.; Chen, Z.; Chen, Y.; Lin, N.; Chen, F.; Wang, X. Accurately mapping the location of the binding site for the interaction between hepatitis B virus X protein and cytochrome c oxidase III. *Int. J. Mol. Med.* **2015**, *35*, 319–324. [CrossRef]

42. Lee, H.; Kim, H.; Lee, S.A.; Won, Y.S.; Kim, H.I.; Inn, K.S.; Kim, B.J. Upregulation of endoplasmic reticulum stress and reactive oxygen species by naturally occurring mutations in hepatitis B virus core antigen. *J. Gen. Virol.* **2015**, *96*, 1850–1854. [CrossRef]

43. Lee, I.K.; Lee, S.A.; Kim, H.; Won, Y.S.; Kim, B.J. Induction of endoplasmic reticulum-derived oxidative stress by an occult infection related S surface antigen variant. *World J. Gastroenterol.* **2015**, *21*, 6872–6883. [CrossRef] [PubMed]

44. Smirnova, O.A.; Ivanova, O.N.; Bartosch, B.; Valuev-Elliston, V.T.; Mukhtarov, F.; Kochetkov, S.N.; Ivanov, A.V. Hepatitis C Virus NS5A Protein Triggers Oxidative Stress by Inducing NADPH Oxidases 1 and 4 and Cytochrome P450 2E1. *Oxid. Med. Cell. Longev.* **2016**, *2016*, 8341937. [CrossRef] [PubMed]

45. Williams, V.; Brichler, S.; Khan, E.; Chami, M.; Deny, P.; Kremsdorf, D.; Gordien, E. Large hepatitis delta antigen activates STAT-3 and NF-kappaB via oxidative stress. *J. Viral Hepat.* **2012**, *19*, 744–753. [CrossRef] [PubMed]

46. Abdalla, M.Y.; Ahmad, I.M.; Spitz, D.R.; Schmidt, W.N.; Britigan, B.E. Hepatitis C virus-core and non structural proteins lead to different effects on cellular antioxidant defenses. *J. Med. Virol.* **2005**, *76*, 489–497. [CrossRef]

47. Carvajal-Yepes, M.; Himmelsbach, K.; Schaedler, S.; Ploen, D.; Krause, J.; Ludwig, L.; Weiss, T.; Klingel, K.; Hildt, E. Hepatitis C virus impairs the induction of cytoprotective Nrf2 target genes by delocalization of small Maf proteins. *J. Biol. Chem.* **2011**, *286*, 8941–8951. [CrossRef] [PubMed]

48. Ivanov, A.V.; Smirnova, O.A.; Ivanova, O.N.; Masalova, O.V.; Kochetkov, S.N.; Isaguliants, M.G. Hepatitis C virus proteins activate NRF2/ARE pathway by distinct ROS-dependent and independent mechanisms in HUH7 cells. *PLoS ONE* **2011**, *6*, e24957. [CrossRef]

49. Jiang, Y.; Bao, H.; Ge, Y.; Tang, W.; Cheng, D.; Luo, K.; Gong, G.; Gong, R. Therapeutic targeting of GSK3beta enhances the Nrf2 antioxidant response and confers hepatic cytoprotection in hepatitis C. *Gut* **2015**, *64*, 168–179. [CrossRef]

50. Smirnova, O.A.; Ivanova, O.N.; Mukhtarov, F.S.; Tunitskaya, V.L.; Jansons, J.; Isaguliants, M.G.; Kochetkov, S.N.; Ivanov, A.V. Analysis of the Domains of Hepatitis C Virus Core and NS5A Proteins that Activate the Nrf2/ARE Cascade. *Acta Nat.* **2016**, *8*, 123–127. [CrossRef]

51. Tang, W.; Lazaro, C.A.; Campbell, J.S.; Parks, W.T.; Katze, M.G.; Fausto, N. Responses of nontransformed human hepatocytes to conditional expression of full-length hepatitis C virus open reading frame. *Am. J. Pathol.* **2007**, *171*, 1831–1846. [CrossRef]

52. Blackham, S.; Baillie, A.; Al-Hababi, F.; Remlinger, K.; You, S.; Hamatake, R.; McGarvey, M.J. Gene expression profiling indicates the roles of host oxidative stress, apoptosis, lipid metabolism, and intracellular transport genes in the replication of hepatitis C virus. *J. Virol.* **2010**, *84*, 5404–5414. [CrossRef] [PubMed]

53. Walters, K.A.; Syder, A.J.; Lederer, S.L.; Diamond, D.L.; Paeper, B.; Rice, C.M.; Katze, M.G. Genomic analysis reveals a potential role for cell cycle perturbation in HCV-mediated apoptosis of cultured hepatocytes. *PLoS Pathog.* **2009**, *5*, e1000269. [CrossRef] [PubMed]

54. Schaedler, S.; Krause, J.; Himmelsbach, K.; Carvajal-Yepes, M.; Lieder, F.; Klingel, K.; Nassal, M.; Weiss, T.S.; Werner, S.; Hildt, E. Hepatitis B virus induces expression of antioxidant response element-regulated genes by activation of Nrf2. *J. Biol. Chem.* **2010**, *285*, 41074–41086. [CrossRef] [PubMed]

55. Liu, B.; Fang, M.; He, Z.; Cui, D.; Jia, S.; Lin, X.; Xu, X.; Zhou, T.; Liu, W. Hepatitis B virus stimulates G6PD expression through HBx-mediated Nrf2 activation. *Cell Death Dis.* **2015**, *6*, e1980. [CrossRef]

56. Huang, Y.; Zhang, Y.; Lin, Z.; Han, M.; Cheng, H. Altered serum copper homeostasis suggests higher oxidative stress and lower antioxidant capability in patients with chronic hepatitis B. *Medicine* **2018**, *97*, e11137. [CrossRef]

57. Nakashima, T.; Sumida, Y.; Yoh, T.; Kakisaka, Y.; Nakajima, Y.; Ishikawa, H.; Mitsuyoshi, H.; Kashima, K.; Nakamura, H.; Yodoi, J. Thioredoxin levels in the sera of untreated viral hepatitis patients and those treated with glycyrrhizin or ursodeoxycholic acid. *Antioxid. Redox Signal.* **2000**, *2*, 687–694. [CrossRef]

58. Wang, Q.; Na, B.; Ou, J.H.; Pulliam, L.; Yen, T.S. Hepatitis B virus alters the antioxidant system in transgenic mice and sensitizes hepatocytes to Fas signaling. *PLoS ONE* **2012**, *7*, e36818. [CrossRef]

59. Qi, L.; Zou, Z.Q.; Wang, L.Y.; Gao, S.; Fan, Y.C.; Long, B.; Guo, Y.M.; Xu, A.L.; Han, J.; Li, T.; et al. Methylation of the glutathione-S-transferase M3 gene promoter is associated with oxidative stress in acute-on-chronic hepatitis B liver failure. *Tohoku J. Exp. Med.* **2012**, *228*, 43–51. [CrossRef]

60. Niu, D.; Zhang, J.; Ren, Y.; Feng, H.; Chen, W.N. HBx genotype D represses GSTP1 expression and increases the oxidative level and apoptosis in HepG2 cells. *Mol. Oncol.* **2009**, *3*, 67–76. [CrossRef]

61. Cho, I.J.; Ki, S.H.; Brooks, C., 3rd; Kim, S.G. Role of hepatitis B virus X repression of C/EBPbeta activity in the down-regulation of glutathione S-transferase A2 gene: Implications in other phase II detoxifying enzyme expression. *Xenobiotica* **2009**, *39*, 182–192. [CrossRef]

62. Ding, C.; Wei, H.; Sun, R.; Zhang, J.; Tian, Z. Hepatocytes proteomic alteration and seroproteome analysis of HBV-transgenic mice. *Proteomics* **2009**, *9*, 87–105. [CrossRef] [PubMed]

63. Yi, Y.S.; Park, S.G.; Byeon, S.M.; Kwon, Y.G.; Jung, G. Hepatitis B virus X protein induces TNF-alpha expression via down-regulation of selenoprotein P in human hepatoma cell line, HepG2. *Biochim. Biophys. Acta* **2003**, *1638*, 249–256. [CrossRef]

64. Chen, M.; Du, D.; Zheng, W.; Liao, M.; Zhang, L.; Liang, G.; Gong, M. Small hepatitis delta antigen selectively binds to target mRNA in hepatic cells: A potential mechanism by which hepatitis D virus downregulates glutathione S-transferase P1 and induces liver injury and hepatocarcinogenesis. *Biochem. Cell Biol.* **2019**, *97*, 130–139. [CrossRef]

65. Jaeschke, H. Reactive oxygen and mechanisms of inflammatory liver injury. *J. Gastroenterol. Hepatol.* **2000**, *15*, 718–724. [CrossRef]

66. Gong, G.; Waris, G.; Tanveer, R.; Siddiqui, A. Human hepatitis C virus NS5A protein alters intracellular calcium levels, induces oxidative stress, and activates STAT-3 and NF-kappa B. *Proc. Natl. Acad. Sci. USA* **2001**, *98*, 9599–9604. [CrossRef] [PubMed]

67. Lin, W.; Tsai, W.L.; Shao, R.X.; Wu, G.; Peng, L.F.; Barlow, L.L.; Chung, W.J.; Zhang, L.; Zhao, H.; Jang, J.Y.; et al. Hepatitis C virus regulates transforming growth factor beta1 production through the generation of reactive oxygen species in a nuclear factor kappaB-dependent manner. *Gastroenterology* **2010**, *138*, 2509–2518.e1. [CrossRef] [PubMed]

68. Bataller, R.; Lemon, S.M. Fueling fibrosis in chronic hepatitis C. *Proc. Natl. Acad. Sci. USA* **2012**, *109*, 14293–14294. [CrossRef]

69. Presser, L.D.; Haskett, A.; Waris, G. Hepatitis C virus-induced furin and thrombospondin-1 activate TGF-beta1: Role of TGF-beta1 in HCV replication. *Virology* **2011**, *412*, 284–296. [CrossRef]

70. Ramos-Tovar, E.; Muriel, P. Free radicals, antioxidants, nuclear factor-E2-related factor-2 and liver damage. *J. Appl. Toxicol.* **2020**, *40*, 151–168. [CrossRef]

71. Higgs, M.R.; Lerat, H.; Pawlotsky, J.M. Hepatitis C virus-induced activation of beta-catenin promotes c-Myc expression and a cascade of pro-carcinogenetic events. *Oncogene* **2013**, *32*, 4683–4693. [CrossRef]

72. Nishimura, T.; Kohara, M.; Izumi, K.; Kasama, Y.; Hirata, Y.; Huang, Y.; Shuda, M.; Mukaidani, C.; Takano, T.; Tokunaga, Y.; et al. Hepatitis C virus impairs p53 via persistent overexpression of 3beta-hydroxysterol Delta24-reductase. *J. Biol. Chem.* **2009**, *284*, 36442–36452. [CrossRef] [PubMed]

73. Mankouri, J.; Dallas, M.L.; Hughes, M.E.; Griffin, S.D.; Macdonald, A.; Peers, C.; Harris, M. Suppression of a pro-apoptotic K+ channel as a mechanism for hepatitis C virus persistence. *Proc. Natl. Acad. Sci. USA* **2009**, *106*, 15903–15908. [CrossRef] [PubMed]

74. Tanaka, N.; Moriya, K.; Kiyosawa, K.; Koike, K.; Gonzalez, F.J.; Aoyama, T. PPARalpha activation is essential for HCV core protein-induced hepatic steatosis and hepatocellular carcinoma in mice. *J. Clin. Investig.* **2008**, *118*, 683–694. [CrossRef] [PubMed]

75. Seo, Y.L.; Heo, S.; Jang, K.L. Hepatitis C virus core protein overcomes H_2O_2-induced apoptosis by downregulating p14 expression via DNA methylation. *J. Gen. Virol.* **2015**, *96*, 822–832. [CrossRef]

76. O'Reilly, M.A. Redox activation of p21Cip1/WAF1/Sdi1: A multifunctional regulator of cell survival and death. *Antioxid. Redox Signal.* **2005**, *7*, 108–118. [CrossRef]

77. Inoue, T.; Kato, K.; Kato, H.; Asanoma, K.; Kuboyama, A.; Ueoka, Y.; Yamaguchi, S.; Ohgami, T.; Wake, N. Level of reactive oxygen species induced by p21Waf1/CIP1 is critical for the determination of cell fate. *Cancer Sci.* **2009**, *100*, 1275–1283. [CrossRef]

78. Villeneuve, N.F.; Sun, Z.; Chen, W.; Zhang, D.D. Nrf2 and p21 regulate the fine balance between life and death by controlling ROS levels. *Cell Cycle* **2009**, *8*, 3255–3256. [CrossRef]

79. Majumder, M.; Ghosh, A.K.; Steele, R.; Ray, R.; Ray, R.B. Hepatitis C virus NS5A physically associates with p53 and regulates p21/waf1 gene expression in a p53-dependent manner. *J. Virol.* **2001**, *75*, 1401–1407. [CrossRef]

80. Wang, F.; Yoshida, I.; Takamatsu, M.; Ishido, S.; Fujita, T.; Oka, K.; Hotta, H. Complex formation between hepatitis C virus core protein and p21Waf1/Cip1/Sdi1. *Biochem. Biophys. Res. Commun.* **2000**, *273*, 479–484. [CrossRef]

81. Elpek, G.O. Molecular pathways in viral hepatitis-associated liver carcinogenesis: An update. *World J. Clin. Cases* **2021**, *9*, 4890–4917. [CrossRef]

82. Park, C.Y.; Oh, S.H.; Kang, S.M.; Lim, Y.S.; Hwang, S.B. Hepatitis delta virus large antigen sensitizes to TNF-alpha-induced NF-kappaB signaling. *Mol. Cells* **2009**, *28*, 49–55. [CrossRef]

83. Williams, V.; Brichler, S.; Radjef, N.; Lebon, P.; Goffard, A.; Hober, D.; Fagard, R.; Kremsdorf, D.; Deny, P.; Gordien, E. Hepatitis delta virus proteins repress hepatitis B virus enhancers and activate the alpha/beta interferon-inducible MxA gene. *J. Gen. Virol.* **2009**, *90*, 2759–2767. [CrossRef]

84. Zampino, R.; Marrone, A.; Restivo, L.; Guerrera, B.; Sellitto, A.; Rinaldi, L.; Romano, C.; Adinolfi, L.E. Chronic HCV infection and inflammation: Clinical impact on hepatic and extra-hepatic manifestations. *World J. Hepatol.* **2013**, *5*, 528–540. [CrossRef]

85. McNab, F.; Mayer-Barber, K.; Sher, A.; Wack, A.; O'Garra, A. Type I interferons in infectious disease. *Nat. Rev. Immunol.* **2015**, *15*, 87–103. [CrossRef] [PubMed]

86. Brezgin, S.; Kostyusheva, A.; Bayurova, E.; Volchkova, E.; Gegechkori, V.; Gordeychuk, I.; Glebe, D.; Kostyushev, D.; Chulanov, V. Immunity and Viral Infections: Modulating Antiviral Response via CRISPR-Cas Systems. *Viruses* **2021**, *13*, 1373. [CrossRef] [PubMed]

87. Heim, M.H.; Thimme, R. Innate and adaptive immune responses in HCV infections. *J. Hepatol.* **2014**, *61*, S14–S25. [CrossRef] [PubMed]

88. Jung, S.; Altstetter, S.M.; Protzer, U. Innate immune recognition and modulation in hepatitis D virus infection. *World J. Gastroenterol.* **2020**, *26*, 2781–2791. [CrossRef] [PubMed]

89. Pugnale, P.; Pazienza, V.; Guilloux, K.; Negro, F. Hepatitis delta virus inhibits alpha interferon signaling. *Hepatology* **2009**, *49*, 398–406. [CrossRef]

90. Lupberger, J.; Duong, F.H.; Fofana, I.; Zona, L.; Xiao, F.; Thumann, C.; Durand, S.C.; Pessaux, P.; Zeisel, M.B.; Heim, M.H.; et al. Epidermal growth factor receptor signaling impairs the antiviral activity of interferon-alpha. *Hepatology* **2013**, *58*, 1225–1235. [CrossRef]

91. Hosel, M.; Quasdorff, M.; Ringelhan, M.; Kashkar, H.; Debey-Pascher, S.; Sprinzl, M.F.; Bockmann, J.H.; Arzberger, S.; Webb, D.; von Olshausen, G.; et al. Hepatitis B Virus Activates Signal Transducer and Activator of Transcription 3 Supporting Hepatocyte Survival and Virus Replication. *Cell. Mol. Gastroenterol. Hepatol.* **2017**, *4*, 339–363. [CrossRef]

92. Yang, Y.; Zheng, B.; Han, Q.; Zhang, C.; Tian, Z.; Zhang, J. Targeting blockage of STAT3 inhibits hepatitis B virus-related hepatocellular carcinoma. *Cancer Biol. Ther.* **2016**, *17*, 449–456. [CrossRef] [PubMed]

93. Iwamoto, M.; Saso, W.; Sugiyama, R.; Ishii, K.; Ohki, M.; Nagamori, S.; Suzuki, R.; Aizaki, H.; Ryo, A.; Yun, J.H.; et al. Epidermal growth factor receptor is a host-entry cofactor triggering hepatitis B virus internalization. *Proc. Natl. Acad. Sci. USA* **2019**, *116*, 8487–8492. [CrossRef] [PubMed]

94. Lupberger, J.; Zeisel, M.B.; Xiao, F.; Thumann, C.; Fofana, I.; Zona, L.; Davis, C.; Mee, C.J.; Turek, M.; Gorke, S.; et al. EGFR and EphA2 are host factors for hepatitis C virus entry and possible targets for antiviral therapy. *Nat. Med.* **2011**, *17*, 589–595. [CrossRef] [PubMed]

95. Zona, L.; Lupberger, J.; Sidahmed-Adrar, N.; Thumann, C.; Harris, H.J.; Barnes, A.; Florentin, J.; Tawar, R.G.; Xiao, F.; Turek, M.; et al. HRas signal transduction promotes hepatitis C virus cell entry by triggering assembly of the host tetraspanin receptor complex. *Cell Host Microbe* **2013**, *13*, 302–313. [CrossRef]

96. Van Renne, N.; Roca Suarez, A.A.; Duong, F.H.T.; Gondeau, C.; Calabrese, D.; Fontaine, N.; Ababsa, A.; Bandiera, S.; Croonen-borghs, T.; Pochet, N.; et al. miR-135a-5p-mediated downregulation of protein tyrosine phosphatase receptor delta is a candidate driver of HCV-associated hepatocarcinogenesis. *Gut* **2018**, *67*, 953–962. [CrossRef]

97. Mori, R.; Wauman, J.; Icardi, L.; Van der Heyden, J.; De Cauwer, L.; Peelman, F.; De Bosscher, K.; Tavernier, J. TYK2-induced phosphorylation of Y640 suppresses STAT3 transcriptional activity. *Sci. Rep.* **2017**, *7*, 15919. [CrossRef] [PubMed]

98. Wang, C.C.; Cheng, P.N.; Kao, J.H. Systematic review: Chronic viral hepatitis and metabolic derangement. *Aliment. Pharmacol. Ther.* **2020**, *51*, 216–230. [CrossRef]

99. Hui, R.W.H.; Seto, W.K.; Cheung, K.S.; Mak, L.Y.; Liu, K.S.H.; Fung, J.; Wong, D.K.; Lai, C.L.; Yuen, M.F. Inverse relationship between hepatic steatosis and hepatitis B viremia: Results of a large case-control study. *J. Viral Hepat.* **2018**, *25*, 97–104. [CrossRef]

100. Joo, E.J.; Chang, Y.; Yeom, J.S.; Ryu, S. Hepatitis B virus infection and decreased risk of nonalcoholic fatty liver disease: A cohort study. *Hepatology* **2017**, *65*, 828–835. [CrossRef]

101. Seto, W.K. Chronic hepatitis B and metabolic risk factors: A call for rigorous longitudinal studies. *World J. Gastroenterol.* **2019**, *25*, 282–286. [CrossRef]

102. Patel, A.; Harrison, S.A. Hepatitis C virus infection and nonalcoholic steatohepatitis. *Gastroenterol. Hepatol.* **2012**, *8*, 305–312.

103. Wrensch, F.; Crouchet, E.; Ligat, G.; Zeisel, M.B.; Keck, Z.Y.; Foung, S.K.H.; Schuster, C.; Baumert, T.F. Hepatitis C Virus (HCV)-Apolipoprotein Interactions and Immune Evasion and Their Impact on HCV Vaccine Design. *Front. Immunol.* **2018**, *9*, 1436. [CrossRef]

104. Benga, W.J.; Krieger, S.E.; Dimitrova, M.; Zeisel, M.B.; Parnot, M.; Lupberger, J.; Hildt, E.; Luo, G.; McLauchlan, J.; Baumert, T.F.; et al. Apolipoprotein E interacts with hepatitis C virus nonstructural protein 5A and determines assembly of infectious particles. *Hepatology* **2010**, *51*, 43–53. [CrossRef]

105. Chang, K.S.; Jiang, J.; Cai, Z.; Luo, G. Human apolipoprotein e is required for infectivity and production of hepatitis C virus in cell culture. *J. Virol.* **2007**, *81*, 13783–13793. [CrossRef] [PubMed]

106. Herker, E.; Harris, C.; Hernandez, C.; Carpentier, A.; Kaehlcke, K.; Rosenberg, A.R.; Farese, R.V., Jr.; Ott, M. Efficient hepatitis C virus particle formation requires diacylglycerol acyltransferase-1. *Nat. Med.* **2010**, *16*, 1295–1298. [CrossRef]

107. Li, Q.; Zhang, Y.Y.; Chiu, S.; Hu, Z.; Lan, K.H.; Cha, H.; Sodroski, C.; Zhang, F.; Hsu, C.S.; Thomas, E.; et al. Integrative functional genomics of hepatitis C virus infection identifies host dependencies in complete viral replication cycle. *PLoS Pathog.* **2014**, *10*, e1004163. [CrossRef] [PubMed]

108. Levy, P.L.; Duponchel, S.; Eischeid, H.; Molle, J.; Michelet, M.; Diserens, G.; Vermathen, M.; Vermathen, P.; Dufour, J.F.; Dienes, H.P.; et al. Hepatitis C virus infection triggers a tumor-like glutamine metabolism. *Hepatology* **2017**, *65*, 789–803. [CrossRef] [PubMed]

109. He, G.; Karin, M. NF-kappaB and STAT3—Key players in liver inflammation and cancer. *Cell Res.* **2011**, *21*, 159–168. [CrossRef]

110. Park, E.J.; Lee, J.H.; Yu, G.Y.; He, G.; Ali, S.R.; Holzer, R.G.; Osterreicher, C.H.; Takahashi, H.; Karin, M. Dietary and genetic obesity promote liver inflammation and tumorigenesis by enhancing IL-6 and TNF expression. *Cell* **2010**, *140*, 197–208. [CrossRef]

111. Chang, Z.; Wang, Y.; Zhou, X.; Long, J.E. STAT3 roles in viral infection: Antiviral or proviral? *Future Virol.* **2018**, *13*, 557–574. [CrossRef]

112. Hiscott, J.; Kwon, H.; Genin, P. Hostile takeovers: Viral appropriation of the NF-kappaB pathway. *J. Clin. Investig.* **2001**, *107*, 143–151. [CrossRef] [PubMed]

113. Waris, G.; Huh, K.W.; Siddiqui, A. Mitochondrially associated hepatitis B virus X protein constitutively activates transcription factors STAT-3 and NF-kappa B via oxidative stress. *Mol. Cell. Biol.* **2001**, *21*, 7721–7730. [CrossRef] [PubMed]

114. Yoshida, T.; Hanada, T.; Tokuhisa, T.; Kosai, K.; Sata, M.; Kohara, M.; Yoshimura, A. Activation of STAT3 by the hepatitis C virus core protein leads to cellular transformation. *J. Exp. Med.* **2002**, *196*, 641–653. [CrossRef] [PubMed]

115. Du, L.; Ma, Y.; Liu, M.; Yan, L.; Tang, H. Peroxisome Proliferators Activated Receptor (PPAR) agonists activate hepatitis B virus replication in vivo. *Virol J.* **2017**, *14*, 96. [CrossRef]

116. Hu, W.; Wang, X.; Ding, X.; Li, Y.; Zhang, X.; Xie, P.; Yang, J.; Wang, S. MicroRNA-141 represses HBV replication by targeting PPARA. *PLoS ONE* **2012**, *7*, e34165. [CrossRef]

117. Dubuquoy, L.; Louvet, A.; Hollebecque, A.; Mathurin, P.; Dharancy, S. Peroxisome proliferator-activated receptors in HBV-related infection. *PPAR Res.* **2009**, *2009*, 145124. [CrossRef]

118. Wang, M.D.; Wu, H.; Huang, S.; Zhang, H.L.; Qin, C.J.; Zhao, L.H.; Fu, G.B.; Zhou, X.; Wang, X.M.; Tang, L.; et al. HBx regulates fatty acid oxidation to promote hepatocellular carcinoma survival during metabolic stress. *Oncotarget* **2016**, *7*, 6711–6726. [CrossRef]

119. Wu, Y.L.; Peng, X.E.; Zhu, Y.B.; Yan, X.L.; Chen, W.N.; Lin, X. Hepatitis B Virus X Protein Induces Hepatic Steatosis by Enhancing the Expression of Liver Fatty Acid Binding Protein. *J. Virol.* **2016**, *90*, 1729–1740. [CrossRef]

120. Pawlak, M.; Lefebvre, P.; Staels, B. Molecular mechanism of PPARalpha action and its impact on lipid metabolism, inflammation and fibrosis in non-alcoholic fatty liver disease. *J. Hepatol.* **2015**, *62*, 720–733. [CrossRef]

121. Friedman, S.L.; Neuschwander-Tetri, B.A.; Rinella, M.; Sanyal, A.J. Mechanisms of NAFLD development and therapeutic strategies. *Nat. Med.* **2018**, *24*, 908–922. [CrossRef]

122. Haga, Y.; Kanda, T.; Sasaki, R.; Nakamura, M.; Nakamoto, S.; Yokosuka, O. Nonalcoholic fatty liver disease and hepatic cirrhosis: Comparison with viral hepatitis-associated steatosis. *World J. Gastroenterol.* **2015**, *21*, 12989–12995. [CrossRef] [PubMed]

123. Yang, F.; Yan, S.; He, Y.; Wang, F.; Song, S.; Guo, Y.; Zhou, Q.; Wang, Y.; Lin, Z.; Yang, Y.; et al. Expression of hepatitis B virus proteins in transgenic mice alters lipid metabolism and induces oxidative stress in the liver. *J. Hepatol.* **2008**, *48*, 12–19. [CrossRef] [PubMed]

124. Hajjou, M.; Norel, R.; Carver, R.; Marion, P.; Cullen, J.; Rogler, L.E.; Rogler, C.E. cDNA microarray analysis of HBV transgenic mouse liver identifies genes in lipid biosynthetic and growth control pathways affected by HBV. *J. Med. Virol.* **2005**, *77*, 57–65. [CrossRef]

125. Hertzel, A.V.; Bernlohr, D.A. The mammalian fatty acid-binding protein multigene family: Molecular and genetic insights into function. *Trends Endocrinol. Metab.* **2000**, *11*, 175–180. [CrossRef]

126. Liu, G.; Liu, G.; Cui, X.; Xu, Y. Transcriptomic Data Analyses Reveal a Reprogramed Lipid Metabolism in HCV-Derived Hepatocellular Cancer. *Front. Cell Dev. Biol.* **2020**, *8*, 581863. [CrossRef]

127. Shieh, Y.S.; Chang, Y.S.; Hong, J.R.; Chen, L.J.; Jou, L.K.; Hsu, C.C.; Her, G.M. Increase of hepatic fat accumulation by liver specific expression of Hepatitis B virus X protein in zebrafish. *Biochim. Biophys. Acta* **2010**, *1801*, 721–730. [CrossRef]

128. Na, T.Y.; Shin, Y.K.; Roh, K.J.; Kang, S.A.; Hong, I.; Oh, S.J.; Seong, J.K.; Park, C.K.; Choi, Y.L.; Lee, M.O. Liver X receptor mediates hepatitis B virus X protein-induced lipogenesis in hepatitis B virus-associated hepatocellular carcinoma. *Hepatology* **2009**, *49*, 1122–1131. [CrossRef] [PubMed]

129. Kim, K.; Kim, K.H.; Kim, H.H.; Cheong, J. Hepatitis B virus X protein induces lipogenic transcription factor SREBP1 and fatty acid synthase through the activation of nuclear receptor LXRalpha. *Biochem. J.* **2008**, *416*, 219–230. [CrossRef]

130. Kim, K.H.; Shin, H.J.; Kim, K.; Choi, H.M.; Rhee, S.H.; Moon, H.B.; Kim, H.H.; Yang, U.S.; Yu, D.Y.; Cheong, J. Hepatitis B virus X protein induces hepatic steatosis via transcriptional activation of SREBP1 and PPARgamma. *Gastroenterology* **2007**, *132*, 1955–1967. [CrossRef]

131. Mota, S.; Mendes, M.; Freitas, N.; Penque, D.; Coelho, A.V.; Cunha, C. Proteome analysis of a human liver carcinoma cell line stably expressing hepatitis delta virus ribonucleoproteins. *J. Proteomics* **2009**, *72*, 616–627. [CrossRef]

132. Petta, S.; Camma, C.; Di Marco, V.; Macaluso, F.S.; Maida, M.; Pizzolanti, G.; Belmonte, B.; Cabibi, D.; Di Stefano, R.; Ferraro, D.; et al. Hepatic steatosis and insulin resistance are associated with severe fibrosis in patients with chronic hepatitis caused by HBV or HCV infection. *Liver Int.* **2011**, *31*, 507–515. [CrossRef] [PubMed]

133. Verrier, E.R.; Weiss, A.; Bach, C.; Heydmann, L.; Turon-Lagot, V.; Kopp, A.; El Saghire, H.; Crouchet, E.; Pessaux, P.; Garcia, T.; et al. Combined small molecule and loss-of-function screen uncovers estrogen receptor alpha and CAD as host factors for HDV infection and antiviral targets. *Gut* **2020**, *69*, 158–167. [CrossRef] [PubMed]

134. Hatting, M.; Tavares, C.D.J.; Sharabi, K.; Rines, A.K.; Puigserver, P. Insulin regulation of gluconeogenesis. *Ann. N. Y. Acad. Sci.* **2018**, *1411*, 21–35. [CrossRef] [PubMed]

135. Wang, X.; Lin, Y.; Kemper, T.; Chen, J.; Yuan, Z.; Liu, S.; Zhu, Y.; Broering, R.; Lu, M. AMPK and Akt/mTOR signalling pathways participate in glucose-mediated regulation of hepatitis B virus replication and cellular autophagy. *Cell. Microbiol.* **2020**, *22*, e13131. [CrossRef]

136. Banerjee, S.; Saito, K.; Ait-Goughoulte, M.; Meyer, K.; Ray, R.B.; Ray, R. Hepatitis C virus core protein upregulates serine phosphorylation of insulin receptor substrate-1 and impairs the downstream akt/protein kinase B signaling pathway for insulin resistance. *J. Virol.* **2008**, *82*, 2606–2612. [CrossRef] [PubMed]

137. Gao, T.T.; Qin, Z.L.; Ren, H.; Zhao, P.; Qi, Z.T. Inhibition of IRS-1 by hepatitis C virus infection leads to insulin resistance in a PTEN-dependent manner. *Virol. J.* **2015**, *12*, 12. [CrossRef] [PubMed]

138. Parvaiz, F.; Manzoor, S.; Iqbal, J.; Sarkar-Dutta, M.; Imran, M.; Waris, G. Hepatitis C virus NS5A promotes insulin resistance through IRS-1 serine phosphorylation and increased gluconeogenesis. *World J. Gastroenterol.* **2015**, *21*, 12361–12369. [CrossRef]

139. Arai, T.; Kano, F.; Murata, M. Translocation of forkhead box O1 to the nuclear periphery induces histone modifications that regulate transcriptional repression of PCK1 in HepG2 cells. *Genes Cells* **2015**, *20*, 340–357. [CrossRef]

140. Bose, S.K.; Shrivastava, S.; Meyer, K.; Ray, R.B.; Ray, R. Hepatitis C virus activates the mTOR/S6K1 signaling pathway in inhibiting IRS-1 function for insulin resistance. *J. Virol.* **2012**, *86*, 6315–6322. [CrossRef]

141. Deng, L.; Shoji, I.; Ogawa, W.; Kaneda, S.; Soga, T.; Jiang, D.P.; Ide, Y.H.; Hotta, H. Hepatitis C virus infection promotes hepatic gluconeogenesis through an NS5A-mediated, FoxO1-dependent pathway. *J. Virol.* **2011**, *85*, 8556–8568. [CrossRef]

142. Shin, H.J.; Park, Y.H.; Kim, S.U.; Moon, H.B.; Park, D.S.; Han, Y.H.; Lee, C.H.; Lee, D.S.; Song, I.S.; Lee, D.H.; et al. Hepatitis B virus X protein regulates hepatic glucose homeostasis via activation of inducible nitric oxide synthase. *J. Biol. Chem.* **2011**, *286*, 29872–29881. [CrossRef] [PubMed]

143. Li, H.; Zhu, W.; Zhang, L.; Lei, H.; Wu, X.; Guo, L.; Chen, X.; Wang, Y.; Tang, H. The metabolic responses to hepatitis B virus infection shed new light on pathogenesis and targets for treatment. *Sci. Rep.* **2015**, *5*, 8421. [CrossRef] [PubMed]

144. Michalopoulos, G.K.; Bhushan, B. Liver regeneration: Biological and pathological mechanisms and implications. *Nat. Rev. Gastroenterol. Hepatol.* **2021**, *18*, 40–55. [CrossRef] [PubMed]

145. Tomas, A.; Futter, C.E.; Eden, E.R. EGF receptor trafficking: Consequences for signaling and cancer. *Trends Cell Biol.* **2014**, *24*, 26–34. [CrossRef]

146. Mailly, L.; Xiao, F.; Lupberger, J.; Wilson, G.K.; Aubert, P.; Duong, F.H.T.; Calabrese, D.; Leboeuf, C.; Fofana, I.; Thumann, C.; et al. Clearance of persistent hepatitis C virus infection in humanized mice using a claudin-1-targeting monoclonal antibody. *Nat. Biotechnol.* **2015**, *33*, 549–554. [CrossRef]

147. Diao, J.; Pantua, H.; Ngu, H.; Komuves, L.; Diehl, L.; Schaefer, G.; Kapadia, S.B. Hepatitis C virus induces epidermal growth factor receptor activation via CD81 binding for viral internalization and entry. *J. Virol.* **2012**, *86*, 10935–10949. [CrossRef] [PubMed]

148. Mankouri, J.; Griffin, S.; Harris, M. The hepatitis C virus non-structural protein NS5A alters the trafficking profile of the epidermal growth factor receptor. *Traffic* **2008**, *9*, 1497–1509. [CrossRef] [PubMed]

149. Igloi, Z.; Kazlauskas, A.; Saksela, K.; Macdonald, A.; Mankouri, J.; Harris, M. Hepatitis C virus NS5A protein blocks epidermal growth factor receptor degradation via a proline motif- dependent interaction. *J. Gen. Virol.* **2015**, *96*, 2133–2144. [CrossRef]

150. Stindt, S.; Cebula, P.; Albrecht, U.; Keitel, V.; Schulte am Esch, J.; Knoefel, W.T.; Bartenschlager, R.; Haussinger, D.; Bode, J.G. Hepatitis C Virus Activates a Neuregulin-Driven Circuit to Modify Surface Expression of Growth Factor Receptors of the ErbB Family. *PLoS ONE* **2016**, *11*, e0148711. [CrossRef]

151. Iwamoto, M.; Saso, W.; Nishioka, K.; Ohashi, H.; Sugiyama, R.; Ryo, A.; Ohki, M.; Yun, J.H.; Park, S.Y.; Ohshima, T.; et al. The machinery for endocytosis of epidermal growth factor receptor coordinates the transport of incoming hepatitis B virus to the endosomal network. *J. Biol. Chem.* **2020**, *295*, 800–807. [CrossRef]

152. Burckstummer, T.; Kriegs, M.; Lupberger, J.; Pauli, E.K.; Schmittel, S.; Hildt, E. Raf-1 kinase associates with Hepatitis C virus NS5A and regulates viral replication. *FEBS Lett.* **2006**, *580*, 575–580. [CrossRef] [PubMed]

153. Feng, D.Y.; Sun, Y.; Cheng, R.X.; Ouyang, X.M.; Zheng, H. Effect of hepatitis C virus nonstructural protein NS3 on proliferation and MAPK phosphorylation of normal hepatocyte line. *World J. Gastroenterol.* **2005**, *11*, 2157–2161. [CrossRef] [PubMed]

154. Hayashi, J.; Aoki, H.; Kajino, K.; Moriyama, M.; Arakawa, Y.; Hino, O. Hepatitis C virus core protein activates the MAPK/ERK cascade synergistically with tumor promoter TPA, but not with epidermal growth factor or transforming growth factor alpha. *Hepatology* **2000**, *32*, 958–961. [CrossRef]

155. Zhao, L.J.; Wang, L.; Ren, H.; Cao, J.; Li, L.; Ke, J.S.; Qi, Z.T. Hepatitis C virus E2 protein promotes human hepatoma cell proliferation through the MAPK/ERK signaling pathway via cellular receptors. *Exp. Cell Res.* **2005**, *305*, 23–32. [CrossRef] [PubMed]

156. Hoxhaj, G.; Manning, B.D. The PI3K-AKT network at the interface of oncogenic signalling and cancer metabolism. *Nat. Rev. Cancer* **2020**, *20*, 74–88. [CrossRef]

157. Chen, C.Y.; Chen, J.; He, L.; Stiles, B.L. PTEN: Tumor Suppressor and Metabolic Regulator. *Front. Endocrinol.* **2018**, *9*, 338. [CrossRef]

158. Hemmings, B.A.; Restuccia, D.F. PI3K-PKB/Akt pathway. *Cold Spring Harb. Perspect. Biol.* **2012**, *4*, a011189. [CrossRef]

159. Cheng, D.; Zhang, L.; Yang, G.; Zhao, L.; Peng, F.; Tian, Y.; Xiao, X.; Chung, R.T.; Gong, G. Hepatitis C virus NS5A drives a PTEN-PI3K/Akt feedback loop to support cell survival. *Liver Int.* **2015**, *35*, 1682–1691. [CrossRef]

160. Rawat, S.; Bouchard, M.J. The hepatitis B virus (HBV) HBx protein activates AKT to simultaneously regulate HBV replication and hepatocyte survival. *J. Virol.* **2015**, *89*, 999–1012. [CrossRef]

161. Xiang, K.; Wang, B. Role of the PI3KAKTmTOR pathway in hepatitis B virus infection and replication. *Mol. Med. Rep.* **2018**, *17*, 4713–4719. [CrossRef]

162. Sun, Y.; Yu, M.; Qu, M.; Ma, Y.; Zheng, D.; Yue, Y.; Guo, S.; Tang, L.; Li, G.; Zheng, W.; et al. Hepatitis B virus-triggered PTEN/beta-catenin/c-Myc signaling enhances PD-L1 expression to promote immune evasion. *Am. J. Physiol. Gastrointest Liver Physiol.* **2020**, *318*, G162–G173. [CrossRef] [PubMed]

163. Perugorria, M.J.; Olaizola, P.; Labiano, I.; Esparza-Baquer, A.; Marzioni, M.; Marin, J.J.G.; Bujanda, L.; Banales, J.M. Wnt-beta-catenin signalling in liver development, health and disease. *Nat. Rev. Gastroenterol. Hepatol.* **2019**, *16*, 121–136. [CrossRef] [PubMed]

164. Wang, W.; Pan, Q.; Fuhler, G.M.; Smits, R.; Peppelenbosch, M.P. Action and function of Wnt/beta-catenin signaling in the progression from chronic hepatitis C to hepatocellular carcinoma. *J. Gastroenterol.* **2017**, *52*, 419–431. [CrossRef] [PubMed]

165. Liu, J.; Ding, X.; Tang, J.; Cao, Y.; Hu, P.; Zhou, F.; Shan, X.; Cai, X.; Chen, Q.; Ling, N.; et al. Enhancement of canonical Wnt/beta-catenin signaling activity by HCV core protein promotes cell growth of hepatocellular carcinoma cells. *PLoS ONE* **2011**, *6*, e27496. [CrossRef]

166. Street, A.; Macdonald, A.; McCormick, C.; Harris, M. Hepatitis C virus NS5A-mediated activation of phosphoinositide 3-kinase results in stabilization of cellular beta-catenin and stimulation of beta-catenin-responsive transcription. *J. Virol.* **2005**, *79*, 5006–5016. [CrossRef]

167. Roehlen, N.; Crouchet, E.; Baumert, T.F. Liver Fibrosis: Mechanistic Concepts and Therapeutic Perspectives. *Cells* **2020**, *9*, 875. [CrossRef]

168. Chusri, P.; Kumthip, K.; Hong, J.; Zhu, C.; Duan, X.; Jilg, N.; Fusco, D.N.; Brisac, C.; Schaefer, E.A.; Cai, D.; et al. HCV induces transforming growth factor beta1 through activation of endoplasmic reticulum stress and the unfolded protein response. *Sci. Rep.* **2016**, *6*, 22487. [CrossRef]

169. Zou, L.L.; Li, J.R.; Li, H.; Tan, J.L.; Wang, M.X.; Liu, N.N.; Gao, R.M.; Yan, H.Y.; Wang, X.K.; Dong, B.; et al. TGF-beta isoforms inhibit hepatitis C virus propagation in transforming growth factor beta/SMAD protein signalling pathway dependent and independent manners. *J. Cell. Mol. Med.* **2021**, *25*, 3498–3510. [CrossRef]

170. Choi, S.H.; Hwang, S.B. Modulation of the transforming growth factor-beta signal transduction pathway by hepatitis C virus nonstructural 5A protein. *J. Biol. Chem.* **2006**, *281*, 7468–7478. [CrossRef]

171. Liang, G.; Liu, G.; Kitamura, K.; Wang, Z.; Chowdhury, S.; Monjurul, A.M.; Wakae, K.; Koura, M.; Shimadu, M.; Kinoshita, K.; et al. TGF-beta suppression of HBV RNA through AID-dependent recruitment of an RNA exosome complex. *PLoS Pathog.* **2015**, *11*, e1004780. [CrossRef]

172. Choi, S.H.; Jeong, S.H.; Hwang, S.B. Large hepatitis delta antigen modulates transforming growth factor-beta signaling cascades: Implication of hepatitis delta virus-induced liver fibrosis. *Gastroenterology* **2007**, *132*, 343–357. [CrossRef]

173. Liang, Y.J.; Sun, C.P.; Hsu, Y.C.; Chen, Y.W.; Wang, I.A.; Su, C.W.; Tao, M.H.; Wu, J.C. Statin inhibits large hepatitis delta antigen-Smad3 -twist-mediated epithelial-to-mesenchymal transition and hepatitis D virus secretion. *J. Biomed. Sci.* **2020**, *27*, 65. [CrossRef] [PubMed]

174. Wedemeyer, H.; Manns, M.P. Epidemiology, pathogenesis and management of hepatitis D: Update and challenges ahead. *Nat. Rev. Gastroenterol. Hepatol.* **2010**, *7*, 31–40. [CrossRef]

175. Klohn, M.; Schrader, J.A.; Bruggemann, Y.; Todt, D.; Steinmann, E. Beyond the Usual Suspects: Hepatitis E Virus and Its Implications in Hepatocellular Carcinoma. *Cancers* **2021**, *13*, 5867. [CrossRef] [PubMed]

176. Khuroo, M.S.; Khuroo, M.S.; Khuroo, N.S. Hepatitis E: Discovery, global impact, control and cure. *World J. Gastroenterol.* **2016**, *22*, 7030–7045. [CrossRef] [PubMed]

177. Pottier, C.; Fresnais, M.; Gilon, M.; Jerusalem, G.; Longuespee, R.; Sounni, N.E. Tyrosine Kinase Inhibitors in Cancer: Breakthrough and Challenges of Targeted Therapy. *Cancers* **2020**, *12*, 731. [CrossRef]

178. Fuchs, B.C.; Hoshida, Y.; Fujii, T.; Wei, L.; Yamada, S.; Lauwers, G.Y.; McGinn, C.M.; DePeralta, D.K.; Chen, X.; Kuroda, T.; et al. Epidermal growth factor receptor inhibition attenuates liver fibrosis and development of hepatocellular carcinoma. *Hepatology* **2014**, *59*, 1577–1590. [CrossRef]

179. El-Far, Y.M.; Khodir, A.E.; Emarah, Z.A.; Ebrahim, M.A.; Al-Gayyar, M.M.H. Chemopreventive and hepatoprotective effects of genistein via inhibition of oxidative stress and the versican/PDGF/PKC signaling pathway in experimentally induced hepatocellular carcinoma in rats by thioacetamide. *Redox Rep.* **2022**, *27*, 9–20. [CrossRef]

180. Faure-Dupuy, S.; Riedl, T.; Rolland, M.; Hizir, Z.; Reisinger, F.; Neuhaus, K.; Schuehle, S.; Remouchamps, C.; Gillet, N.; Schonung, M.; et al. Control of APOBEC3B induction and cccDNA decay by NF-kappaB and miR-138-5p. *JHEP Rep.* **2021**, *3*, 100354. [CrossRef]

181. Zeisel, M.B.; Lupberger, J.; Fofana, I.; Baumert, T.F. Host-targeting agents for prevention and treatment of chronic hepatitis C—Perspectives and challenges. *J. Hepatol.* **2013**, *58*, 375–384. [CrossRef]

Annex V: Profibrotic Signaling and HCC Risk during Chronic Viral Hepatitis: Biomarker Development

<u>Review</u>

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Review

Profibrotic Signaling and HCC Risk during Chronic Viral Hepatitis: Biomarker Development

Alessia Virzì [1,2], Victor Gonzalez-Motos [1,2], Simona Tripon [1,2,3], Thomas F. Baumert [1,2,3,4] and Joachim Lupberger [1,2,*]

1   Université de Strasbourg, 67000 Strasbourg, France; virzi@unistra.fr (A.V.);
    gonzalezmotos@unistra.fr (V.G.-M.); simona.tripon@chru-strasbourg.fr (S.T.);
    thomas.baumert@unistra.fr (T.F.B.)
2   Institut National de la Santé et de la Recherche Médicale, U1110, Institut de Recherche sur les Maladies
    Virales et Hépatiques (IVH), 67000 Strasbourg, France
3   Institut Hospitalo-Universitaire, Pôle Hépato-Digestif, Nouvel Hôpital Civil, 67091 Strasbourg, France
4   Institut Universitaire de France (IUF), 75231 Paris, France
*   Correspondence: joachim.lupberger@unistra.fr

Abstract: Despite breakthroughs in antiviral therapies, chronic viral hepatitis B and C are still the major causes of liver fibrosis and hepatocellular carcinoma (HCC). Importantly, even in patients with controlled infection or viral cure, the cancer risk cannot be fully eliminated, highlighting a persisting oncogenic pressure imposed by epigenetic imprinting and advanced liver disease. Reliable and minimally invasive biomarkers for early fibrosis and for residual HCC risk in HCV-cured patients are urgently needed. Chronic infection with HBV and/or HCV dysregulates oncogenic and profibrogenic signaling within the host, also displayed in the secretion of soluble factors to the blood. The study of virus-dysregulated signaling pathways may, therefore, contribute to the identification of reliable minimally invasive biomarkers for the detection of patients at early-stage liver disease potentially complementing existing noninvasive methods in clinics. With a focus on virus-induced signaling events, this review provides an overview of candidate blood biomarkers for liver disease and HCC risk associated with chronic viral hepatitis and epigenetic viral footprints.

Keywords: HBV; HCV; biomarkers; liver disease; HCC; cure; risk

1. Introduction

Chronic liver disease is a major health problem and globally associated with > 2 million deaths per year [1]. The most important etiologies are chronic viral hepatitis, alcohol abuse and metabolic dysfunction-associated fatty liver disease (MAFLD) [2], sharing a similar pattern of liver disease progression from chronic inflammation, fibrosis to terminal complications, such as decompensated liver cirrhosis and liver cancer [3–6]. Globally, every fourth cancer-associated death is associated with liver cancer, most frequently hepatocellular carcinoma (HCC), with a fast-rising incidence [7]. HCC typically arises in the background of cirrhosis; however, in HCV patients, about 10% of cases can develop in a noncirrhotic liver [8].

Despite tremendous advances in antiviral therapies, chronic viral hepatitis B and C are still the major etiology for chronic liver disease. Worldwide, an estimated 180 million people live with hepatitis B virus (HBV) and 75 million with hepatitis C virus (HCV), and for most, testing and treatment remain beyond reach [9]. Both viruses share similar as well as distinct mechanisms contributing to liver disease and cancer. In Europe, it is estimated that 10–15% of HCCs are caused by HBV infection, while 70% are caused by HCV infection, HCV being the major risk factor for HCC development [10]. Both viruses contribute to liver fibrosis and HCC risk by multiple factors involving a dysregulation of host signal transduction through viral proteins, miRNAs, virus-induced growth factor and

cytokine expression or antiviral responses that cumulate in a pro-fibrotic and pro-oncogenic environment in the liver [11–17]. Liver fibrosis is characterized by an excessive production of the extracellular matrix by hepatic stellate cells and myofibroblasts in response to the inflammation and oxidative stress induced by viral infection [18].

The most important measure to reduce HCC risk is to eliminate the underlying etiology. However, even though viral infection with HBV or HCV can be controlled or cured, the risk of developing HCC cannot be fully eradicated due to multiple reasons, especially in patients with already advanced liver disease. The mechanisms are not well understood, but evidence points towards epigenetic viral footprints that maintain dysregulated pro-oncogenic signal transduction. It is thus crucial to be able to identify patients with elevated HCC risk to stratify for a more frequent liver screening. Today, liver disease diagnosis and HCC risk assessment relies on a combination of imaging, blood markers and liver biopsies. While liver tissue allows a detailed transcriptomic HCC risk assessment linked to predictive transcriptomic signature [19,20], liver biopsies are associated with a significant risk for the patients and, therefore, are not applicable for a tighter screening [21]. An impressive number of approaches and "liquid biomarker" candidates for liver disease are underway, studying extracellular vesicles, circulating tumor cells and cell-free nucleic acids (reviewed in detail [22]) to improve prognostic power, minimize the risk for the patients and provide additional tools for the screening of patients at risk. With a focus on virus-induced signaling events, this review provides an overview of the candidate blood biomarkers of fibrotic liver disease and HCC risk associated with chronic viral hepatitis.

2. Viral Hepatitis B and C

Although HBV and HCV are hepatotropic, causing similar liver disease, they are very different viruses. HBV is an hepatotropic DNA virus of the Hepadnaviridae family, which specifically infects hepatocytes via the recently discovered functional receptor sodium taurocholate co-transporting polypeptide (NTCP) [23] and epidermal growth factor signaling [24]. Following endocytosis, the nucleocapsid is released into the cytoplasm, and the partially double-stranded viral relaxed circular DNA (rcDNA) is repaired and converted to covalently closed circular DNA (cccDNA) in the nucleus (for a more detailed review, see [25]). cccDNA is a replicative intermediate of the HBV life cycle, and it is crucial for HBV persistence within the hepatocytes. It serves as a template for the transcription of HBV RNA species and consequently for the translation of HBV proteins, i.e., three surface proteins (L-HBsAg, M-HBsAg and S-HBsAg), core (HBc), E antigen (HBeAg), X protein (HBx) and viral polymerase (Pol). Among them, HBx is believed to have key roles related to HBV replication and signaling pathways [26].

HCV is a single-stranded, positive-sense RNA virus of the Flaviviridae family that requires multiple host entry factors, including receptor tyrosine kinase signaling. After endocytosis, the RNA genome is translated into a viral polyprotein at the endoplasmic reticulum, leading to a massive reshaping of host membranes to a replication complex termed the membranous web. De novo virus assembles at lipid droplets, which are accumulated at the replication complex (for a more detailed review, see [27,28]). HCV does not integrate into the host genome nor possesses a latent viral phase. Thus, HCV requires a constant modulation of the host cell to evade the antiviral response and to maintain its viral cycle [29–31]. In contrast to HBV, which is considered to be a stealth virus that does not cause big changes in the host transcriptomics [32], HCV massively re-orchestrates signaling pathways. A multiomics analysis of HCV infection studying gene set enrichment analysis (GSEA) revealed that almost half of the ~2000 studied gene sets from the molecular signature database (MSigDB) were dysregulated by HCV infection involving pro-oncogenic pathways regulating proliferation (EGF/MAPK), inflammation and stress (STAT3, NF-κB), hypoxia and angiogenesis (VEGFR) and fibrosis (TGF-β) [16,17]. Such massive remodulation of the signaling landscape holds the potential to derive novel minimally invasive biomarkers.

3. Antiviral Therapies

The most important measure to treat liver disease and prevent HCC formation is the removal of the underlying etiology. The challenges to help patients with viral hepatitis are heterogenous: while an efficient preventive HBV vaccine is available, established chronic HBV infection can only be controlled but rarely eliminated due to a persistent chromosome-like viral DNA species and genome integrations [33]. A "functional cure" of HBV infection is defined by a sustained loss of hepatitis B surface antigen (HBsAg) in the blood, with or without seroconversion to anti-HBsAg. However, it is not always achieved in individuals with HBV. For this reason, the achievement of a sustained HBsAg seroclearance, even after suspension of the antiviral therapy, is nowadays considered the most realistic endpoint for the cure of individuals with chronic HBV. On the other hand, "virological cure", defined as the complete eradication of the virus, is too hard to reach, and it does not represent a reasonable therapeutic goal to date. This is due to the integration of HBV DNA into the host genome and the persistence of cccDNA within the hepatocytes [34]. Importantly, a constant activation state of fibrotic signaling pathways is believed to persist even in patients with undetectable HBV serum viral loads after therapy [35–37].

For HCV, no vaccine is in reach, but the novel generation of antiviral therapies with direct acting antivirals (DAAs) can efficiently cure HCV infection [38]. A new generation of interferon-free HCV drug regimens (sofosbuvir/velpatasvir and glecaprevir/pibrentasvir) is pan-genotypic and, therefore, can be used to treat individuals without identifying their HCV genotype and subtype [38]. Efficiency is very high (90–98% after 12 weeks; ASTRAL-3 trial) even in patients with difficult to treat genotype 3 and advanced liver disease [38]. However, the high price for HCV cure is still a barrier to guarantee treatment programs worldwide. Even when prices vary across countries, there is no adjustment of DAA prices with population income or viral infected population, making its accessibility more complicated for the poorest sectors of society [39]. HCV cure markedly decreases but cannot fully eliminate HCC risk, especially in patients with already advanced liver disease [40].

4. Fibrosis and HCC Screening in the Clinics—State of the Art

The majority of HCCs arise from liver cirrhosis, and thus the current HCC risk assessment is largely coupled to the staging of liver fibrosis. Despite the screening programs in cirrhotic patients, often the HCCs are diagnosed at an advanced stage. Less than 30–40% of them are eligible for a curative treatment using surgical approaches or radiological ablation [41]. Currently, the trans-abdominal ultrasound surveillance of patients at risk is the standard technique to detect HCC. However, the sensitivity of this method is not good enough to detect small tumors. For this reason, other methods, e.g., computed tomography (CT) or magnetic resonance imaging (MRI), are added, increasing cost, complexity and time for early detection and diagnosis. Magnetic resonance elastography (MRE) can be thought of as quantitative, noninvasive palpation. The use of this technique has become widespread in the diagnosis and staging of liver fibrosis [42]. However, early detection of HCC is complicated because of the co-existence with a chronic liver disease. The performance of noninvasive methods is represented by the area under the receiver operator characteristic (AUROC) curve, which provides information regarding the sensitivity and specificity of the method. Based on this, several tests have been proposed, such as the fibrosis-4 index or fibrotest, which may help clinicians in determining prognosis and risk for future complications [43]. Another noninvasive method that helps in discriminating cirrhotic patients from noncirrhotic patients is the measurement of liver stiffness using transient elastography (TE), as the fibrotic tissue is much stiffer than healthy tissue. In patients with advanced fibrosis, the liver function and patient prognosis is classified with the Child–Pugh score summarizing biological and clinical features, i.e., bilirubin, albumin, prothrombin time, ascites and hepatic encephalopathy. Child–Pugh comprises 3 classes of severity: A, good liver functionality (median 2-year survival: 85%); B, moderate liver

functionality (median 2-year survival: 57%); C, poor liver functionality (median 2-year survival: 35%) [44].

Several patient-derived transcriptomic signatures that associate with HCC risk and which are specific for certain etiologies or reflect a hepatic state of struggle in the liver independently from the underlying etiology have been identified in recent years (for a more detailed review, see [45]). Although a translation into minimally invasive biomarkers is explored, these signatures largely depend on liver tissue from resections or liver biopsies, which are still the gold standard to diagnose liver disease and assess HCC risk. However, liver biopsies are costly, exhausting for the patients and associated with a significant risk, reasons why this procedure is not applicable for a tighter screening [21].

Current guidelines recommend the screening of HCC in at-risk patients using ultrasonography (US) of the liver every 6 months with or without serum alpha-fetoprotein (AFP) [46,47], the most commonly used biomarker for liver disease detection. Additional conventional serum biomarkers are lectin-binding AFP-3 (AFP-L3) and des-carboxyprothrombin (DCP), which are still evaluated [48–52]. A Japanese prospective study demonstrated that a combination of DCP with AFP level is useful to detect HCC development and recurrence in chronic liver disease patients [53]. This was recently consolidated by a retrospective study, which showed that combining DCP and AFP serum levels in NUC-treated HBV Caucasian cirrhotic individuals, represents a potential surveillance strategy for HCC [49]. Additional candidate biomarkers for HCC in the blood have been suggested, i.e., proprotein convertase subtilisin/kexin type 9 (PCSK9) [54,55], glypican 3 (GPC3), squamous cell carcinoma antigen (SCCA), cytokeratine-19, osteopontin (OPN), Golgi protein-73 (GP73), alpha-L-fucosidase (AFU) [56], heat shock 70 kD protein (HSP-70) [57], annexin A2, midkine (MDK), aldo-keto reductase family 1 member B10 (AKR1B10) [58], and HCC-responsive miRNAs and cell-free DNA (for a more detailed review, see [59]). However, even if these candidate biomarkers are promising, to date, none of them have been adopted in the current clinical practice, and they need to be externally validated.

Additional scoring systems have been predictive for HCC risk in patients with chronic liver disease. The GALAD score [60,61] is derived from the combination of different parameters and single biomarkers, such as gender, age, AFP-L3, AFP and DCP, and has been validated in several patient cohorts [62]. The ALBI score evaluates the liver function of patients with HCC of different stages based on albumin and bilirubin levels in the blood [63]. However, the complex mathematical calculation of the ALBI score has limited its use, and new scores based on it have been developed, e.g., EZ-ALBI [64] or the modified ALBI (mALBI) which is used in clinical practice [65]. The enhanced liver fibrosis (ELF) score assesses a range of liver disease in conjunction with liver biopsy [66]. It provides a single score combining in an algorithm the measurement of three indirect biomarkers: HA, PIIINP and TIMP-1. The algorithm detects accurately liver fibrosis in patients with chronic HCV [67]; however, it is limited in low disease prevalence [68].

5. Signaling Pathways Associated with Candidate Serum Biomarkers

Signal transduction is an essential process involved in almost every step of cellular homeostasis. Signaling is tightly controlled, transmitting signals between cellular compartments and regulating gene transcriptional responses. Thus, the chronic dysregulation of signaling pathways is involved in the majority of diseases, including cancer [69]. Viruses including HBV and HCV make use of host signaling to maintain their life cycles or to evade the host antiviral response. The resulting persistent dysregulation of host signaling pathways by chronic viral infection promotes viral pathogenesis and malignant transformation [70]. Therefore, the study of virus-dysregulated signaling pathways may contribute to identify efficient minimally invasive biomarkers for liver disease (Figure 1).
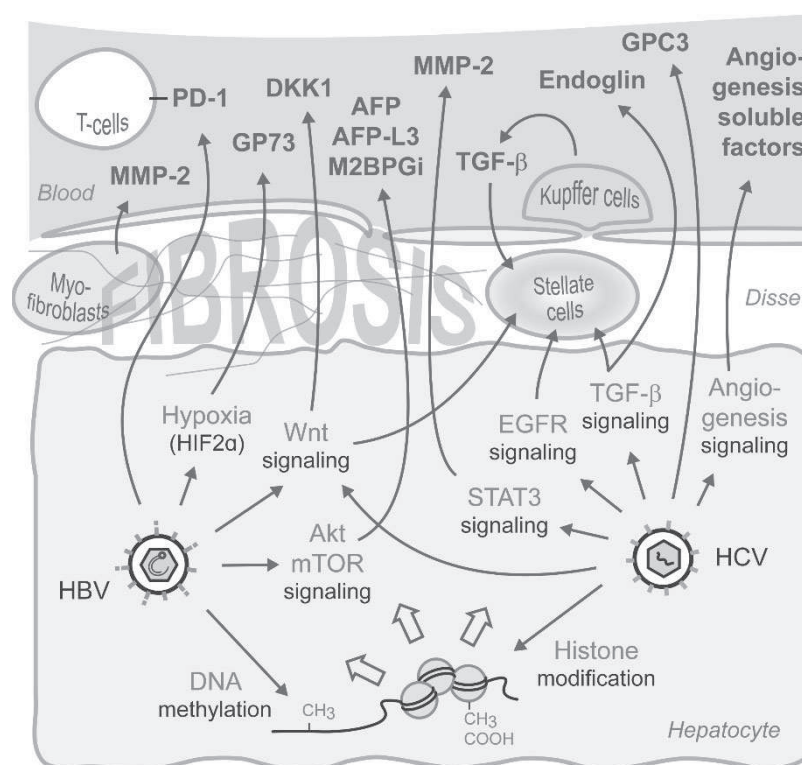
Figure 1. Dysregulation of signaling pathways by chronic viral hepatitis and epigenetic imprinting impact the secretion of circulating candidate biomarkers to the blood. Abbreviations: AFP, alpha-fetoprotein; AFP-L3, Lectin-Binding AFP-3; Akt, AKT Serine/Threonine Kinase; DKK1, Dickkopf WNT Signaling Pathway Inhibitor 1; Disse, Space of Disse; EGFR, Epidermal Growth Factor Receptor; GPC3, Glypican 3; GP73, Golgi Membrane Protein 1; HIF2α, Hypoxia-Inducible Factor 2 Alpha; M2BPGi, mac-2-binding protein glycan isomer; MMP-2, Matrix Metallopeptidase 2; mTOR, Mechanistic Target Of Rapamycin Kinase; PD-1, Programmed Cell Death 1; STAT3, Signal Transducer and Activator of Transcription 3; TGF-β, Transforming Growth Factor Beta; Wnt, Wnt Family Member.

Despite differences in terms of structure and life cycle, HBV and HCV are believed to share common pathways which influence hepatic fibrosis and promote hepatocarcinogenesis [71,72]. It became evident that HBV infection does not trigger the innate immune response and thus behaves as a stealth virus in the liver [73,74]. Nevertheless, chronic HBV infection impacts host signaling with potential relevance to markers of liver disease progression. The HCC biomarker AFP is also a regulator of growth signaling via PI3K/AKT signaling in hepatoma cells [75]. The viral protein HBx induces the expression of AFP, potentially driving the malignant transformation of hepatocytes in vivo via activation of the PI3K/AKT/mTOR pathway [76,77]. Moreover, members of the mTOR signaling pathway and eukaryotic translation initiation factors (eIFs) have recently been identified as potential biomarkers for HCC, and their expression patterns depend on different HCC aetiologias, such as HBV, HCV and non-virus-related HCC [78]. Another potential HBV-responsive risk marker is the protein Dickkopf WNT Signaling Pathway Inhibitor 1 (DKK1) involved in embryonic development as an inhibitor of Wnt signaling. DKK1 is a secreted protein whose mechanism of action is centered in binding and isolating the low-density lipoprotein receptor-related protein 6 (LRP6) co-receptor avoiding its role in activating the Wnt signaling pathway. DKK1 promotes HCC development by the modulation of the Wnt/β-catenin signaling pathway [79] and interaction with TGF-β signaling [80,81]. High serum levels of DKK1 may distinguish HCC associated with chronic HBV infection from HCC associated with nonviral liver cirrhosis. Moreover, DKK1 may allow early-stage HCC detection even in patients with AFP negative status [82]. DKK1 is also gaining interest as a potential biomarker for HCV-associated HCC. Although HCV core protein promotes the activation

of the Wnt signaling protein and the suppression of Wnt pathway inhibitors [83,84], DKK1 abundance seems to be significantly decreased in the blood of patients with HCV [85]. However, DKK1 is spiking in patients with HCV who also have HCC [86].

GP73 has been suggested as a serum biomarker for liver cirrhosis in individuals with chronic HBV [87]. Moreover, GP73 seems to be a good predictor of liver inflammation and fibrosis in HBV patients with normal or slightly raised alanine aminotransferase (ALT) [88]. The biological function of GP73 is not completely understood but it is assumed to be involved in protein secretion and signaling. Moreover, its expression is linked to different pathological conditions [89]. HBV modulates various signaling pathways converging in GP73 modulation [90,91]. For example, it promotes GP73 expression by the activation of hypoxia-inducible factor-2α (HIF-2α) signaling [91], which is a hallmark of chronic infection and HCC development. Moreover, GP73 influences the immune response to HBV infection, as an increased production of GP73 can be observed in HBV-stimulated leukocytes [90], in peripheral blood mononuclear cells isolated from healthy donors and in macrophages derived from human acute monocytic leukemia cells (THP-1). In the same study using hepatoma cell lines, the authors demonstrated that GP73 represses the expression of the p50 subunit of NF-κB, promoting HBV replication and thus highlighting the role of GP73 as a potential antiviral modulator [90]. Immune dysregulation and T-cell exhaustion are among the major hallmarks of chronic HBV infection and the associated pathological development [92]. On this basis, the presence of HBV-specific T cells has been recently proposed as an immunological biomarker for safely monitoring therapy in chronic HBV patients [93] and programmed cell death protein (PD-1) expression as a potential marker for liver fibrosis in patients with chronic HBV [93].

Recently, mac-2-binding protein glycan isomer (M2BPGi) has been shown to enhance the aggressiveness of HCC via the activation of the mTOR signaling pathway [94], harboring potential as a minimally invasive biomarker. Glycoproteomic analysis has revealed that mac-2-binding protein (M2BP), an extracellular matrix protein that interacts with collagens, fibronectin and integrin [95], may undergo specific changes in its glycan structure correlating with fibrosis development [95,96]. M2BPGi has been suggested as a valid predictor of fibrosis and HCC in HBV patients [97–100]. Moreover, according to a prospective study conducted in China, M2BPGi serum level decreases in chronic HBV patients treated with nucleos(t)ide analogues (NAs), indicating its potential role in predicting HCC development in NA-treated populations [101]. In addition, M2BP-modified molecules have been studied as biomarkers of fibrosis in patients with chronic HCV infection [102]. Its cut-off values differ between etiologies, and M2BP levels decrease after viral cure [103]. Interestingly, M2BPGi has been introduced as a novel and noninvasive biomarker for the assessment of liver fibrosis in chronic HCV patients treated with DAAs [104].

Signaling pathways play a pivotal role during viral hepatitis and liver fibrosis. Epidermal growth factor receptor (EGFR) signaling is required by HBV and HCV for entry [24,105], where it orchestrates entry factor complex formation and endocytosis [24,106]. Importantly, HCV itself promotes EGFR signaling [12,16,107] to maintain its life cycle and to attenuate the host antiviral response [106] with important consequences for liver disease progression. Indeed, EGFR signaling has been identified as a major driver of liver fibrosis and HCC in animal models and patients [13,19]. Additionally, TGF-β signaling is induced by HCV infection in hepatocytes [16] and in activated Kupffer cells, which are resident liver macrophages activated during liver injury [108]. Like EGF and Wnt signaling, TGF-β is a major regulator of cell proliferation, differentiation and apoptosis. It is essential for the induction of epithelial–mesenchymal transition (EMT) and the activation of stellate cells [108]. TGF-β is a cytokine suppressing tumor activity at early stages by arresting cell growth and inducing apoptosis. However, at later tumor stages, it promotes the proliferation and survival of malignant cells (for a more detailed review, see [109]). HCV infection induces TGF-β signaling indirectly via NF-kB and unfolded protein response (UPR) [110] and directly via the interaction of HCV core protein with SMAD3 [111]. HCV core protein

increases intrahepatic and circulating levels of endoglin, which is a TGF-β1 co-receptor associated with progressive hepatic fibrosis during chronic HCV infection [112].

Persistent oxidative stress is an important factor in virus-induced liver fibrosis. Especially HCV infection, and its massive reorganization of cellular membranes to the replication complex, is a major cause of UPR and oxidative stress [113]. HCV protein core, NS3 and NS5, block heme oxygenase-1 (HO-1) in hepatocytes accumulating oxygen radicals in the cell [114]. This activates NF-kB and STAT3 [113], which are key players in inflammation and cancer [115]. During HCV infection, STAT3 activity is further intensified by a suppression of negative regulators, i.e., the STAT3 phosphatase PTPRD via miR135a-5p [17] and SOCS3 by enhancing EGFR signaling [106]. Consequently, STAT3 signaling impairs peroxisomal function, leading to an accumulation of very-long-chain fatty acids and peroxides in the HCV-infected hepatocyte [16]. Moreover, HCV-induced STAT3 signaling also triggers the upregulation and secretion of the metalloprotease MMP-2 [116], which is involved in remodeling the extracellular matrix and has been previously suggested as a prognostic marker for liver fibrosis [117].

HCV infection induces hypoxia in infected cells and stabilizes HIFs [118], which is also a hallmark of HCC development linked to a stimulation of angiogenesis. In patients with HCV-associated cirrhosis and HCC, several angiogenesis soluble factors were significantly upregulated in the blood plasma, including TIMP-1, TIMP-2, HGF, angiopoietin 1, angiopoietin 2, VEGFA, IP-10, PDGF, KGF and FGF. AUROC analysis highlighted especially the potential of angiopoietin 2, a growth factor that belongs to the angiopoietin/Tie signaling pathway [119–121]. Additionally, CCL20, a secreted chemokine detected in HCCs, promotes blood vessel formation during chronic HCV infection [122]. CCL20 and VEGF correlate in patients with cirrhosis and HCV-induced HCC, highlighting their potential as biomarkers for HCV-induced HCC [123–125]. Moreover, the serum level of TIMP-1 has been found to be significantly correlated with fibrosis development in chronic HBV patients [126]. Interestingly, a previous study demonstrated that TIMP-1 and hyaluronic acid (HA) are good predictors of advanced liver fibrosis in chronic hepatitis B and D patients [127] and bases for the earlier mentioned ELF score.

Glypican-3 (GPC3) is an heparan sulfate proteoglycan that regulates cell morphology via the Hippo/YAP pathway. In a normal liver, the HCV entry factor CD81 interacts with GPC3 and inhibits the Hippo/YAP pathway. HCV E2 protein mimics the role of CD81 stimulating Hippo/YAP by engaging GPC3. In a chronically inflamed liver, HCV is thus likely to promote hepatic neoplasia by the growth of early CD81-negative neoplastic hepatocytes, which are resistant to HCV infection [128]. Given its upregulation in the blood of patients with HCV-associated HCC, GPC3 had been suggested as a biomarker [129].

6. Virus-Induced Epigenetic Changes as Biomarkers

Epigenetic imprinting acts as a memory for environmental influences and disease [130]. This has long-term consequences to the cellular homeostasis and pathogenesis relevant not only for therapeutic strategies but which may also be instrumental to identify specific biomarkers. Epigenetic modifications comprise DNA methylation and posttranslational modification of histones, which directly regulates the accessibility of genes to the transcriptional machinery but also posttranscriptional regulation via noncoding RNAs [131]. The discovery of new epigenetic modulators is paving the way to the identification of new epigenetic biomarkers for the development of diagnostic and prognostic tools for hepatic fibrosis. It has been demonstrated that aberrant epigenetic signatures associated with fibrosis and HCC are released into the blood stream, providing blood-based biomarkers that could be used for advancing the diagnosis and prognosis of liver-associated diseases [132,133]. During chronic viral hepatitis, specific DNA methylation patterns in the genes of peripheral blood mononuclear cells (PBMCs) suggest a role in the progression of liver disease to HCC [134]. Methylation of cytosine-phospho-guanine (CpG) dinucleotides island in regulatory gene elements correlates with the silencing of the gene expression. Thus, a methylome analysis can identify dysregulated disease-relevant signaling pathways. This

strategy highlighted a role of dysregulated IL-15, IL-8, as well as nitric oxide signaling in PBMCs from HBV patients and cirrhotic livers causing reprogramming of the immune and inflammatory responses [135]. HBV causes a virus-specific DNA methylation pattern in the hepatocyte DNA [136], which, however, in a study from 2015 on primary human hepatocytes had only limited overlap with the transcriptional pattern [137]. Circulating methylated DNA fragments are explored as a noninvasive diagnostic tool for early-stage liver cancer prevention [138–140]. Interestingly, this also includes methylated fragments of the SOCS3 gene [139], which is a negative regulator of the IL-6/STAT3 signaling pathway.

Epigenetic regulation of gene expression by HCV has been observed at the histone level. Such epigenetic footprints have been identified in cell lines and patients with chronic HCV infection and NASH [141–143], suggesting a lasting dysregulation of signaling pathways even after the underlying cause has eased. Indeed, comparative ChIP-seq and RNA-seq analysis of DAA-cured HCV patients identified epigenetic histone modifications. These were associated with the dysregulated pro-oncogenic transcriptional pattern, suggesting a persistently dysregulated signal transduction after viral cure [141]. Comparative analysis with HCV-infected and DAA-cured human liver chimeric mice highlighted an HCV-specific viral footprint, since these mice do not develop liver fibrosis, which also involves the STAT3 phosphatase PTPRD [141]. Moreover, a liver fibrosis-specific footprint of cancer-risk genes has been identified in HCV- and NASH-associated fibrotic patients' livers [142]. However, a translation of these footprint signatures into blood born biomarkers is pending.

HCV infection and liver disease largely impact miRNA expression [11], which influences signaling pathway activity and liver disease progression [17]. Circulating miRNAs harbor the potential of being developed into minimally invasive biomarkers [144,145]. miRNAs are enriched and well protected in extracellular vesicles (EVs) in the blood. Exosomal miRNAs have been evaluated in clinics, such as miR-122 and miR-21, for the early detection and prediction of HCC [146], and let-7s for the detection of liver fibrosis in patients with chronic hepatitis C infection [145,147].

7. Discussion and Perspectives

Although HBV and HCV cause both liver disease progression and HCC, the clinical challenges differ substantially. HBV infection can only be controlled but not eradicated because of a persisting chromosome-like cccDNA and genome integration [36]. HCV infection is now curable; however, chronic infection is leaving an epigenetic footprint that manifests the dysregulation of the pro-oncogenic signal beyond viral eradication. In both cases, patients remain at considerable risk to develop HCC over the years, which highlights the importance of reliable and minimally invasive biomarkers to stratify these risk patients for tighter HCC screening. A combination of circulating signaling components, secondary markers (e.g., gut microbiome [148,149]), with noninvasive imaging biomarkers will hold the biggest potential. However, it is important to remark that although some of these signaling-linked candidate biomarkers show promising results, almost all of them are still in development, and only AFP has reached phase V (Table 1). Moreover, it is generally difficult to draw a line between biomarkers derived from the pro-fibrotic signaling events and carcinogenesis markers during end stage liver disease. While the dysregulated signaling and epigenetics in diseased livers partially overlap between etiologies, e.g., HCV and MAFLD [13,16,141,142,150], hepatocarcinogenesis is a highly heterogenous event even within a specific etiology [151,152]. This, we need novel concepts, which differentiate those markers that "simply" predict increased fibrogenesis from those that are definitively associated with risk of carcinogenesis.

Table 1. Promising minimally invasive biomarker candidates with links to virus-induced signaling (HBV, HCV) and predictive of liver fibrosis and HCC. Biomarker research is categorized into phases I–V [153]: phase V (evaluates the effect of the biomarker screening in the burden of the disease in the population), phase IV (prospective evaluation of the biomarker to assess its clinical performance), phase III (testing in patients before their diagnosis to determine the performance of the biomarker in detecting pre-clinical disease), phase II (testing in patients at high risk to determine the performance of the biomarker in distinguishing between patients with and without the disease), phase I (discovery of new biomarkers by investigating gene expression and protein levels in pathological tissue and patient samples).

Biomarker	Specificity	Viral Etiology	Development Status	Reference
AFP	HCC	HBV, HCV	Phase V	[41,46,47,51,52,77,101]
DCP	HCC	HBV, HCV	Phase IV	[48–53]
M2BPGi	HCC	HBV, HCV	Phase IV	[95–104]
MDK	HCC	HCV	Phase III	[81,154]
OPN	HCC, fibrosis	HBV, HCV	Phase III	[155–157]
Annexin A2	HCC, fibrosis	HBV, HCV	Phase II	[158,159]
DKK1	HCC	HBV, HCV	Phase II	[81,82,85,86]
GPC3	HCC	HCV	Phase II	[128,129]
HSP-70	HCC	HBV, HCV	Phase II	[57]
PCSK9	HCC	HCV	Phase II	[54,55]
SCCA	HCC, fibrosis	HCV	Phase II	[160,161]
TIMP-1	Fibrosis	HBV, HCV	Phase II	[119,126]
Angiopoietin-2	HCC, fibrosis	HCV	Phase I	[119–121]
CCL20	HCC	HCV	Phase I	[122,123]
Endoglin	Fibrosis	HCV	Phase I	[112]
VEGF	HCC, fibrosis	HCV	Phase I	[119,123–125]

While HCV infection was rendered a curable disease due to efficient direct antiviral therapies, an important unmet medical need is to identify the fraction of patients with elevated HCC risk. In future, etiology-specific epigenetic markers, including histone modifications and miRNAs, will provide new perspectives for translation into correlating secreted biomarkers, which may be used for personalized approaches targeting specific groups of patients, e.g., biomarkers for HCC risk in HCV-cured patients.

References

1. Asrani, S.K.; Devarbhavi, H.; Eaton, J.; Kamath, P.S. Burden of Liver Diseases in the World. *J. Hepatol.* **2019**, *70*, 151–171. [CrossRef] [PubMed]
2. Tilg, H.; Effenberger, M. From NAFLD to MAFLD: When Pathophysiology Succeeds. *Nat. Rev. Gastroenterol. Hepatol.* **2020**, *17*, 387–388. [CrossRef]

3.   Baumert, T.F.; Berg, T.; Lim, J.K.; Nelson, D.R. Status of Direct-Acting Antiviral Therapy for Hepatitis C Virus Infection and Remaining Challenges. *Gastroenterology* **2019**, *156*, 431–445. [CrossRef] [PubMed]

4.   Chung, R.T.; Baumert, T.F. Curing Chronic Hepatitis C—The Arc of a Medical Triumph. *N. Engl. J. Med.* **2014**, *370*, 1576–1578. [CrossRef] [PubMed]

5.   Friedman, S.L.; Neuschwander-Tetri, B.A.; Rinella, M.; Sanyal, A.J. Mechanisms of NAFLD Development and Therapeutic Strategies. *Nat. Med.* **2018**, *24*, 908–922. [CrossRef]

6.   Villanueva, A. Hepatocellular Carcinoma. *N. Engl. J. Med.* **2019**, *380*, 1450–1462. [CrossRef]

7.   Tang, A.; Hallouch, O.; Chernyak, V.; Kamaya, A.; Sirlin, C.B. Epidemiology of Hepatocellular Carcinoma: Target Population for Surveillance and Diagnosis. *Abdom. Radiol.* **2018**, *43*, 13–25. [CrossRef]

8.   Desai, A.; Sandhu, S.; Lai, J.-P.; Sandhu, D.S. Hepatocellular Carcinoma in Non-cirrhotic Liver: A Comprehensive Review. *World J. Hepatol.* **2019**, *11*, 1–18. [CrossRef] [PubMed]

9.   WHO. *Global Hepatitis Report*; WHO: Geneva, Switzerland, 2017.

10.   Bray, F.; Ferlay, J.; Soerjomataram, I.; Siegel, R.L.; Torre, L.A.; Jemal, A. Global Cancer Statistics 2018: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. *CA Cancer J. Clin.* **2018**, *68*, 394–424. [CrossRef]

11.   Bandiera, S.; Pernot, S.; El Saghire, H.; Durand, S.C.; Thumann, C.; Crouchet, E.; Ye, T.; Fofana, I.; Oudot, M.A.; Barths, J.; et al. Hepatitis C Virus-Induced Upregulation of MicroRNA miR-146a-5p in Hepatocytes Promotes Viral Infection and Deregulates Metabolic Pathways Associated with Liver Disease Pathogenesis. *J. Virol.* **2016**, *90*, 6387–6400. [CrossRef]

12.   Diao, J.; Pantua, H.; Ngu, H.; Komuves, L.; Diehl, L.; Schaefer, G.; Kapadia, S.B. Hepatitis C Virus Induces Epidermal Growth Factor Receptor Activation via CD81 Binding for Viral Internalization and Entry. *J. Virol.* **2012**, *86*, 10935–10949. [CrossRef]

13.   Fuchs, B.C.; Hoshida, Y.; Fujii, T.; Wei, L.; Yamada, S.; Lauwers, G.Y.; McGinn, C.M.; Deperalta, D.K.; Chen, X.; Kuroda, T.; et al. Epidermal Growth Factor Receptor Inhibition Attenuates Liver Fibrosis and Development of Hepatocellular Carcinoma. *Hepatology* **2014**, *59*, 1577–1590. [CrossRef]

14.   Igloi, Z.; Kazlauskas, A.; Saksela, K.; Macdonald, A.; Mankouri, J.; Harris, M. Hepatitis C virus NS5A Protein Blocks Epidermal Growth Factor Receptor Degradation via a Proline Motif-Dependent Interaction. *J. Gen. Virol.* **2015**, *96*, 2133–2144. [CrossRef]

15.   Lou, W.; Liu, J.; Ding, B.; Chen, D.; Xu, L.; Ding, J.; Jiang, D.; Zhou, L.; Zheng, S.; Fan, W. Identification of Potential miRNA–mRNA Regulatory Network Contributing to Pathogenesis of HBV-Related HCC. *J. Transl. Med.* **2019**, *17*, 1–14. [CrossRef]

16.   Lupberger, J.; Croonenborghs, T.; Suarez, A.A.R.; Van Renne, N.; Jühling, F.; Oudot, M.A.; Virzì, A.; Bandiera, S.; Jamey, C.; Meszaros, G.; et al. Combined Analysis of Metabolomes, Proteomes, and Transcriptomes of Hepatitis C Virus–Infected Cells and Liver to Identify Pathways Associated with Disease Development. *Gastroenterology* **2019**, *157*, 537–551.e9. [CrossRef]

17.   Van Renne, N.; Suarez, A.A.R.; Duong, F.H.T.; Gondeau, C.; Calabrese, D.; Fontaine, N.; Ababsa, A.; Bandiera, S.; Croonenborghs, T.; Pochet, N.; et al. miR-135a-5p-Mediated Downregulation of Protein Tyrosine Phosphatase Receptor Delta Is a Candidate Driver of HCV-Associated Hepatocarcinogenesis. *Gut* **2017**, *67*, 953–962. [CrossRef]

18.   Yavuz, B.G.; Pestana, R.C.; AbuGabal, Y.I.; Krishnan, S.; Chen, J.; Hassan, M.M.; Wolff, R.A.; Rashid, A.; Amin, H.M.; Kaseb, A.O. Origin and Role of Hepatic Myofibroblasts in Hepatocellular Carcinoma. *Oncotarget* **2020**, *11*, 1186–1201. [CrossRef] [PubMed]

19.   Hoshida, Y.; Villanueva, A.; Kobayashi, M.; Peix, J.; Chiang, D.Y.; Camargo, A.; Gupta, S.; Moore, J.; Wrobel, M.J.; Lerner, J.; et al. Gene Expression in Fixed Tissues and Outcome in Hepatocellular Carcinoma. *N. Engl. J. Med.* **2008**, *359*, 1995–2004. [CrossRef]

20.   Hoshida, Y.; Villanueva, A.; SanGiovanni, A.; Sole, M.; Hur, C.; Andersson, K.L.; Chung, R.T.; Gould, J.; Kojima, K.; Gupta, S.; et al. Prognostic Gene Expression Signature for Patients with Hepatitis C–Related Early-Stage Cirrhosis. *Gastroenterology* **2013**, *144*, 1024–1030. [CrossRef] [PubMed]

21.   Castera, L.; Friedrich-Rust, M.; Loomba, R. Noninvasive Assessment of Liver Disease in Patients with Nonalcoholic Fatty Liver Disease. *Gastroenterology* **2019**, *156*, 1264–1281.e4. [CrossRef] [PubMed]

22.   Mann, J.; Reeves, H.L.; Feldstein, E.A. Liquid Biopsy for Liver Diseases. *Gut* **2018**, *67*, 2204–2212. [CrossRef] [PubMed]

23.   Yan, H.; Zhong, G.; Xu, G.; He, W.; Jing, Z.; Gao, Z.; Huang, Y.; Qi, Y.; Peng, B.; Wang, H.; et al. Sodium Taurocholate Cotransporting Polypeptide Is a Functional Receptor for Human Hepatitis B and D Virus. *eLife* **2012**, *1*, e00049. [CrossRef] [PubMed]

24.   Iwamoto, M.; Saso, W.; Sugiyama, R.; Ishii, K.; Ohki, M.; Nagamori, S.; Suzuki, R.; Aizaki, H.; Ryo, A.; Yun, J.-H.; et al. Epidermal Growth Factor Receptor Is a Host-Entry Cofactor Triggering Hepatitis B Virus Internalization. *Proc. Natl. Acad. Sci. USA* **2019**, *116*, 8487–8492. [CrossRef] [PubMed]

25.   Tsukuda, S.; Watashi, K. Hepatitis B Virus Biology and Life Cycle. *Antivir. Res.* **2020**, *182*, 104925. [CrossRef] [PubMed]

26.   Lucifora, J.; Arzberger, S.; Durantel, D.; Belloni, L.; Strubin, M.; Levrero, M.; Zoulim, F.; Hantz, O.; Protzer, U. Hepatitis B Virus X Protein Is Essential to Initiate and Maintain Virus Replication after Infection. *J. Hepatol.* **2011**, *55*, 996–1003. [CrossRef]

27.   Colpitts, C.C.; Tsai, P.-L.; Zeisel, M.B. Hepatitis C Virus Entry: An Intriguingly Complex and Highly Regulated Process. *Int. J. Mol. Sci.* **2020**, *21*, 2091. [CrossRef] [PubMed]

28.   Tabata, K.; Neufeldt, C.J.; Bartenschlager, R. Hepatitis C Virus Replication. *Cold Spring Harb. Perspect. Med.* **2019**, *10*, a037093. [CrossRef] [PubMed]

29.   Cheng, Y.; Sun, F.; Wang, L.; Gao, M.; Xie, Y.; Sun, Y.; Liu, H.; Yuan, Y.; Yi, W.; Huang, Z.; et al. Virus-Induced p38 MAPK Activation Facilitates Viral Infection. *Theranostics* **2020**, *10*, 12223–12240. [CrossRef]

30.   Negash, A.A.; Olson, R.M.; Griffin, S.; Gale, M. Modulation of Calcium Signaling Pathway by Hepatitis C Virus Core Protein Stimulates NLRP3 Inflammasome Activation. *PLoS Pathog.* **2019**, *15*, e1007593. [CrossRef] [PubMed]

31. Virzì, A.; Suarez, A.A.R.; Baumert, T.F.; Lupberger, J. Oncogenic Signaling Induced by HCV Infection. *Viruses* **2018**, *10*, 538. [CrossRef] [PubMed]

32. König, A.; Yang, J.; Jo, E.; Park, K.H.P.; Kim, H.; Than, T.T.; Song, X.; Qi, X.; Dai, X.; Park, S.; et al. Efficient Long-Term Amplification of Hepatitis B Virus Isolates after Infection of Slow Proliferating HepG2-NTCP Cells. *J. Hepatol.* **2019**, *71*, 289–300. [CrossRef]

33. Fanning, G.C.; Zoulim, F.; Hou, J.; Bertoletti, A. Therapeutic Strategies for Hepatitis B Virus Infection: Towards a Cure. *Nat. Rev. Drug Discov.* **2019**, *18*, 827–844. [CrossRef] [PubMed]

34. Lok, A.S.; Zoulim, F.; Dusheiko, G.; Ghany, M.G. Hepatitis B Cure: From Discovery to Regulatory Approval. *Hepatology* **2017**, *66*, 1296–1313. [CrossRef]

35. Mu, D.; Yuan, F.-C.; Chen, Y.; Jiang, X.-Y.; Yan, L.; Jiang, L.-Y.; Gong, J.-P.; Zhang, D.-Z.; Ren, H.; Liao, Y. Baseline Value of Intrahepatic HBV DNA Over cccDNA Predicts Patient's Response to Interferon Therapy. *Sci. Rep.* **2017**, *7*, 5937. [CrossRef] [PubMed]

36. Nassal, M. HBV cccDNA: Viral Persistence Reservoir and Key Obstacle for a Cure of Chronic Hepatitis B. *Gut* **2015**, *64*, 1972–1984. [CrossRef] [PubMed]

37. Zhang, X.; Lu, W.; Zheng, Y.; Wang, W.; Bai, L.; Chen, L.; Feng, Y.; Zhang, Z.; Yuan, Z. In Situ Analysis of Intrahepatic Virological Events in Chronic Hepatitis B Virus Infection. *J. Clin. Investig.* **2016**, *126*, 1079–1092. [CrossRef] [PubMed]

38. European Association for the Study of the Liver. Electronic Address Eee, Clinical Practice Guideline Panel C, Panel m, Representative EGB. EASL Clinical Practice Guidelines: Drug-Induced Liver Injury. *J. Hepatol.* **2020**, *73*, 1170–1218. [CrossRef]

39. Lu, C.Y.; Ross-Degnan, D.; Zhang, F.; LeCates, R.; Lupton, C.; Sherman, M.; Wagner, A. Cost Burden of Hepatitis C Virus Treatment in Commercially Insured Patients. *Am J Manag Care* **2019**, *25*, e379–e387.

40. Baumert, T.F.; Jühling, F.; Ono, A.; Hoshida, Y. Hepatitis C-Related Hepatocellular Carcinoma in the Era of New Generation Antivirals. *BMC Med.* **2017**, *15*, 1–10. [CrossRef] [PubMed]

41. Chan, S.L.; Mo, F.; Johnson, P.J.; Siu, D.Y.W.; Chan, M.H.M.; Lau, W.Y.; Lai, P.B.S.; Lam, C.W.K.; Yeo, W.; Yu, S.C.H. Performance of Serum α-Fetoprotein Levels in the Diagnosis of Hepatocellular Carcinoma in Patients with a Hepatic Mass. *HPB* **2014**, *16*, 366–372. [CrossRef]

42. Manduca, A.; Bayly, P.J.; Ehman, R.L.; Kolipaka, A.; Royston, T.J.; Sack, I.; Sinkus, R.; Van Beers, B.E. MR Elastography: Principles, Guidelines, and Terminology. *Magn. Reson. Med.* **2021**, *85*, 2377–2390. [CrossRef]

43. European Association for the Study of the Liver; Asociación Latinoamericana para el Estudio del Hígado. EASL-ALEH Clinical Practice Guidelines: Non-Invasive Tests for Evaluation of Liver Disease Severity and Prognosis. *J. Hepatol.* **2015**, *63*, 237–264. [CrossRef]

44. Tsoris, A.; Marlar, C.A. *Use of the Child Pugh Score in Liver Disease*; StatPearls: Treasure Island, FL, USA, 2020.

45. Kubota, N.; Fujiwara, N.; Hoshida, Y. Clinical and Molecular Prediction of Hepatocellular Carcinoma Risk. *J. Clin. Med.* **2020**, *9*, 3843. [CrossRef]

46. European Association for the Study of the Liver. Electronic Address Eee, European Association for the Study of the L. EASL Clinical Practice Guidelines: Management of Hepatocellular Carcinoma. *J. Hepatol.* **2018**, *69*, 182–236. [CrossRef]

47. Heimbach, J.K.; Kulik, L.M.; Finn, R.S.; Sirlin, C.B.; Abecassis, M.M.; Roberts, L.R.; Zhu, A.X.; Murad, M.H.; Marrero, J.A. AASLD Guidelines for the Treatment of Hepatocellular Carcinoma. *Hepatology* **2018**, *67*, 358–380. [CrossRef] [PubMed]

48. Ertle, J.M.; Heider, D.; Wichert, M.; Keller, B.; Kueper, R.; Hilgard, P.; Gerken, G.; Schlaak, J.F. A Combination of A-Fetoprotein and Des-γ-Carboxy Prothrombin Is Superior in Detection of Hepatocellular Carcinoma. *Digestion* **2013**, *87*, 121–131. [CrossRef]

49. Loglio, A.; Iavarone, M.; Facchetti, F.; Di Paolo, D.; Perbellini, R.; Lunghi, G.; Ceriotti, F.; Galli, C.; Sandri, M.T.; Viganò, M.; et al. The Combination of PIVKA-II and AFP Improves the Detection Accuracy for HCC in HBV Caucasian Cirrhotics on Long-Term Oral Therapy. *Liver Int.* **2020**, *40*, 1987–1996. [CrossRef] [PubMed]

50. Ji, J.; Wang, H.; Li, Y.; Zheng, L.; Yin, Y.; Zou, Z.; Zhou, F.; Zhou, W.; Shen, F.; Gao, C. Diagnostic Evaluation of Des-Gamma-Carboxy Prothrombin versus α-Fetoprotein for Hepatitis B Virus-Related Hepatocellular Carcinoma in China: A Large-Scale, Multicentre Study. *PLoS ONE* **2016**, *11*, e0153227. [CrossRef]

51. Song, T.; Wang, L.; Su, B.; Zeng, W.; Jiang, T.; Zhang, T.; Sun, G.; Wu, H. Diagnostic Value of Alpha-Fetoprotein, Lens Culinaris Agglutinin-Reactive Alpha-Fetoprotein, and Des-Gamma-Carboxyprothrombin in Hepatitis B Virus-Related Hepatocellular Carcinoma. *J. Int. Med. Res.* **2020**, *48*, 0300060519889270. [CrossRef]

52. Wang, X.; Zhang, Y.; Yang, N.; He, H.; Tao, X.; Kou, C.; Jiang, J. Evaluation of the Combined Application of AFP, AFP-L3%, and DCP for Hepatocellular Carcinoma Diagnosis: A Meta-Analysis. *BioMed Res. Int.* **2020**, *2020*, 1–10. [CrossRef]

53. Ikoma, J.; Kaito, M.; Ishihara, T.; Nakagawa, N.; Kamei, A.; Fujita, N.; Iwasa, M.; Tamaki, S.; Watanabe, S.; Adachi, Y. Early Diagnosis of Hepatocellular Carcinoma Using a Sensitive Assay for Serum Des-Gamma-Carboxy Prothrombin: A Prospective Study. *Hepatogastroenterology* **2002**, *49*, 235–238.

54. Fasolato, S.; Pigozzo, S.; Pontisso, P.; Angeli, P.; Ruscica, M.; Savarino, E.; De Martin, S.; Lupo, M.G.; Ferri, N. PCSK9 Levels Are Raised in Chronic HCV Patients with Hepatocellular Carcinoma. *J. Clin. Med.* **2020**, *9*, 3134. [CrossRef]

55. Wiciński, M.; Żak, J.; Malinowski, B.; Popek, G.; Grześk, G. PCSK9 Signaling Pathways and Their Potential Importance in Clinical Practice. *EPMA J.* **2017**, *8*, 391–402. [CrossRef] [PubMed]

56. Zhang, S.-Y.; Lin, B.-D.; Li, B.-R. Evaluation of the Diagnostic Value of Alpha-L-Fucosidase, Alpha-Fetoprotein and Thymidine Kinase 1 with Roc and Logistic Regression for Hepatocellular Carcinoma. *FEBS Open Bio* **2015**, *5*, 240–244. [CrossRef]

57. Moudi, B.; Heidari, Z.; Mahmoudzadeh-Sagheb, H.; Alavian, S.-M.; Lankarani, K.B.; Farrokh, P.; Nyengaard, J.R. Concomitant Use of Heat-Shock Protein 70, Glutamine Synthetase and Glypican-3 Is Useful in Diagnosis of HBV-Related Hepatocellular Carcinoma with Higher Specificity and Sensitivity. *Eur. J. Histochem.* **2018**, *62*, 2859. [CrossRef] [PubMed]

58. Murata, A.; Genda, T.; Ichida, T.; Amano, N.; Sato, S.; Tsuzura, H.; Sato, S.; Narita, Y.; Kanemitsu, Y.; Shimada, Y.; et al. Pretreatment AKR1B10 Expression Predicts the Risk of Hepatocellular Carcinoma Development after Hepatitis C Virus Eradication. *World J. Gastroenterol.* **2016**, *22*, 7569–7578. [CrossRef] [PubMed]

59. Lou, J.; Zhang, L.; Lv, S.; Zhang, C.; Jiang, S. Biomarkers for Hepatocellular Carcinoma. *Biomark. Cancer* **2017**, *9*, 1–9. [CrossRef] [PubMed]

60. Best, J.; Bechmann, L.P.; Sowa, J.-P.; Sydor, S.; Dechêne, A.; Pflanz, K.; Bedreli, S.; Schotten, C.; Geier, A.; Berg, T.; et al. GALAD Score Detects Early Hepatocellular Carcinoma in an International Cohort of Patients with Nonalcoholic Steatohepatitis. *Clin. Gastroenterol. Hepatol.* **2020**, *18*, 728–735.e4. [CrossRef]

61. Johnson, P.J.; Pirrie, S.J.; Cox, T.F.; Berhane, S.; Teng, M.; Palmer, D.; Morse, J.; Hull, D.; Patman, G.; Kagebayashi, C.; et al. The Detection of Hepatocellular Carcinoma Using a Prospectively Developed and Validated Model Based on Serological Biomarkers. *Cancer Epidemiol. Biomark. Prev.* **2014**, *23*, 144–153. [CrossRef]

62. Yang, J.D.; Addissie, B.D.; Mara, K.C.; Harmsen, W.S.; Dai, J.; Zhang, N.; Wongjarupong, N.; Ali, H.M.; Ali, H.A.; Hassan, F.A.; et al. GALAD Score for Hepatocellular Carcinoma Detection in Comparison with Liver Ultrasound and Proposal of GALADUS Score. *Cancer Epidemiol. Biomark. Prev.* **2019**, *28*, 531–538. [CrossRef]

63. Johnson, P.J.; Berhane, S.; Kagebayashi, C.; Satomura, S.; Teng, M.; Reeves, H.L.; O'Beirne, J.; Fox, R.; Skowronska, A.; Palmer, D.; et al. Assessment of Liver Function in Patients With Hepatocellular Carcinoma: A New Evidence-Based Approach—The ALBI Grade. *J. Clin. Oncol.* **2015**, *33*, 550–558. [CrossRef] [PubMed]

64. Kariyama, K.; Nouso, K.; Hiraoka, A.; Wakuta, A.; Oonishi, A.; Kuzuya, T.; Toyoda, H.; Tada, T.; Tsuji, K.; Itobayashi, E.; et al. EZ-ALBI Score for Predicting Hepatocellular Carcinoma Prognosis. *Liver Cancer* **2020**, *9*, 734–743. [CrossRef]

65. Dieter, P.; Altin, J.G.; Decker, K.; Bygrave, F.L. Possible Involvement of Eicosanoids in the Zymosan and Arachidonic-Acid-Induced Oxygen Uptake, Glycogenolysis and Ca^{2+} Mobilization in the Perfused Rat Liver. *JBIC J. Biol. Inorg. Chem.* **1987**, *165*, 455–460. [CrossRef] [PubMed]

66. Rosenberg, W.; Voelker, M.; Thiel, R.; Becka, M.; Burtb, A.; Schuppan, D.; Hubscher, S.; Roskams, T.; Pinzani, M.; Arthur, M.J. Serum Markers Detect the Presence of Liver Fibrosis: A Cohort Study. *Gastroenterology* **2004**, *127*, 1704–1713. [CrossRef] [PubMed]

67. Parkes, J.; Guha, I.N.; Roderick, P.; Harris, S.; Cross, R.; Manos, M.M.; Irving, W.; Zaitoun, A.; Wheatley, M.; Ryder, S.; et al. Enhanced Liver Fibrosis (ELF) Test Accurately Identifies Liver Fibrosis in Patients with Chronic Hepatitis C. *J. Viral Hepat.* **2010**, *18*, 23–31. [CrossRef]

68. Vali, Y.; Lee, J.; Boursier, J.; Spijker, R.; Löffler, J.; Verheij, J.; Brosnan, M.J.; Böcskei, Z.; Anstee, Q.M.; Bossuyt, P.M.; et al. Enhanced Liver Fibrosis Test for the Non-invasive Diagnosis of Fibrosis in Patients With NAFLD: A Systematic Review and Meta-Analysis. *J. Hepatol.* **2020**, *73*, 252–262. [CrossRef]

69. Lemmon, M.A.; Schlessinger, J. Cell Signaling by Receptor Tyrosine Kinases. *Cell* **2010**, *141*, 1117–1134. [CrossRef]

70. Krump, N.A.; You, J. Molecular Mechanisms of Viral Oncogenesis in Humans. *Nat. Rev. Genet.* **2018**, *16*, 684–698. [CrossRef]

71. Cong, M.; Iwaisako, K.; Jiang, C.; Kisseleva, T. Cell Signals Influencing Hepatic Fibrosis. *Int. J. Hepatol.* **2012**, *2012*, 1–18. [CrossRef]

72. Szabó, E.; Páska, C.; Novák, P.K.; Schaff, Z.; Kiss, A. Similarities and Differences in Hepatitis B and C Virus Induced Hepatocarcinogenesis. *Pathol. Oncol. Res.* **2004**, *10*, 5–11. [CrossRef]

73. Faure-Dupuy, S.; Lucifora, J.; Durantel, D. Interplay between the Hepatitis B Virus and Innate Immunity: From an Understanding to the Development of Therapeutic Concepts. *Viruses* **2017**, *9*, 95. [CrossRef] [PubMed]

74. Suslov, A.; Boldanova, T.; Wang, X.; Wieland, S.; Heim, M.H. Hepatitis B Virus Does Not Interfere with Innate Immune Responses in the Human Liver. *Gastroenterology* **2018**, *154*, 1778–1790. [CrossRef] [PubMed]

75. Li, M.; Li, H.; Li, C.; Wang, S.; Jiang, W.; Liu, Z.; Zhou, S.; Liu, X.; McNutt, M.A.; Li, G. Alpha-Fetoprotein: A New Member of Intracellular Signal Molecules in Regulation of the PI3K/AKT Signaling in Human Hepatoma Cell Lines. *Int. J. Cancer* **2010**, *128*, 524–532. [CrossRef] [PubMed]

76. Zhu, M.; Guo, J.; Li, W.; Lu, Y.; Fu, S.; Xie, X.; Xia, H.; Dong, X.; Chen, Y.; Quan, M.; et al. Hepatitis B Virus X Protein Induces Expression of Alpha-Fetoprotein and Activates PI3K/MTOR Signaling Pathway in Liver Cells. *Oncotarget* **2015**, *6*, 12196–12208. [CrossRef] [PubMed]

77. Xue, J.; Cao, Z.; Cheng, Y.; Wang, J.; Liu, Y.; Yang, R.; Li, H.; Jiang, W.; Li, G.; Zhao, W.; et al. Acetylation of Alpha-Fetoprotein Promotes Hepatocellular Carcinoma Progression. *Cancer Lett.* **2020**, *471*, 12–26. [CrossRef]

78. Golob-Schwarzl, N.; Krassnig, S.; Toeglhofer, A.M.; Park, Y.N.; Gogg-Kamerer, M.; Vierlinger, K.; Schröder, F.; Rhee, H.; Schicho, R.; Fickert, P.; et al. New Liver Cancer Biomarkers: PI3K/AKT/mTOR Pathway Members and Eukaryotic Translation Initiation Factors. *Eur. J. Cancer* **2017**, *83*, 56–70. [CrossRef]

79. Zhang, R.; Lin, H.-M.; Broering, R.; Shi, X.-D.; Yu, X.-H.; Xu, L.-B.; Wu, W.-R.; Liu, C. Dickkopf-1 Contributes to Hepatocellular Carcinoma Tumorigenesis by Activating the Wnt/β-Catenin Signaling Pathway. *Signal Transduct. Target. Ther.* **2019**, *4*, 1–10. [CrossRef]

80. Fezza, M.; Moussa, M.; Aoun, R.; Haber, R.; Hilal, G. DKK1 Promotes Hepatocellular Carcinoma Inflammation, Migration and Invasion: Implication of TGF-β1. *PLoS ONE* **2019**, *14*, e0223252. [CrossRef]

81. Zekri, A.-R.N.; El Kassas, M.; El Salam, E.S.; Hassan, R.M.; Mohanad, M.; Gabr, R.M.; Lotfy, M.M.; Abdel-Zaher, R.A.T.; Bahnassy, A.A.; Ahmed, O.S. The Possible Role of Dickkopf-1, Golgi Protein-73 and Midkine as Predictors of Hepatocarcinogenesis: A Review and an Egyptian Study. *Sci. Rep.* **2020**, *10*, 1–11. [CrossRef]

82. Shen, Q.; Fan, J.; Yang, X.-R.; Tan, Y.; Zhao, W.; Xu, Y.; Wang, N.; Niu, Y.; Wu, Z.; Zhou, J.; et al. Serum DKK1 as a Protein Biomarker for the Diagnosis of Hepatocellular Carcinoma: A Large-Scale, Multicentre Study. *Lancet Oncol.* **2012**, *13*, 817–826. [CrossRef]

83. Liu, J.; Wang, Z.; Tang, J.; Tang, R.; Shan, X.; Zhang, W.; Chen, Q.; Zhou, F.; Chen, K.; Huang, A.; et al. Hepatitis C Virus Core Protein Activates Wnt/β-Catenin Signaling through Multiple Regulation of Upstream Molecules in the SMMC-7721 Cell Line. *Arch. Virol.* **2011**, *156*, 1013–1023. [CrossRef]

84. Umer, M.; Qureshi, S.A.; Hashmi, Z.Y.; Raza, A.; Ahmad, J.; Rahman, M.; Iqbal, M. Promoter Hypermethylation of Wnt Pathway Inhibitors in Hepatitis C Virus-Induced Multistep Hepatocarcinogenesis. *Virol. J.* **2014**, *11*, 117. [CrossRef] [PubMed]

85. Eldeeb, M.K.; Magour, G.M.; Bedair, R.N.; Shamseya, M.M.; Hammouda, M.A. Study of Dickkopf-1 (DKK-1) in Patients with Chronic Viral Hepatitis C-Related Liver Cirrhosis with and without Hepatocellular Carcinoma. *Clin. Exp. Hepatol.* **2020**, *6*, 85–91. [CrossRef] [PubMed]

86. Essa, E.S.; Montaser, A.B.; Badawy, M.T.; Essa, A.S.; Mokhtar, M.S. DKK1 in Relation to HCV Induced Liver Cirrhosis and HCV Induced HCC Curative Resection. *Acta Gastro-Enterol. Belg.* **2016**, *79*, 309–313.

87. Qiao, Y.; Chen, J.; Li, X.; Wei, H.; Xiao, F.; Chang, L.; Zhang, R.; Hao, X.; Wei, H. Serum gp73 is Also a Biomarker for Diagnosing Cirrhosis in Population with Chronic HBV Infection. *Clin. Biochem.* **2014**, *47*, 216–222. [CrossRef]

88. Wei, M.; Xu, Z.; Pan, X.; Zhang, X.; Liu, L.; Yang, B.; Chen, Y. Serum GP73-An Additional Biochemical Marker for Liver Inflammation in Chronic HBV Infected Patients with Normal or Slightly Raised ALT. *Sci. Rep.* **2019**, *9*, 1–7. [CrossRef]

89. Xia, Y.; Zhang, Y.; Shen, M.; Xu, H.; Li, Z.; He, N. Golgi Protein 73 and Its Diagnostic Value in Liver Diseases. *Cell Prolif.* **2019**, *52*, e12538. [CrossRef] [PubMed]

90. Liu, L.; Zhu, J.; Yang, J.; Li, X.; Yuan, J.; Wu, J.; Liu, Z. GP73 Facilitates Hepatitis B Virus Replication by Repressing the NF-kappaB Signaling Pathway. *J. Med. Virol.* **2020**, *92*, 3327–3335. [CrossRef]

91. Yang, S.; Zeng, C.; Fang, X.; He, Q.; Liu, L.; Bao, S.; Pan, X.; Xiong, Z. Hepatitis B Virus Upregulates GP73 Expression by Activating the HIF-2α Signaling Pathway. *Oncol. Lett.* **2018**, *15*, 5264–5270. [CrossRef]

92. Chen, Y.; Tian, Z. HBV-Induced Immune Imbalance in the Development of HCC. *Front. Immunol.* **2019**, *10*, 2048. [CrossRef]

93. Rivino, L.; Le Bert, N.; Gill, U.S.; Kunasegaran, K.; Cheng, Y.; Tan, D.Z.; Becht, E.; Hansi, N.K.; Foster, G.R.; Su, T.-H.; et al. Hepatitis B Virus–Specific T Cells Associate with Viral Control upon Nucleos(t)ide-Analogue Therapy Discontinuation. *J. Clin. Investig.* **2018**, *128*, 668–681. [CrossRef]

94. Dolgormaa, G.; Harimoto, N.; Ishii, N.; Yamanaka, T.; Hagiwara, K.; Tsukagoshi, M.; Igarashi, T.; Watanabe, A.; Kubo, N.; Araki, K.; et al. Mac-2-Binding Protein Glycan Isomer Enhances the Aggressiveness of Hepatocellular Carcinoma by Activating mTOR Signaling. *Br. J. Cancer* **2020**, *123*, 1145–1153. [CrossRef] [PubMed]

95. Kuno, A.; Ikehara, Y.; Tanaka, Y.; Ito, K.; Matsuda, A.; Sekiya, S.; Hige, S.; Sakamoto, M.; Kage, M.; Mizokami, M.; et al. A Serum "Sweet-Doughnut" Protein Facilitates Fibrosis Evaluation and Therapy Assessment in Patients with Viral Hepatitis. *Sci. Rep.* **2013**, *3*, 1065. [CrossRef] [PubMed]

96. Narimatsu, H. Development of M2BPGi: A Novel Fibrosis Serum GlyCo-biomarker for Chronic Hepatitis/Cirrhosis Diagnostics. *Expert Rev. Proteom.* **2015**, *12*, 683–693. [CrossRef] [PubMed]

97. Jekarl, D.W.; Choi, H.; Lee, S.; Kwon, J.H.; Lee, S.W.; Yu, H.; Kim, M.; Kim, Y.; Sung, P.S.; Yoon, S.K. Diagnosis of Liver Fibrosis with Wisteria Floribunda Agglutinin-Positive Mac-2 Binding Protein (WFA-M2BP) Among Chronic Hepatitis B Patients. *Ann. Lab. Med.* **2018**, *38*, 348–354. [CrossRef]

98. Jun, T.; Hsu, Y.-C.; Ogawa, S.; Huang, Y.-T.; Yeh, M.-L.; Tseng, C.-H.; Huang, C.-F.; Tai, C.-M.; Dai, C.-Y.; Huang, J.-F.; et al. Mac-2 Binding Protein Glycosylation Isomer as a Hepatocellular Carcinoma Marker in Patients with Chronic Hepatitis B or C Infection. *Hepatol. Commun.* **2019**, *3*, 493–503. [CrossRef]

99. Liu, J.; Hu, H.-H.; Lee, M.-H.; Korenaga, M.; Jen, C.-L.; Batrla-Utermann, R.; Lu, S.-N.; Wang, L.-Y.; Mizokami, M.; Chen, C.-J.; et al. Serum Levels of M2BPGi as Short-Term Predictors of Hepatocellular Carcinoma in Untreated Chronic Hepatitis B Patients. *Sci. Rep.* **2017**, *7*, 1–10. [CrossRef]

100. Mak, L.-Y.; To, W.-P.; Wong, D.K.-H.; Fung, J.; Liu, F.; Seto, W.-K.; Lai, C.-L.; Yuen, M.-F. Serum Mac-2 Binding Protein Glycosylation Isomer Level Predicts Hepatocellular Carcinoma Development in E-negative Chronic Hepatitis B Patients. *World J. Gastroenterol.* **2019**, *25*, 1398–1408. [CrossRef]

101. Hsu, Y.-C.; Jun, T.; Huang, Y.-T.; Yeh, M.-L.; Lee, C.-L.; Ogawa, S.; Cho, S.-H.; Lin, J.-T.; Yu, M.-L.; Nguyen, M.H.; et al. Serum M2BPGi Level and Risk of Hepatocellular Carcinoma after Oral Anti-viral Therapy in Patients with Chronic Hepatitis B. *Aliment. Pharmacol. Ther.* **2018**, *48*, 1128–1137. [CrossRef] [PubMed]

102. Yamasaki, K.; Tateyama, M.; Abiru, S.; Komori, A.; Nagaoka, S.; Saeki, A.; Hashimoto, S.; Sasaki, R.; Bekki, S.; Kugiyama, Y.; et al. Elevated Serum Levels of Wisteria Floribunda Agglutinin-Positive Human Mac-2 Binding Protein Predict the Development of Hepatocellular Carcinoma in Hepatitis C Patients. *Hepatology* **2014**, *60*, 1563–1570. [CrossRef] [PubMed]

103. Shirabe, K.; Bekki, Y.; Gantumur, D.; Araki, K.; Ishii, N.; Kuno, A.; Narimatsu, H.; Mizokami, M. Mac-2 Binding Protein Glycan Isomer (M2BPGI) Is a New Serum Biomarker for Assessing Liver Fibrosis: More Than a Biomarker of Liver Fibrosis. *J. Gastroenterol.* **2018**, *53*, 819–826. [CrossRef] [PubMed]

104. Saleh, A.S.; Salama, M.M.; Alhusseini, M.M.; A Mohamed, G. M2BPGi for Assessing Liver Fibrosis in Patients with Hepatitis C Treated with Direct-Acting Antivirals. *World J. Gastroenterol.* **2020**, *26*, 2864–2876. [CrossRef] [PubMed]

105. Lupberger, J.; Zeisel, M.B.; Xiao, F.; Thumann, C.; Fofana, I.; Zona, L.; Davis, C.; Mee, C.J.; Turek, M.; Gorke, S.; et al. EGFR and EphA2 are Host Factors for Hepatitis C Virus Entry and Possible Targets for Antiviral Therapy. *Nat. Med.* **2011**, *17*, 589–595. [CrossRef] [PubMed]

106. Zona, L.; Lupberger, J.; SidAhmed-Adrar, N.; Thumann, C.; Harris, H.J.; Barnes, A.; Florentin, J.; Tawar, R.G.; Xiao, F.; Turek, M.; et al. HRas Signal Transduction Promotes Hepatitis C Virus Cell Entry by Triggering Assembly of the Host Tetraspanin Receptor Complex. *Cell Host Microbe* **2013**, *13*, 302–313. [CrossRef]

107. Mailly, L.; Xiao, F.; Lupberger, J.; Wilson, G.K.; Aubert, P.; Duong, F.H.T.; Calabrese, D.; Leboeuf, C.; Fofana, I.; Thumann, C.; et al. Clearance of Persistent Hepatitis C Virus Infection in Humanized Mice Using a Claudin-1-Targeting Monoclonal Antibody. *Nat. Biotechnol.* **2015**, *33*, 549–554. [CrossRef]

108. Roehlen, N.; Crouchet, E.; Baumert, T.F. Liver Fibrosis: Mechanistic Concepts and Therapeutic Perspectives. *Cells* **2020**, *9*, 875. [CrossRef] [PubMed]

109. Seoane, J.; Gomis, R.R. TGF-β Family Signaling in Tumor Suppression and Cancer Progression. *Cold Spring Harb. Perspect. Biol.* **2017**, *9*, a022277. [CrossRef] [PubMed]

110. Chusri, P.; Kumthip, K.; Hong, J.; Zhu, C.; Duan, X.; Jilg, N.; Fusco, D.N.; Brisac, C.; Schaefer, E.A.; Cai, D.; et al. HCV Induces Transforming Growth Factor β1 through Activation of Endoplasmic Reticulum Stress and the Unfolded Protein Response. *Sci. Rep.* **2016**, *6*, 22487. [CrossRef]

111. Cheng, P.-L.; Chang, M.-H.; Chao, C.-H.; Lee, Y.-H.W. Hepatitis C Viral Proteins Interact with Smad3 and Differentially Regulate TFG-β/Smad3-Mediated Transcriptional Activation. *Oncogene* **2004**, *23*, 7821–7838. [CrossRef]

112. Clemente, M.; Nunez, O.; Lorente, R.; Rincon, D.; Matilla, A.; Salcedo, M.; Catalina, M.V.; Ripoll, C.; Iacono, O.L.; Banares, R.; et al. Increased Intrahepatic and Circulating Levels of Endoglin, a TFG-β1 Co-receptor, in Patients with Chronic Hepatitis C Virus Infection: Relationship to Histological and Serum Markers of Hepatic Fibrosis. *J. Viral Hepat.* **2006**, *13*, 625–632. [CrossRef]

113. Tardif, K.D.; Waris, G.; Siddiqui, A. Hepatitis C Virus, ER Stress, and Oxidative Stress. *Trends Microbiol.* **2005**, *13*, 159–163. [CrossRef]

114. Abdalla, M.Y.; Mathahs, M.M.; Ahmad, I.M. Reduced Heme Oxygenase-1 Expression in Steatotic Livers Infected with Hepatitis C Virus. *Eur. J. Intern. Med.* **2012**, *23*, 649–655. [CrossRef] [PubMed]

115. He, G.; Karin, M. NF-κB and STAT3–Key Players in Liver Inflammation and Cancer. *Cell Res.* **2010**, *21*, 159–168. [CrossRef] [PubMed]

116. Li, Y.; Zhang, Q.; Liu, Y.; Luo, Z.; Kang, L.; Qu, J.; Liu, W.; Xia, X.; Wu, K.; Wu, J. Hepatitis C Virus Activates Bcl-2 and MMP-2 Expression through Multiple Cellular Signaling Pathways. *J. Virol.* **2012**, *86*, 12531–12543. [CrossRef]

117. Abdel-Latif, M.S. Plasma Levels of Matrix Metalloproteinase (MMP)-2, MMP-9 and Tumor Necrosis Factor-α in Chronic Hepatitis C Virus Patients. *Open Microbiol. J.* **2015**, *9*, 136–140. [CrossRef]

118. Wilson, G.K.; Brimacombe, C.L.; Rowe, I.A.; Reynolds, G.M.; Fletcher, N.F.; Stamataki, Z.; Bhogal, R.H.; Simões, M.L.; Ashcroft, M.; Afford, S.C.; et al. A Dual Role for Hypoxia Inducible Factor-1α in the Hepatitis C Virus Lifecycle and Hepatoma Migration. *J. Hepatol.* **2012**, *56*, 803–809. [CrossRef] [PubMed]

119. Mas, V.R.; Maluf, D.G.; Archer, K.J.; Yanek, K.C.; Fisher, R.A. Angiogenesis Soluble Factors as Hepatocellular Carcinoma Noninvasive Markers for Monitoring Hepatitis C Virus Cirrhotic Patients Awaiting Liver Transplantation. *Transplantation* **2007**, *84*, 1262–1271. [CrossRef] [PubMed]

120. Hernández-Bartolomé, Á; López-Rodríguez, R.; Borque, M.J.; González-Moreno, L.; Real-Martínez, Y.; García-Buey, L.; Moreno-Otero, R.; Sanz-Cameno, P. Angiopoietin-2/Angiopoietin-1 as Non-invasive Biomarker of Cirrhosis in Chronic Hepatitis C. *World J. Gastroenterol.* **2016**, *22*, 9744–9751. [CrossRef]

121. Hernández-Bartolomé, Á.; López-Rodríguez, R.; García-Buey, L.; Martín-Vílchez, S.; Rodríguez-Muñoz, Y.; Borque, M.J.; González-Moreno, L.; Real-Martínez, Y.; Mendoza-Ridruejo, J.; Martín-Pérez, E.; et al. Intrahepatic Angiopoietin-2 Correlates with Chronic Hepatitis C Progression and Is Induced in Hepatitis C Virus Replicon Systems. *Liver Int.* **2016**, *37*, 1148–1156. [CrossRef]

122. Benkheil, M.; Van Haele, M.; Roskams, T.; Laporte, M.; Noppen, S.; Abbasi, K.; Delang, L.; Neyts, J.; Liekens, S. CCL20, A Direct-Acting Pro-angiogenic Chemokine Induced by Hepatitis C Virus (HCV): Potential Role in HCV-Related Liver Cancer. *Exp. Cell Res.* **2018**, *372*, 168–177. [CrossRef]

123. Soliman, H.H.; Nagy, H.; Kotb, N.; El-Din, M.A.A. The Role of Chemokine CC Ligand 20 in Patients with Liver Cirrhosis and Hepatocellular Carcinoma. *Int. J. Biol. Markers* **2012**, *27*, 125–131. [CrossRef]

124. Oliveira, D.A.R.; Castanhole-Nunes, M.M.; Biselli-Chicote, P.M.; Pavarino, É.C.; da Silva, R.C.M.A.; Da Silva, R.F.; Goloni-Bertollo, E.M. Differential Expression of Angiogenesis-Related miRNAs and VEGFA in Cirrhosis and Hepatocellular Carcinoma. *Arch. Med. Sci.* **2020**, *16*, 1150–1157. [CrossRef]

125. Salum, G.M.; El Din, N.G.B.; Ibrahim, M.K.; Anany, M.A.; Dawood, R.M.; Khairy, A.; El Awady, M.K. Vascular Endothelial Growth Factor Expression in Hepatitis C Virus-Induced Liver Fibrosis: A Potential Biomarker. *J. Interf. Cytokine Res.* **2017**, *37*, 310–316. [CrossRef]

126. Zhu, C.L.; Li, W.T.; Li, Y.; Gao, R.T. Serum Levels of Tissue Inhibitor of Metalloproteinase-1 Are Correlated with Liver Fibrosis in Patients with Chronic Hepatitis B. *J. Dig. Dis.* **2012**, *13*, 558–563. [CrossRef]

127. Seven, G.; Karatayli, S.C.; Kose, S.K.; Yakut, M.; Kabacam, G.; Toruner, M.; Heper, A.O.; Voelker, M.; Erden, E.; Bozdayi, A.M.; et al. Serum Connective Tissue Markers as Predictors of Advanced Fibrosis in Patients with Chronic Hepatitis B and D. *Turk. J. Gastroenterol.* **2011**, *22*, 305–314. [CrossRef]

128. Xue, Y.; Mars, W.M.; Bowen, W.; Singhi, A.D.; Stoops, J.; Michalopoulos, G.K. Hepatitis C Virus Mimics Effects of Glypican-3 on CD81 and Promotes Development of Hepatocellular Carcinomas via Activation of Hippo Pathway in Hepatocytes. *Am. J. Pathol.* **2018**, *188*, 1469–1477. [CrossRef] [PubMed]

129. Shimizu, Y.; Mizuno, S.; Fujinami, N.; Suzuki, T.; Saito, K.; Konishi, M.; Takahashi, S.; Gotohda, N.; Tada, T.; Toyoda, H.; et al. Plasma and Tumoral Glypican-3 Levels Are Correlated in Patients with Hepatitis C Virus-Related Hepatocellular Carcinoma. *Cancer Sci.* **2019**, *111*, 334–342. [CrossRef]

130. Jaenisch, R.; Bird, A. Epigenetic Regulation of Gene Expression: How the Genome Integrates Intrinsic and Environmental Signals. *Nat. Genet.* **2003**, *33*, 245–254. [CrossRef] [PubMed]

131. Hardy, T.; Mann, A.D. Epigenetics in Liver Disease: From Biology to Therapeutics. *Gut* **2016**, *65*, 1895–1905. [CrossRef] [PubMed]

132. Moran-Salvador, E.; Mann, J. Epigenetics and Liver Fibrosis. *Cell. Mol. Gastroenterol. Hepatol.* **2017**, *4*, 125–134. [CrossRef]

133. Wilson, C.L.; Mann, D.A.; Borthwick, L.A. Epigenetic Reprogramming in Liver Fibrosis and Cancer. *Adv. Drug Deliv. Rev.* **2017**, *121*, 124–132. [CrossRef]

134. Zhang, Y.; Petropoulos, S.; Liu, J.; Cheishvili, D.; Zhou, R.; Dymov, S.; Li, K.; Li, N.; Szyf, M. The Signature of Liver Cancer in Immune Cells DNA Methylation. *Clin. Epigenetics* **2018**, *10*, 8. [CrossRef]

135. Li, K.; Qin, L.; Jiang, S.; Zhang, C.; Liu, G.; Sun, J.; Sun, H.; Zhao, Y.; Li, N.; Zhang, Y.; et al. The Signature of HBV-Related Liver Disease in Peripheral Blood Mononuclear Cell DNA Methylation. *Clin. Epigenetics* **2020**, *12*, 1–13. [CrossRef]

136. Vivekanandan, P.; Daniel, H.D.-J.; Kannangai, R.; Martinez-Murillo, F.; Torbenson, M. Hepatitis B Virus Replication Induces Methylation of both Host and Viral DNA. *J. Virol.* **2010**, *84*, 4321–4329. [CrossRef]

137. Ancey, P.-B.; Testoni, B.; Gruffaz, M.; Cros, M.-P.; Durand, G.; Le Calvez-Kelm, F.; Durantel, D.; Herceg, Z.; Hernandez-Vargas, H. Genomic Responses to Hepatitis B Virus (HBV) Infection in Primary Human Hepatocytes. *Oncotarget* **2015**, *6*, 44877–44891. [CrossRef]

138. Dong, X.; Hou, Q.; Chen, Y.; Wang, X. Diagnostic Value of the Methylation of Multiple Gene Promoters in Serum in Hepatitis B Virus-Related Hepatocellular Carcinoma. *Dis. Markers* **2017**, *2017*, 1–6. [CrossRef]

139. Wei, L.; Huang, Y.; Zhao, R.; Zhang, J.; Liu, Q.; Liang, W.; Ding, X.; Gao, B.; Li, B.; Sun, C.; et al. Detection of Promoter Methylation Status of Suppressor of Cytokine Signaling 3 (SOCS3) in Tissue and Plasma from Chinese Patients with Different Hepatic Diseases. *Clin. Exp. Med.* **2018**, *18*, 79–87. [CrossRef]

140. Wu, H.-C.; Yang, H.-I.; Wang, Q.; Chen, C.-J.; Santella, R.M. Plasma DNA Methylation Marker and Hepatocellular Carcinoma Risk Prediction Model for the General Population. *Carcinogenesis* **2017**, *38*, 1021–1028. [CrossRef]

141. Hamdane, N.; Jühling, F.; Crouchet, E.; El Saghire, H.; Thumann, C.; Oudot, M.A.; Bandiera, S.; Saviano, A.; Ponsolles, C.; Suarez, A.A.R.; et al. HCV-Induced Epigenetic Changes Associated with Liver Cancer Risk Persist After Sustained Virologic Response. *Gastroenterology* **2019**, *156*, 2313–2329.e7. [CrossRef]

142. Juhling, F.; Hamdane, N.; Crouchet, E.; Li, S.; El Saghire, H.; Mukherji, A.; Fujiwara, N.; Oudot, M.A.; Thumann, C.; Saviano, A.; et al. Targeting Clinical Epigenetic Reprogramming for Chemoprevention of Metabolic and Viral Hepatocellular Carcinoma. *Gut* **2020**, *70*, 157–169. [CrossRef]

143. Perez, S.; Kaspi, A.; Domovitz, T.; Davidovich, A.; Lavi-Itzkovitz, A.; Meirson, T.; Holmes, J.A.; Dai, C.-Y.; Huang, C.-F.; Chung, R.T.; et al. Hepatitis C Virus Leaves an Epigenetic Signature Post Cure of Infection by Direct-Acting Antivirals. *PLoS Genet.* **2019**, *15*, e1008181. [CrossRef] [PubMed]

144. Shaker, O.G.; Senousy, M.A. Serum microRNAs as Predictors for Liver Fibrosis Staging in Hepatitis C Virus-Associated Chronic Liver Disease Patients. *J. Viral Hepat.* **2017**, *24*, 636–644. [CrossRef]

145. Wang, X.; He, Y.; Mackowiak, B.; Gao, B. MicroRNAs as Regulators, Biomarkers and Therapeutic Targets in Liver Diseases. *Gut* **2020**, *10*, 1136. [CrossRef]

146. Thietart, S.; Rautou, P.-E. Extracellular Vesicles as Biomarkers in Liver Diseases: A Clinician's Point of View. *J. Hepatol.* **2020**, *73*, 1507–1525. [CrossRef]

147. Matsuura, K.; De Giorgi, V.; Schechterly, C.; Wang, R.Y.; Farci, P.; Tanaka, Y.; Alter, H.J. Circulating Let-7 Levels in Plasma and Extracellular Vesicles Correlate with Hepatic Fibrosis Progression in Chronic Hepatitis C. *Hepatology* **2016**, *64*, 732–745. [CrossRef]

148. Rattan, P.; Minacapelli, C.D.; Rustgi, V. The Microbiome and Hepatocellular Carcinoma. *Liver Transplant.* **2020**, *26*, 1316–1327. [CrossRef]

149. Yu, L.-X.; Schwabe, L.-X.Y.R.F. The Gut Microbiome and Liver Cancer: Mechanisms and Clinical Translation. *Nat. Rev. Gastroenterol. Hepatol.* **2017**, *14*, 527–539. [CrossRef]

150. Hoshida, Y.; Nijman, S.M.; Kobayashi, M.; Chan, J.A.; Brunet, J.-P.; Chiang, D.Y.; Villanueva, A.; Newell, P.; Ikeda, K.; Hashimoto, M.; et al. Integrative Transcriptome Analysis Reveals Common Molecular Subclasses of Human Hepatocellular Carcinoma. *Cancer Res.* **2009**, *69*, 7385–7392. [CrossRef]

151. Guichard, C.; Amaddeo, G.; Imbeaud, S.; Ladeiro, Y.; Pelletier, L.; Ben Maad, I.; Calderaro, J.; Bioulac-Sage, P.; Letexier, M.; Degos, F.; et al. Integrated Analysis of Somatic Mutations and Focal Copy-Number Changes Identifies Key Genes and Pathways in Hepatocellular Carcinoma. *Nat. Genet.* **2012**, *44*, 694–698. [CrossRef]

152. Schulze, K.; Imbeaud, S.; Letouzé, E.; Alexandrov, L.B.; Calderaro, J.; Rebouissou, S.; Couchy, G.; Meiller, C.; Shinde, J.; Soysouvanh, F.; et al. Exome Sequencing of Hepatocellular Carcinomas Identifies New Mutational Signatures and Potential Therapeutic Targets. *Nat. Genet.* **2015**, *47*, 505–511. [CrossRef]
153. Sengupta, S.; Parikh, N.D. Biomarker Development for Hepatocellular Carcinoma Early Detection: Current and Future Perspectives. *Hepatic Oncol.* **2017**, *4*, 111–122. [CrossRef]
154. Saad, Y.; El-Serafy, M.; Eldin, M.S.; Abdellatif, Z.; Khatab, H.; Elbaz, T.; Elgarem, H. New Genetic Markers for Diagnosis of Hepatitis C Related Hepatocellular Carcinoma in Egyptian Patients. *J. Gastrointest. Liver Dis.* **2013**, *22*, 419–425.
155. Hodeib, H.; Elshora, O.; Selim, A.; Sabry, N.M.; El-Ashry, H.M. Serum Midkine and Osteopontin Levels as Diagnostic Biomarkers of Hepatocellular Carcinoma. *Electron. Physician* **2017**, *9*, 3492–3498. [CrossRef] [PubMed]
156. Sobhy, A.M.M.F.; Azeem, A.H.; Ashmawy, A.M.; Khalifa, H.O. Significance of Biglycan and Osteopontin as Non-invasive Markers of Liver Fibrosis in Patients with Chronic Hepatitis B Virus and Chronic Hepatitis C Virus. *J. Investig. Med.* **2018**, *67*, 681–685. [CrossRef] [PubMed]
157. Zhu, M.; Zheng, J.; Wu, F.; Kang, B.; Liang, J.; Heskia, F.; Zhang, X.; Shan, Y. OPN is a Promising Serological Biomarker for Hepatocellular Carcinoma Diagnosis. *J. Med. Virol.* **2020**, *92*, 3596–3603. [CrossRef] [PubMed]
158. Kolgelier, S.; Demir, N.A.; Inkaya, A.C.; Sumer, S.; Ozcimen, S.; Demir, L.S.; Pehlivan, F.S.; Arslan, M.; Arpaci, A. Serum Levels of Annexin A2 as a Candidate Biomarker for Hepatic Fibrosis in Patients with Chronic Hepatitis B. *Hepat. Mon.* **2015**, *15*, e30655. [CrossRef] [PubMed]
159. Tang, L.; Liu, J.-X.; Zhang, Z.-J.; Xu, C.-Z.; Zhang, X.-N.; Huang, W.-R.; Zhou, D.-H.; Wang, R.-R.; Chen, X.-D.; Xiao, M.-B.; et al. High Expression of Anxa2 and Stat3 Promote Progression of Hepatocellular Carcinoma and Predict Poor Prognosis. *Pathol. Res. Pr.* **2019**, *215*, 152386. [CrossRef] [PubMed]
160. Ali, L.H.; Higazi, A.M.; Moness, H.M.; Farag, N.M.; Saad, Z.M.; Moukareb, A.H.; Soliman, W.; El Sagheer, G.; El Hamid, S.R.A.; Hamid, H.A. Clinical Significances and Diagnostic Utilities of Both miR-215 and Squamous Cell Carcinoma Antigen-IgM versus Alpha-Fetoprotein in Egyptian Patients with Hepatitis C Virus-Induced Hepatocellular Carcinoma. *Clin. Exp. Gastroenterol.* **2019**, *12*, 51–66. [CrossRef]
161. Gallotta, A.; Paneghetti, L.; Mrázová, V.; Bednárová, A.; Kružlicová, D.; Frecer, V.; Miertus, S.; Biasiolo, A.; Martini, A.; Pontisso, P.; et al. Development of a Novel Diagnostic Algorithm to Predict NASH in HCV-Positive Patients. *Int. J. Biol. Markers* **2018**, *33*, 231–236. [CrossRef]

Annex VI: Oncogenic Signaling Induced by HCV Infection

Review

Virzì A, Roca Suarez AA, Baumert TF, Lupberger J.

I contribute to conceptualize and write the review. The figures were prepared together with the other authors.

Oncogenic Signaling Induced by HCV Infection

Alessia Virzì [1,2] , **Armando Andres Roca Suarez** [1,2] , **Thomas F. Baumert** [1,2,3]
and Joachim Lupberger [1,2,*]

[1]   Inserm, U1110, Institut de Recherche sur les Maladies Virales et Hépatiques, 67000 Strasbourg, France;
       virzi@unistra.fr (A.V.); andres.roca-suarez@etu.unistra.fr (A.A.R.S.); thomas.baumert@unistra.fr (T.F.B.)

[2]   Université de Strasbourg, 67000 Strasbourg, France

[3]   Pôle Hépato-digestif, Institut Hospitalo-universitaire, Hôpitaux Universitaires de Strasbourg,
       67000 Strasbourg, France

*   Correspondence: joachim.lupberger@unistra.fr; Tel.: +33-3-6885-3715; Fax: +33-3-6885-5508

Abstract: The liver is frequently exposed to toxins, metabolites, and oxidative stress, which can challenge organ function and genomic stability. Liver regeneration is therefore a highly regulated process involving several sequential signaling events. It is thus not surprising that individual oncogenic mutations in hepatocytes do not necessarily lead to cancer and that the genetic profiles of hepatocellular carcinomas (HCCs) are highly heterogeneous. Long-term infection with hepatitis C virus (HCV) creates an oncogenic environment by a combination of viral protein expression, persistent liver inflammation, oxidative stress, and chronically deregulated signaling events that cumulate as a tipping point for genetic stability. Although novel direct-acting antivirals (DAA)-based treatments efficiently eradicate HCV, the associated HCC risk cannot be fully eliminated by viral cure in patients with advanced liver disease. This suggests that HCV may persistently deregulate signaling pathways beyond viral cure and thereby continue to perturb cancer-relevant gene function. In this review, we summarize the current knowledge about oncogenic signaling pathways derailed by chronic HCV infection. This will not only help to understand the mechanisms of hepatocarcinogenesis but will also highlight potential chemopreventive strategies to help patients with a high-risk profile of developing HCC.

Keywords: signaling; cancer; HCV; HCC; chemoprevention; liver disease

1. Introduction

Tumor-inducing viruses represent a considerable field of study for the comprehension of molecular carcinogenesis. Several oncogenes were first discovered in association with retroviruses and then associated with most forms of cancer [1,2]. The study of virus-coded oncogenes also led to the discovery of canonical signaling pathways and the understanding of elementary cellular processes. Several viruses are considered as oncogenic viruses as they are associated with human cancer, e.g., human papilloma virus (HPV), Epstein–Barr virus (EBV), human herpes virus 8 (HHV8), Merkel cell polyomavirus (MCPyV), human T-lymphotropic virus (HTLV-1), hepatitis B virus (HBV), and hepatitis C virus (HCV) [3].

Infection with oncogenic viruses generally leads to the disruption of genetic and epigenetic homeostasis and DNA repair mechanisms. In addition, some viruses stimulate the proliferation of cancer stem cells (CSCs), which are involved in cancer initiation, progression, and chemotherapy resistance [3]. Oncogenic viruses have a direct and indirect impact on carcinogenesis [4]. At least four HCV proteins (core, NS3, NS5A, and NS5B) seem to deregulate potentially oncogenic signaling pathways [5]. At the same time, it is beyond question that HCV creates a procarcinogenic environment in the liver by inducing a chronic inflammatory state [6]. In addition, liver disease progression can

be favored by several cofactors, including alcohol consumption and coinfection with other viruses such as HBV and human immunodeficiency virus (HIV) [7]. Moreover, HCV infection is implicated in extrahepatic cancers, including B-cell non-Hodgkin lymphomas (NHL) [8] and cancers of the oral cavity, oropharynx, intrahepatic bile duct, pancreas, and kidney [9–15]. Although the molecular links between HCV and extrahepatic cancers are not well understood, it has been suggested that some of the possible mechanisms behind this association could be related to a chronic immune stimulation in the presence of HCV or to the infection of extrahepatic cell types [16].

The study of the HCV life cycle revealed several host dependencies of the virus that involve signaling molecules [17–21]. However, it soon became evident that HCV not only requires signaling processes but also actively manipulates host signal transduction with considerable impact on liver pathogenesis. Numerous studies have described signaling cascades that are altered by chronic HCV infection and are potentially involved in carcinogenesis (Figure 1). In the present review, we classify these pathways in three cancer-relevant categories according to their role in cell proliferation/survival, differentiation/adhesion/angiogenesis, inflammatory response, and dissect potential clinical strategies for hepatocellular carcinomas (HCC) chemoprevention and therapy.
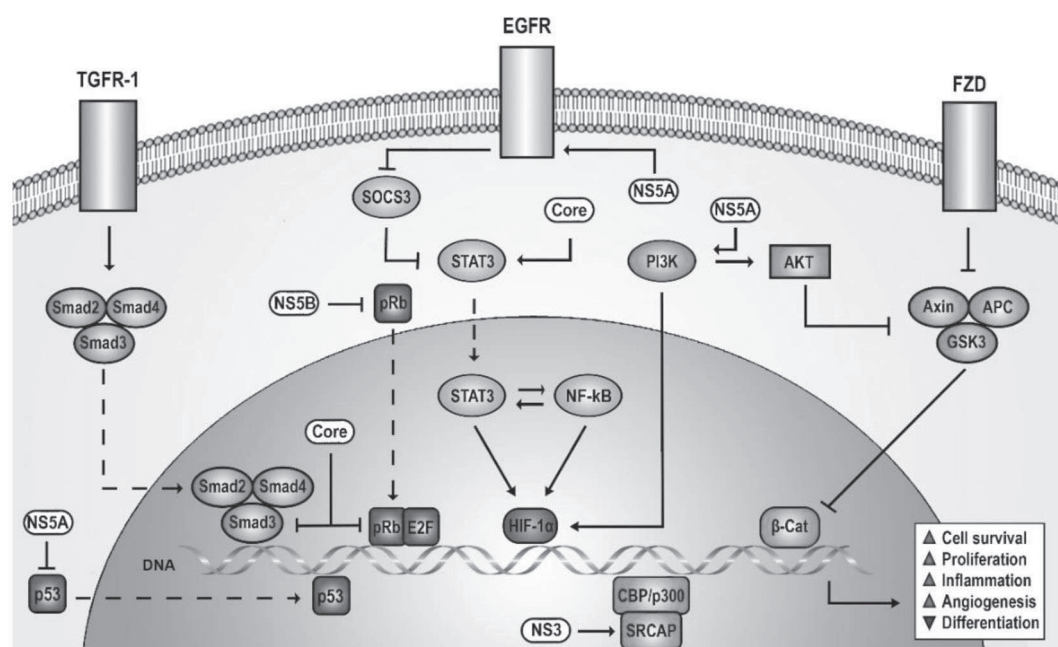
Figure 1. Hepatitis C Virus (HCV)-induced oncogenic signaling. HCV infection creates a procarcinogenic effect through the simultaneous dysregulation of cell survival, proliferation, inflammatory, angiogenic, and differentiation signaling pathways. The tight control of target genes involved in transcriptional regulation and cell cycle progression is altered by HCV via different strategies. Forcing p53 in the cytoplasm, NS5A prevents the gene expression of cyclin-dependent kinase inhibitor p21 (not shown). This cytoplasmic-retention strategy is also shared by NS5B, which traps pRb in the cytoplasm. Consequently, E2F is free to act as transcriptional activator for cell proliferation target genes. Core protein, which is preferentially localized in the cytoplasm, translocates to the nucleus, where it interferes with transforming growth factor-beta (TGF-β) signaling via Smad3 interaction. Epidermal growth factor receptor (EGFR) and mitogen-activated protein kinase (MAPK) signaling are not only required for HCV entry but also represent oncogenic targets for HCV-encoded proteins. Both NS5A and core protein induce the activation of signal transducer and activator of transcription 3 (STAT3) by indirect (inhibiting the suppressor of cytokine signaling 3, SOCS3) and direct mechanisms, respectively.

Following its translocation to the nucleus, STAT3 strongly promotes a proinflammatory environment in cooperation with nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling. Furthermore, STAT3 and NF-κB, together with PI3K, induce hypoxia-inducible factor 1-alpha (HIF-1α) stabilization, which mediates the transcription of several proangiogenic factors (e.g., vascular endothelial growth factor, VEGF). HCV impairs cell differentiation programs by manipulating Wnt and Notch signaling pathways. NS5A induces a sustained Wnt signaling activation through the PI3K/Akt axis. This leads to the inactivation of a downstream degradation complex and the consequent accumulation of β-catenin in the nucleus, where it activates the expression of cell proliferation-related genes. NS3 stimulates downstream components of Notch pathway by the recruitment of CREB-binding protein (CBP)/p300 complex on Snf2-related CBP activator (SRCAP), repressing cell differentiation programs. TGFR-1: TGF-β receptor 1; FZD: Wnt receptor (Frizzled).

2. HCV Creates a Persistent Proliferative and Anti-Apoptotic Signaling Environment

Proliferative signaling pathways of mammalian cells are modulated by extracellular factors that engage precise programs of gene transcription and protein regulation [22,23]. Contact inhibition, controlled availability of growth factors, and other physiological feedback systems ensure a tight regulation of the proliferative signaling pathways. Excessive cell proliferation is the key feature of most types of cancers [24]. In general, growth factor and cytokine signaling pathways essentially induce all the primary steps of tumor progression, which include clonal expansion, invasion, angiogenesis, and metastatic formation [25]. Tumor suppressors, such as the cellular tumor antigen p53 and the retinoblastoma-associated protein (pRb), regulate cell proliferation, and their perturbation promotes a persistent activation of the cell cycle machinery [24]. Although HCC proliferative index is generally low, which is one of the reasons why most cytostatics are considered inefficient, there is a clear correlation of HCC risk and proliferative signals in a pretumor state [26].

2.1. HCV-Induced Receptor Tyrosine Kinase Signaling Contributes to Liver Cancer Risk

Growth factors like epidermal growth factor (EGF), fibroblast growth factor (FGF), hepatocyte growth factor (HGF), and insulin growth factor (IGF) trigger downstream signal transduction by binding to their specific receptor tyrosine kinase receptors [27]. The cascade of events that follow epidermal growth factor receptor (EGFR) is one of the most widely studied signal transduction pathways [28–30]. ErbB-1 and three additional homologous members of the EGFR family (ErbB-2, ErbB-3, ErbB-4), regulate cell proliferation, differentiation, and migration under normal physiological conditions [29]. EGFR itself is critical in epithelial development, and other members of the family have a crucial role in cardiac, mammary glands, and nervous system development and disorders [28,31–33]. The EGFR signaling pathway plays a central role also in embryonic development and in the regeneration of stem cells in skin, liver, and gut [34,35]. Moreover, the EGFR signaling pathway is in the spotlight as a driver of cancer risk and progression [26,36,37].

Viruses have developed sophisticated strategies to manipulate EGFR functions (i.e., perturbing EGFR expression, activity, or recycling) [38]. EGFR is a host factor for HCV entry into hepatocytes by regulating the assembly of the coreceptor complex [17,21], viral internalization [39], and membrane fusion [17]. Furthermore, EGFR signaling pathway tempers the antiviral activity of interferon-alpha (IFN-α) by maintaining phosphorylation of signal transducer and activator of transcription 3 (STAT3) through the suppression of a negative feedback regulator (i.e., suppressor of cytokine signaling 3, SOCS3) [40]. It is evident that HCV has a vital interest in maintaining EGFR signaling. Indeed, HCV not only requires EGFR signaling but also actively induces the activation of this pathway during HCV binding and infection [41,42] and prolongs EGFR signaling by perturbing EGFR degradation via NS5A, as reported upon its ectopic expression [43]. This leads to an increased HCC risk in infected patients as persistent EGF signaling is a key driver of liver disease [26].

2.2. HCV Increases Cell Survival by Cytoplasmic Retention of p53 and pRb

Proliferative signals seem beneficial for HCV to avoid stress-induced growth arrest and apoptosis, both of which would oppose viral replication and survival [44,45]. The tumor suppressors pRb and p53 regulate cell growth control via their action on cell cycle checkpoints and apoptosis programs [22]. Therefore, pRb, p107, and p130 proteins cooperate with various proteins, including transcription factors of the E2F family required for cellular DNA replication [46–48]. The downstream interaction between pRb and E2F causes the inhibition of gene expression by the recruitment of histone deacetylases (HDACs) [49] and other chromatin remodeling factors [50–52]. pRb constitutively inhibits the transcriptional activity of E2Fs, whereas it is deactivated after phosphorylation by cyclin-dependent kinases (CDKs). G1 phase CDKs phosphorylate pRb family proteins, which leads to the activation of genes required for S phase entry (i.e., *cyclin E*) [22,24]. In contrast, p53 maintains genetic integrity of cells by blocking cell proliferation in response to stress and DNA damage by activating cyclin-dependent kinase inhibitors (CKIs) [24]. Therefore, p53 accumulates in the nucleus, where it acts as a transcription factor for cyclin-dependent kinase inhibitor 1 (*CDKN1A*) that codes for p21 [53,54]. Thus, it is not surprising that deregulation of p53 function or signaling is associated with many cancers [24]. For example, pRb is a target of viral oncoproteins encoded by adenovirus [55] and HPV [56]. In addition, HCV has developed strategies to suppress pRb [57–59]. During HCV infection, NS5B protein retains pRb in the cytoplasm of the hepatocyte, leading to its proteasomal degradation via E6-associated protein (E6AP) recruitment and polyubiquitination [57,59]. The isolated expression of HCV core protein impairs pRb expression in immortalized rat embryo fibroblasts and thereby promotes a E2F-1 activity with impact on cell proliferation and apoptosis [60]. The frequency and the geographic distribution of *TP53* (p53) mutations presumably depend on the variability of aetiological and host susceptibility factors [61,62]. HCV and other viruses have sophisticated strategies to modulate or inhibit p53 signaling [63]. HCV core proteins, NS5A, and NS3 associate with p53 and repress its function without initiating its degradation. HCV core protein, however, seems to act as both activator and a repressor of p53 pathway [64–66]. This dual role of core protein may reflect a dose-dependent impact on p53 signaling, depending on the infection model used [67]. In vitro data suggest that the effect of NS3 protein on p53 depends on the HCV genotype [68,69]. Like pRb, virus-induced perturbation of p53 function involves a forced retention in the cytoplasm, which prevents DNA binding of p53. HCV NS5A colocalizes with p53 in the cytoplasmic perinuclear region and sufficiently reduces nuclear p53 concentration to suppress apoptosis. In addition, NS5A expression enforces p53 inhibition via binding to hTAFII32, which is an essential p53 coactivator [70]. In a more indirect manner, HCV proteins perturb the function of essential cofactors of p53 transcriptional activity. Core interacts with DEAD-Box Helicase 3 X-Linked (DDX3X), as observed in an isolated core-expression context [71–73]. DDX3X is a target of p53 [74] and modulates *CDKN1A* promoter activity. Furthermore, NS5B binds and relocalizes p53 coactivator DEAD-Box Helicase 5 (DDX5) to the cytoplasm [75–77]. However, the findings on p53 signaling during HCV infection have to be interpreted with caution as many of the immortalized cell lines used to study HCV present defects in p53 signaling [6]. For example, Huh7-derived cell lines, which are commonly used due to their high permissiveness towards HCV, accumulate a functionally damaged p53 mutant in the nucleus [78].

2.3. HCV Impairs TGF-β Signaling Promoting Epithelial Mesenchymal Transition (EMT)

Cytokines of the transforming growth factor β (TGF-β) superfamily are dimers with conserved structures and exert pleiotropic effects [79]. In physiological conditions, TGF-β acts as a potent growth inhibitor for several types of cells [80–84] and promotes apoptosis in epithelial cells [85]. Consequently, impaired TGF-β may result in cellular hyperproliferation and cancer [86]. In addition, these cytokines stimulate the expression of extracellular matrix components, which promote in vivo fibrosis in different tissues [85,87]. In the liver, TGF-β seems to contribute to all stages of disease development, from early injury through inflammation, fibrosis towards cirrhosis and HCC [88,89]. TGF-β presumably acts as tumor suppressor during the early stage of cancer development but promotes tumor progression,

migration, and invasion in advanced HCCs once the tumor cells have acquired resistance to its suppressive proprieties [89–91]. Members of the TGF-β superfamily interact with two different receptor types, called type I and type II receptors, which are both required for cellular signaling [85,92,93]. TGF-β binds directly to receptor II, which is constitutively active. This event induces the recruitment of receptor I into the complex that subsequently becomes phosphorylated by receptor II and activate downstream signals [92], which includes SMAD proteins [94,95]. Particularly, the activated type I receptor phosphorylates the intracellular substrate R-SMAD (Smad 2/3 or Smad 1/5/8) that crosses the nuclear membrane after binding co-SMAD (Smad4) [85,89]. Smad4 is a critical effector of intracellular signaling and, like TGF-β, has a dual role as tumor suppressor and promoter of HCC [96]. Once in the nucleus, the SMAD complex regulates the transcription of TGF-β-induced target genes together with essential transcriptional cofactors. The SMAD complex induces a specific gene signature by the canonical TGF-β signaling pathway [97], which provokes growth arrest and proapoptotic signals in an early stage. Later, proliferative and antiapoptotic responses gain the upper hand by crosstalk with growth signaling. This noncanonical TGF-β pathway includes modulation of EGFR, mitogen-activated protein kinase (MAPK), phosphoinositide 3 kinase (PI3K)/Akt, Ras, and Rho-like small GTPases signaling pathways [98,99]. TGF-β can induce epithelial to mesenchymal transition (EMT) in human primary hepatocytes, a program that promotes cell invasion and metastasis [100]. During EMT, the epithelial cells lose their phenotypic features and gain invasive properties to become mesenchymal cells. Physiologically, EMT is indispensable in the context of embryonic development. However, there is increasing evidence that it also plays a role in pathological conditions, probably contributing to metastatic carcinoma development as well [101].

HCV has developed strategies targeting TGF-β signaling, presumably to maintain a proliferative antiapoptotic signaling environment that stimulate the HCV life cycle and prevent stress-induced cell death. HCV infection induces unfolded protein response (UPR), which upregulates TGF-β expression via nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) [102,103]. Mainly, the HCV core protein seems to modulate TGF-β signaling (i.e., via an interaction with Smad3) [104,105]. However, HCV does not affect the nuclear translocation of the Smad3/4 complex, suggesting a transient nuclear localization of HCV core protein [105]. An interesting hypothesis suggests that chronic infection provokes the selection of protumorigenic HCV variants in the liver, which strongly interfere with TGF-β signaling. This is supported by the isolation of HCV core variants from HCCs that better resist TGF-β-mediated antiproliferative effects and more intensely promote cell transformation compared to HCV core variants isolated from tissue adjacent to the tumor [105]. Beside its association with SMAD, HCV core expression induces endoglin (CD105) expression on the surface of infected hepatocytes. As a component of the TGF-β receptor complex, endoglin abundance stimulates fibrogenesis and promotes tumor growth and metastasis [106]. Endoglin induces inhibitor of DNA binding 1 (ID1) function via stimulation of ALK-1/SMAD1/5 signaling, which acts as proliferative and antiapoptotic and is a central regulator of CSC development [107]. HCV infection or ectopic expression of viral core enhances the expression of ID1-related markers for survival, proliferation, and CSCs (i.e., BCL2, CyclinD1, HES1, NOTCH1, NANOG, and SOX2 proteins) [106]. Furthermore, endoglin is an angiogenesis marker in patients with HCC [108,109].

3. HCV Manipulates Signaling Circuits of Differentiation, Adhesion, and Angiogenesis

A hallmark of HCC development is the dedifferentiation of hepatocytes, which is accompanied by important changes in intracellular communication and nutrients supply. The identification and understanding of stem cell-like cells in cancers has significantly contributed to the current understanding of tumor formation [110]. Even though CSCs share a few key features of normal tissue stem cells (e.g., unlimited proliferative and differentiation ability), they are potentially able to reproduce many of the elements related to cancer initiation, metastasis, and recurrence after therapy [111–113]. For HCC, a rare population of CSCs, called liver cancer stem cells (LCSCs), is abundant in tumor tissues and support self-renewal malignant transformation and resistance to chemotherapy [114].

Several LCSCs markers have been identified that have impact on the signaling circuitry, and some of them have been proposed as therapeutic targets for liver cancer treatment [115].

3.1. HCV Infection of Hepatocytes Provokes Stem Cell-Like Characteristics

During HCV infection, the virus predisposes cells towards the acquisition of CSC characteristics by the dysregulation of several signaling pathways [116,117]. Many of the characteristic CSC markers (i.e., CD133, CD90, CD44, and EpCAM) are also modulators of signaling pathways, including MAPK pathway, TGF-β mediated EMT, Wnt signaling, which are required to maintain CSC properties [115,118–127]. Other CSCs markers, such as doublecortin-like kinase 1 (DCLK1), impact microtubule filaments, polarized polymers of α and β tubulin heterodimers that are essential for cellular transport, cell division, and differentiation. DCLK1 is overexpressed in the liver of patients with HCV-associated HCC, while its level is very low or absent in normal hepatocytes. Interestingly, HCV replication, inflammation, and cirrhosis contribute to DCLK1 accumulation in the perinuclear region of the hepatocytes, where it colocalizes with NS5A and microtubule filaments [117]. This suggests that HCV-induced DCLK1 activity promotes microtubule filament polymerization and stabilization [117,128]. The maintenance of the CSC state is principally driven by reactivation of embryonic differentiation programs. Of these, especially Wnt, Notch, and Hedgehog signaling pathways potentially play a role in HCV-induced carcinogenesis [129–132].

3.2. HCV Causes Wnt Upregulation and β-Catenin Accumulation

Wnt pathway is a crucial component for embryonic development and tissue homeostasis [133]. Activation of the pathway starts when Wnt ligands bind to Frizzled (FZD) receptor, a seven transmembrane protein containing an extracellular cysteine-rich ligand-binding domain. When FZD receptor is activated, it inhibits the degradation of β-catenin. This leads to β-catenin accumulation and translocation to the nucleus, where it activates regulators of cell proliferation [134], such as *WISP-1, c-MYC*, and *CCND1* [135–137]. Absence of FZD stimulation causes degradation of cytosolic β-catenin by a complex that consists of Axin, adenomatous polyposis coli protein (APC), and two serine/threonine kinases (GSK3β and CK1). Moreover, β-catenin potentiates the expression of ΔN-p73, a repressor of p53 and Tap73 proteins, conferring antiapoptotic and chemoresistance proprieties to HCC cells [134,138–140]. Components of the Wnt signaling are frequently mutated in liver cancer [141], which mostly result in β-catenin stabilization [142]. HCV infection manipulates Wnt signaling in multiple ways via its structural and nonstructural viral proteins. Isolated expression of NS5A has been reported to directly promote Wnt signaling by its interaction with PI3K and subsequent activation of Akt. This induces the phosphorylation and inhibition of glycogen synthase kinase 3β (GSK3β), a key component of β-catenin degradation complex [143]. Furthermore, ectopic expression of HCV core protein induces cell proliferation by forcing the expression of *Wnt-1* and its downstream target gene *WISP2*, which induce Wnt signaling [144].

3.3. HCV Enhances Notch Signaling by Coactivating Hes-1 Promoter

Notch signaling suppresses cell differentiation, and it is involved in the maintenance of CSCs [145,146]. Notch ligands and receptors are both EGF-homologous transmembrane proteins mediating intercellular communication, cell proliferation, differentiation, and apoptosis [147]. Its impact on the cell is defined by the cellular microenvironment and its crosstalk with different signaling pathways [148]. To be activated, Notch receptors undergo a sequence of proteolytical cleavage upon interaction to a cell-bound ligand exposed on the surface of neighboring cells. Subsequently, this leads to the release of the Notch intracellular domain (NICD) and its translocation into the nucleus. Nuclear NICD associates with numerous cofactors and repressors, fine-tuning its transcriptional activity [147]. The complex orchestrates transcription of Notch target genes that regulate cell differentiation, such as hairy enhancer of split (*HES1*) [149], HES-related proteins (*HEY*), Notch-regulated ankyrin repeat protein (*NRARP*) [150], cyclin D1 (*CCND1*) [151], *c-MYC* [152–155],

and receptor tyrosine-protein kinase erbB-2 (*ERBB-2*) [156]. In addition, Notch influences inflammation and metabolism by contributing to the activation of NF-κB [157] and peroxisome proliferator-activated receptor (PPAR) [148].

HCV infection interferes with Notch signaling and thereby contributes to hepatocarcinogenesis. Under isolated expression condition, NS3 protein binds to Snf2-related CBP activator protein (SRCAP) and cooperatively enhances Hes-1 promoter activity [158]. This leads to increased Notch-induced *HES1* expression [159], a transcriptional repressor of cell differentiation [160], suggesting that HCV promotes a dedifferentiated CSC-like state of infected hepatocytes.

3.4. HCV-Induced Liver Damage Promotes Hedgehog Signaling

The Hedgehog pathway (Hh) is involved in the regulation of several morphogenic key functions, such as proliferation, survival, migration, and differentiation [161]. The Hedgehog ligands are essential during morphogenesis and embryogenesis processes as well as for the maintenance of stem cell homeostasis during adulthood [162]. Importantly, Hh pathway plays an essential role in adult liver repair and regeneration [163] and is implicated in several types of liver cancer, such as gallbladder cancer [164], cholangiocarcinoma [165–167], hepatoblastoma [168], and HCC [169]. Probably, the production of Hh ligands is favored by the accumulation of liver damage markers (i.e., platelet-derived growth factor (PDGF), TGF-β, and EGF) [170–172].

In patients with viral hepatitis, the Hh pathway is found to be induced [173], which presumably reflects tissue damage and liver regeneration during chronic infection. Interestingly, the permissiveness of cells to HCV replication seems to positively correlate with Hh pathway activity [174], suggesting that liver regeneration and a profibrotic environment may promote HCV infection. This is supported by the identification of additional key regulators of liver regeneration that are activated by HCV infection, including EGFR [17,41,42] and IL-6/STAT3 [175] signaling. Moreover, the presence of Hh activity promotes EMT in crosstalk with TGF-β and Wnt signaling [176], which once more highlights the relevance of EMT induction for HCV and its consequences for HCV-associated liver pathogenesis and HCC development.

3.5. HCV Promotes Angiogenesis via VEGF and HIF-1α Stabilization

Angiogenesis is a complex growth factor-dependent process responsible for the formation of new vessels from existing vascular trees [177,178]. Physiological angiogenesis is maintained by the balance between proangiogenic and antiangiogenic factors [179]. In pathological conditions, new growth in the vascular web is relevant as the proliferation of cancer cells and metastasis depend on a satisfactory source of oxygen and nutrients as well as waste removal from organs and tissues [180]. Several angiogenic growth factors are elevated in HCC patients, i.e., vascular endothelial growth factor A (VEGF-A), angiopoietin-2 and PDGF [181,182]. HCV infection leads to the development of hepatic angiogenesis, which significantly contributes to HCC progression and invasion [183]. This proangiogenic state is reversed in the livers of patients after viral clearance [184]. Vascular endothelial growth factor (VEGF) is a key regulator of angiogenesis in both normal and neoplastic tissues. Its expression and function are modulated by cytokines and other factors, such as the hypoxia-inducible factor 1α (HIF-1α) [182,185,186]. HCV infection leads to the stabilization of HIF-1α, mediated via oxidative stress and the induction of hypoxia [187]. In addition, the activation of PI3-K/Akt, Erk1/2, NF-κB, and STAT3 is necessary for hypoxia-inducible factor 1-alpha (HIF-1α) stabilization, which leads to the stimulation of VEGF [187]. HCV core protein triggers hepatic angiogenesis by a mechanism that involves crosstalk of multiple pathways, which is reflected by altered marker expression for hepatic angiogenesis, including TGF-β2, VEGF, and CD34 expression [185].

4. HCV Tweaks Signaling of the Inflammatory Response

Inflammation is an essential physiological response to several distressing stimuli, including infection. Inflammation is also tightly linked to the mechanisms of tissue regeneration and cancer.

During chronic inflammation, NF-κB and STAT3 are central regulators of liver inflammation and are frequently associated with increased risk of cancer [188,189]. As part of the immune system, NF-κB contributes to the elimination of transformed cells. In support of this, NF-κB activation during the acute inflammatory response is highly associated with cytotoxic immune cell response [190]. The activation of NF-κB is induced by the IκB kinase (IKK) complex, which mediates phosphorylation and proteasomal degradation of IκB. This allows NF-κB dimers to translocate into the nucleus, where they induce an inflammatory and antiapoptotic response [191]. NF-κB is constitutively active in many types of cancer, promoting tumorigenic processes [192–194]. This suggests a dual role of NF-κB as a tumor suppressor and a tumor promoter, depending on the duration and intensity of tissue inflammation. NF-κB is a transcription factor and a central regulator of inflammation and cell survival. In quiescent cells, NF-κB is inactive, blocked by a tight association with inhibitor of NF-κB (IκB). NF-κB is further regulated by post-translational modifications (e.g., phosphorylation), which are important for its activation and crosstalk with other signaling pathways [195]. Moreover, NF-κB activity is influenced by dynamic protein–protein interactions, forming a tight network of feedback loops and interconnections [196]. In addition, STAT3 possesses a dual role as tumor suppressor and oncogene. It is not only a pivotal transcription factor in acute inflammation, but it is also a key element of liver regeneration [197] by regulating cell proliferation, survival, angiogenesis, and chemotaxis [198,199]. STAT3 is induced by a variety of different ligands, including interleukin 6 (IL-6), cardiotrophin-1 (CT-1), leukemia inhibitory factor (LIF), EGF, oncostatin M (OSM), IFN-α, and IFN-β [200]. Engagement of these ligands to their receptors leads to a subsequent recruitment of Janus kinases (JAK1, 2 and 3) and tyrosine kinase 2 (TYK2) that phosphorylate STAT3 [92,201–203]. Once phosphorylated, STAT3 forms homo- or heterodimers with STAT1 or STAT5 that translocate to the nucleus and bind specific DNA sequences. Without a doubt, STAT3 phosphorylation is necessary for its transcriptional activity. However, unphosphorylated STAT3 also presents biological functions, such as the expression of cell cycle progression genes [204,205]. NF-κB and STAT3 signaling are closely linked. NF-κB-mediated inflammation induces hepatic IL-6 production and STAT3 signaling [206]. Activated STAT3 in cancer cells binds to the NF-κB complex proteins RelA/p65 and the histone acetyltransferase p300 in the nucleus. As a consequence, p300 reversibly acetylates RelA/p65 dimers [207], which cause its nuclear retention [208]. At the same time, NF-κB can also impair oxidative stress, which is an activator of STAT3 [209]. In most HCC tumors, however, STAT3 activity does not coincide with NF-κB activation [210].

HCV Affects the STAT3/NF-κB Circuitry to Maintain a Pro-Inflammatory State

One of the most important examples of inflammation-associated cancers is HCC succeeding chronic HCV infection [211]. Compared to HBV infection, where viral genome integration accounts for the majority of HCCs, HCV-induced HCC is linked to liver disease progression from nonalcoholic fatty liver disease (NAFLD), chronic inflammation, fibrosis, and cirrhosis. This therefore suggests that HCV-induced signals promote liver fibrosis and disease progression following a similar disease pattern observed for other aetiologies. Indeed, HCV causes hepatic inflammation and induces complex alterations in host signal transduction [212]. These include deregulation of cytokine, metabolic, and oxidative stress pathways [213]. HCV-encoded proteins also cover an important role in initiating and maintaining this chronic inflammatory state. For instance, NS5A upregulates the expression of cyclooxygenase-2 (COX-2) [213], which promotes chronic inflammation by the synthesis of prostaglandins. It is therefore not surprising that HCV manipulates regulatory signaling of the inflammatory response, including NF-κB [189] and STAT3 [214], and thereby increases the risk of HCC development. HCV induces chronic hepatic inflammation that is mediated by elevated NF-κB activity. However, the question is whether this is simply a consequence of the cellular defense against infection by HCV or whether the virus has an interest in maintaining an inflammatory state for its own benefit. Several lines of evidence suggest that HCV indeed gains from tweaking the outcome of the inflammatory response. For example, HCV infection enhances tumor necrosis

factor alpha (TNF-α)-induced cell death by suppression of NF-κB activation involving a mechanism dependent on core, NS4B, and NS5B [215]. At the same time, HCV makes use of parts of the NF-κB signaling by activating IKKα which, independent of NF-κB, induces the expression of lipogenic genes that contribute to core-associated lipid droplet formation [20]. The same is true for STAT3, which is a mediator of inflammation and part of the interferon response against viral infection. STAT3 transcriptional activity is elevated upon HCV infection in livers of patients and in cell culture [175] and is associated with poor prognosis in HCCs [189]. STAT3 is activated by HCV-induced oxidative stress via core, NS2, and NS3 proteins [216] and by the innate antiviral immune response in hepatocytes [40]. Additionally, the presence of HCV not only affects the infected hepatocytes but equally affects the liver microenvironment. Exosomes secreted from HCV-infected cells carrying miR-19a induce STAT3 activation in hepatic stellate cells and favor fibrotic gene expression [217]. STAT3 activation in the context of HCV infection has also been linked to the presence of myeloid-derived suppressor cells (MDSCs), a cell type that favors the expansion of T_{reg} lymphocytes and has been associated with an increased tumor burden in HCC patients [218]. The question then arises as to whether the elevated STAT3 signaling is simply a consequence of infection or whether it is beneficial to the virus. Interestingly, HCV core protein also directly associates and activates STAT3 function, which promotes cell transformation [219], suggesting an important role of STAT3 for HCV. Indeed, HCV has a vital interest in maintaining a persistent STAT3 signaling as STAT3 is a cofactor for HCV infection and tempers the antiviral impact of the interferon response [40].

5. Clinical Relevance and Perspectives

Chronic HCV infection is a major cause of HCC, the second most deadly cancer worldwide with only very limited treatment options. HCV-related HCC will remain a major health problem for the next decades, despite the recent development of direct-acting antivirals (DAAs) and their deployment in therapy [220]. Especially in patients with advanced liver disease, the HCC risk cannot be fully reversed after viral cure [221]. This is similar to alcohol-induced liver disease, where the HCC risk during abstinence persists for several years [222]. Although the oncogenic mechanism of alcohol and its carcinogenic metabolite acetaldehyde differ from that of viral hepatitis, it has been suggested that, similar to alcohol [223,224], HCV infection may leave an epigenetic footprint in the host genome. An interesting question is whether this also creates persistent alterations in the host signaling network that maintain an oncogenic pressure to the hepatocyte, like an echo from the chronic infection.

Another point worth mentioning is a suggested increase in tumor recurrence rates in HCC patients after DAA-induced sustained virological response and tumor resection [225,226]. However, these results remain controversial as other groups could not confirm this observation [227,228]. Therefore, whether antiviral treatment in HCC patients leads to a long-term survival benefit is currently unknown, and current guidelines suggest a close surveillance and imaging in these patients [229]. The treatment of HCC is particularly challenging for patient cohorts with moderate and severe liver dysfunction (Child–Pugh Class B or C) in term of toxicity and efficacy as the use of sorafenib for the treatment of Child–Pugh B patients has been questioned [230]. Moreover, the HCC proliferative index is low, which is one of the reasons most cytostatics and small molecules are considered inefficient.

By hijacking the host signaling network, HCV generates a proliferative and antiapoptotic environment, which promotes hepatocyte dedifferentiation and EMT. This forms an optimal environment for the virus to persist but with serious consequences to the host. The signaling pathways deregulated by chronic HCV infection resemble the hallmarks of cancer [231,232], suggesting that HCV-induced oncogenic signaling likely contributes to liver disease progression and hepatocarcinogenesis. Targeting signaling components with therapeutic antibodies or clinical kinase inhibitors in cancer therapy is widely established. The current pharmacological therapy for HCC is essentially based on the multikinase inhibitor sorafenib [233], which is able to increase survival rates of selected HCC patients. Other kinase inhibitors clinically tested include linifanib (VEGFR and PDGFR inhibitor) [234] and erlotinib (EGFR inhibitor) [235]; the latter failed in phase 3 of its clinical

trial [236]. The identification of therapeutic targets in established HCCs is difficult because genetic alterations in tumors are highly heterogeneous [237]. Nevertheless, such approach holds promise in the framework of a personalized treatment, and targeting derailed signaling pathways in patients at risk of developing HCCs can be part of novel chemopreventive strategies. In support of this, an important proof-of-concept was demonstrated in 2014 by Bryan Fuchs and colleagues as erlotinib-attenuated fibrogenesis and HCC development in a rat model [26]. Other HCV-modulated signaling pathways (i.e., NF-κB and STAT3) offer interesting opportunities to therapeutic intervention, as well as prevention, especially in the pathological context of HCC [189].

However, this requires new and well-tolerated compounds that allow a long-term administration of kinase inhibitors to patients with advanced liver disease. A deeper understanding of the signaling network of HCV infection will also contribute to a better understanding of general signaling events involved in liver disease progression, given the gene expression profiles in patients at risk of HCC seem to be independent of the underlying aetiology [238]. In future, well-established HCV infection models will be instrumental in highlighting additional deregulated and druggable signaling pathways that are associated with HCC risk. This will help to overcome the lack of appropriate study models of HCC development and contribute to the discovery of novel drivers and drug targets of liver disease and HCC development.

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References

1. Coffin, J.M.; Varmus, H.E.; Bishop, J.M.; Essex, M.; Hardy, W.D., Jr.; Martin, G.S.; Rosenberg, N.E.; Scolnick, E.M.; Weinberg, R.A.; Vogt, P.K. Proposal for naming host cell-derived inserts in retrovirus genomes. *J. Virol.* **1981**, *40*, 953–957. [PubMed]
2. Vogt, P.K. Retroviral oncogenes: A historical primer. *Nat. Rev. Cancer* **2012**, *12*, 639–648. [CrossRef] [PubMed]
3. Luo, G.G.; Ou, J.H. OncogeniC Viruses and cancer. *Virol. Sin.* **2015**, *30*, 83–84. [CrossRef] [PubMed]
4. Lemon, S.M.; McGivern, D.R. Is hepatitis C Virus carcinogenic? *Gastroenterology* **2012**, *142*, 1274–1278. [CrossRef] [PubMed]
5. Banerjee, A.; Ray, R.B.; Ray, R. Oncogenic potential of hepatitis C Virus proteins. *Viruses* **2010**, *2*, 2108–2133. [CrossRef] [PubMed]
6. McGivern, D.R.; Lemon, S.M. Virus-specific mechanisms of carcinogenesis in hepatitis C Virus associated liver cancer. *Oncogene* **2011**, *30*, 1969–1983. [CrossRef] [PubMed]
7. Manns, M.P.; Buti, M.; Gane, E.; Pawlotsky, J.M.; Razavi, H.; Terrault, N.; Younossi, Z. Hepatitis C Virus infection. *Nat. Rev. Dis. Primers* **2017**, *3*, 17006. [CrossRef] [PubMed]
8. Gisbert, J.P.; Garcia-Buey, L.; Pajares, J.M.; Moreno-Otero, R. Prevalence of hepatitis C Virus infection in b-cell non-hodgkin's lymphoma: Systematic review and meta-analysis. *Gastroenterology* **2003**, *125*, 1723–1732. [CrossRef] [PubMed]
9. Su, F.H.; Chang, S.N.; Chen, P.C.; Sung, F.C.; Huang, S.F.; Chiou, H.Y.; Su, C.T.; Lin, C.C.; Yeh, C.C. Positive association between hepatitis c infection and oral cavity cancer: A nationwide population-based cohort study in taiwan. *PLoS ONE* **2012**, *7*, e48109. [CrossRef] [PubMed]
10. Mahale, P.; Sturgis, E.M.; Tweardy, D.J.; Ariza-Heredia, E.J.; Torres, H.A. Association between hepatitis C Virus and head and neck cancers. *J. Natl. Cancer Inst.* **2016**, *108*, djw035. [CrossRef] [PubMed]
11. El-Serag, H.B.; Engels, E.A.; Landgren, O.; Chiao, E.; Henderson, L.; Amaratunge, H.C.; Giordano, T.P. Risk of hepatobiliary and pancreatic cancers after hepatitis C Virus infection: A population-based study of U.S. Veterans. *Hepatology* **2009**, *49*, 116–123. [CrossRef] [PubMed]

12. Huang, J.; Magnusson, M.; Torner, A.; Ye, W.; Duberg, A.S. Risk of pancreatic cancer among individuals with hepatitis c or hepatitis b virus infection: A nationwide study in sweden. *Br. J. Cancer* **2013**, *109*, 2917–2923. [CrossRef] [PubMed]

13. Gordon, S.C.; Moonka, D.; Brown, K.A.; Rogers, C.; Huang, M.A.; Bhatt, N.; Lamerato, L. Risk for renal cell carcinoma in chronic hepatitis c infection. *Cancer Epidemiol. Biomark. Prev.* **2010**, *19*, 1066–1073. [CrossRef] [PubMed]

14. Mahale, P.; Torres, H.A.; Kramer, J.R.; Hwang, L.Y.; Li, R.; Brown, E.L.; Engels, E.A. Hepatitis C Virus infection and the risk of cancer among elderly us adults: A registry-based case-control study. *Cancer* **2017**, *123*, 1202–1211. [CrossRef] [PubMed]

15. Nyberg, A.H. The association of extrahepatic cancers with chronic hepatitis C Virus infection. *Gastroenterol. Hepatol. (N. Y.)* **2016**, *12*, 185–187.

16. Pol, S.; Vallet-Pichard, A.; Hermine, O. Extrahepatic cancers and chronic HCV infection. *Nat. Rev. Gastroenterol. Hepatol.* **2018**, *15*, 283–290. [CrossRef] [PubMed]

17. Lupberger, J.; Zeisel, M.B.; Xiao, F.; Thumann, C.; Fofana, I.; Zona, L.; Davis, C.; Mee, C.J.; Turek, M.; Gorke, S.; et al. Egfr and EPHA2 are host factors for hepatitis C Virus entry and possible targets for antiviral therapy. *Nat. Med.* **2011**, *17*, 589–595. [CrossRef] [PubMed]

18. Lupberger, J.; Casanova, C.; Fischer, B.; Weiss, A.; Fofana, I.; Fontaine, N.; Fujiwara, T.; Renaud, M.; Kopp, A.; Schuster, C.; et al. PI4K-β and MKNK1 are regulators of hepatitis C Virus IRES-dependent translation. *Sci. Rep.* **2015**, *5*, 13344. [CrossRef] [PubMed]

19. Paul, D.; Madan, V.; Bartenschlager, R. Hepatitis C Virus RNA replication and assembly: Living on the fat of the land. *Cell Host Microbe* **2014**, *16*, 569–579. [CrossRef] [PubMed]

20. Li, Q.; Zhang, Y.Y.; Chiu, S.; Hu, Z.; Lan, K.H.; Cha, H.; Sodroski, C.; Zhang, F.; Hsu, C.S.; Thomas, E.; et al. Integrative functional genomics of hepatitis C Virus infection identifies host dependencies in complete viral replication cycle. *PLoS Pathog.* **2014**, *10*, e1004163. [CrossRef] [PubMed]

21. Zona, L.; Lupberger, J.; Sidahmed-Adrar, N.; Thumann, C.; Harris, H.J.; Barnes, A.; Florentin, J.; Tawar, R.G.; Xiao, F.; Turek, M.; et al. Hras signal transduction promotes hepatitis C Virus cell entry by triggering assembly of the host tetraspanin receptor complex. *Cell Host Microbe* **2013**, *13*, 302–313. [CrossRef] [PubMed]

22. Sherr, C.J.; McCormick, F. The RB and p53 pathways in cancer. *Cancer Cell* **2002**, *2*, 103–112. [CrossRef]

23. Sherr, C.J. Cancer cell cycles. *Science* **1996**, *274*, 1672–1677. [CrossRef] [PubMed]

24. Sever, R.; Brugge, J.S. Signal transduction in cancer. *Cold Spring Harb. Perspect. Med.* **2015**, *5*, a006098. [CrossRef] [PubMed]

25. Witsch, E.; Sela, M.; Yarden, Y. Roles for growth factors in cancer progression. *Physiology (Bethesda)* **2010**, *25*, 85–101. [CrossRef] [PubMed]

26. Fuchs, B.C.; Hoshida, Y.; Fujii, T.; Wei, L.; Yamada, S.; Lauwers, G.Y.; McGinn, C.M.; DePeralta, D.K.; Chen, X.; Kuroda, T.; et al. Epidermal growth factor receptor inhibition attenuates liver fibrosis and development of hepatocellular carcinoma. *Hepatology* **2014**, *59*, 1577–1590. [CrossRef] [PubMed]

27. Pennycuick, A.; Villanueva, A. *Signaling in Hepatocellular Carcinoma*, 3rd ed.; John Wiley & Sons: Hoboken, NJ, USA, 2015.

28. Ferguson, K.M. Structure-based view of epidermal growth factor receptor regulation. *Annu. Rev. Biophys.* **2008**, *37*, 353–373. [CrossRef] [PubMed]

29. Citri, A.; Yarden, Y. EGF-ERBB signalling: Towards the systems level. *Nat. Rev. Mol. Cell Biol.* **2006**, *7*, 505–516. [CrossRef] [PubMed]

30. Yarden, Y.; Sliwkowski, M.X. Untangling the ERBB signalling network. *Nat. Rev. Mol. Cell Biol.* **2001**, *2*, 127–137. [CrossRef] [PubMed]

31. Bublil, E.M.; Yarden, Y. The EGF receptor family: Spearheading a merger of signaling and therapeutics. *Curr. Opin. Cell Biol.* **2007**, *19*, 124–134. [CrossRef] [PubMed]

32. Hynes, N.E.; Lane, H.A. ERBB receptors and cancer: The complexity of targeted inhibitors. *Nat. Rev. Cancer* **2005**, *5*, 341–354. [CrossRef] [PubMed]

33. Mendelsohn, J.; Baselga, J. Epidermal growth factor receptor targeting in cancer. *Semin. Oncol.* **2006**, *33*, 369–385. [CrossRef] [PubMed]

34. Salomon, D.S.; Kim, N.; Saeki, T.; Ciardiello, F. Transforming growth factor-alpha: An oncodevelopmental growth factor. *Cancer Cells* **1990**, *2*, 389–397. [PubMed]

35. Sunnerhagen, M.S.; Persson, E.; Dahlqvist, I.; Drakenberg, T.; Stenflo, J.; Mayhew, M.; Robin, M.; Handford, P.; Tilley, J.W.; Campbell, I.D.; et al. The effect of aspartate hydroxylation on calcium binding to epidermal growth factor-like modules in coagulation factors IX and X. *J. Biol. Chem.* **1993**, *268*, 23339–23344. [PubMed]

36. Huang, W.C.; Chan, S.H.; Jang, T.H.; Chang, J.W.; Ko, Y.C.; Yen, T.C.; Chiang, S.L.; Chiang, W.F.; Shieh, T.Y.; Liao, C.T.; et al. MiRNA-491-5p and GIT1 serve as modulators and biomarkers for oral squamous cell carcinoma invasion and metastasis. *Cancer Res.* **2014**, *74*, 751–764. [CrossRef] [PubMed]

37. Ito, Y.; Takeda, T.; Sasaki, Y.; Sakon, M.; Yamada, T.; Ishiguro, S.; Imaoka, S.; Tsujimoto, M.; Higashiyama, S.; Monden, M.; et al. Expression and clinical significance of the ERBB family in intrahepatic cholangiocellular carcinoma. *Pathol. Res. Pract.* **2001**, *197*, 95–100. [CrossRef] [PubMed]

38. Zheng, K.; Kitazato, K.; Wang, Y. Viruses exploit the function of epidermal growth factor receptor. *Rev. Med. Virol.* **2014**, *24*, 274–286. [CrossRef] [PubMed]

39. Baktash, Y.; Madhav, A.; Coller, K.E.; Randall, G. Single particle imaging of polarized hepatoma organoids upon hepatitis C Virus infection reveals an ordered and sequential entry process. *Cell Host Microbe* **2018**, *23*, 382–394. [CrossRef] [PubMed]

40. Lupberger, J.; Duong, F.H.; Fofana, I.; Zona, L.; Xiao, F.; Thumann, C.; Durand, S.C.; Pessaux, P.; Zeisel, M.B.; Heim, M.H.; et al. Epidermal growth factor receptor signaling impairs the antiviral activity of interferon-α. *Hepatology* **2013**, *58*, 1225–1235. [CrossRef] [PubMed]

41. Diao, J.; Pantua, H.; Ngu, H.; Komuves, L.; Diehl, L.; Schaefer, G.; Kapadia, S.B. Hepatitis C Virus induces epidermal growth factor receptor activation via CD81 binding for viral inteRNAlization and entry. *J. Virol.* **2012**, *86*, 10935–10949. [CrossRef] [PubMed]

42. Mailly, L.; Xiao, F.; Lupberger, J.; Wilson, G.K.; Aubert, P.; Duong, F.H.T.; Calabrese, D.; Leboeuf, C.; Fofana, I.; Thumann, C.; et al. Clearance of persistent hepatitis C Virus infection in humanized mice using a claudin-1-targeting monoclonal antibody. *Nat. Biotechnol.* **2015**, *33*, 549–554. [CrossRef] [PubMed]

43. Igloi, Z.; Kazlauskas, A.; Saksela, K.; Macdonald, A.; Mankouri, J.; Harris, M. Hepatitis C Virus NS5A protein blocks epidermal growth factor receptor degradation via a proline motif- dependent interaction. *J. Gen. Virol.* **2015**, *96*, 2133–2144. [CrossRef] [PubMed]

44. Luong, T.T.D.; Tran, G.V.Q.; Shin, D.J.; Lim, Y.S.; Hwang, S.B. Hepatitis C Virus exploits death receptor 6-mediated signaling pathway to facilitate viral propagation. *Sci. Rep.* **2017**, *7*, 6445. [CrossRef] [PubMed]

45. Kim, S.J.; Syed, G.H.; Khan, M.; Chiu, W.W.; Sohail, M.A.; Gish, R.G.; Siddiqui, A. Hepatitis C Virus triggers mitochondrial fission and attenuates apoptosis to promote viral persistence. *Proc. Natl. Acad. Sci. USA* **2014**, *111*, 6413–6418. [CrossRef] [PubMed]

46. Dyson, N. The regulation of E2F by pRB-family proteins. *Genes Dev.* **1998**, *12*, 2245–2262. [CrossRef] [PubMed]

47. Nevins, J.R. The RB/E2F pathway and cancer. *Hum. Mol. Genet.* **2001**, *10*, 699–703. [CrossRef] [PubMed]

48. Trimarchi, J.M.; Lees, J.A. Sibling rivalry in the E2F family. *Nat. Rev. Mol. Cell Biol.* **2002**, *3*, 11–20. [CrossRef] [PubMed]

49. Ren, B.; Cam, H.; Takahashi, Y.; Volkert, T.; Terragni, J.; Young, R.A.; Dynlacht, B.D. E2F integrates cell cycle progression with DNA repair, replication, and G(2)/M checkpoints. *Genes Dev.* **2002**, *16*, 245–256. [CrossRef] [PubMed]

50. Harbour, J.W.; Dean, D.C. The RB/E2F pathway: Expanding roles and emerging paradigms. *Genes Dev.* **2000**, *14*, 2393–2409. [CrossRef] [PubMed]

51. Rayman, J.B.; Takahashi, Y.; Indjeian, V.B.; Dannenberg, J.H.; Catchpole, S.; Watson, R.J.; Te Riele, H.; Dynlacht, B.D. E2F mediates cell cycle-dependent transcriptional repression in vivo by recruitment of an HDAC1/MSIN3B corepressor complex. *Genes Dev.* **2002**, *16*, 933–947. [CrossRef] [PubMed]

52. Ogawa, H.; Ishiguro, K.; Gaubatz, S.; Livingston, D.M.; Nakatani, Y. A complex with chromatin modifiers that occupies E2F- and MYC-responsive genes in g0 cells. *Science* **2002**, *296*, 1132–1136. [CrossRef] [PubMed]

53. Levine, A.J. P53, the cellular gatekeeper for growth and division. *Cell* **1997**, *88*, 323–331. [CrossRef]

54. El-Deiry, W.S.; Tokino, T.; Velculescu, V.E.; Levy, D.B.; Parsons, R.; Trent, J.M.; Lin, D.; Mercer, W.E.; Kinzler, K.W.; Vogelstein, B. Waf1, a potential mediator of p53 tumor suppression. *Cell* **1993**, *75*, 817–825. [CrossRef]

55. Whyte, P.; Buchkovich, K.J.; Horowitz, J.M.; Friend, S.H.; Raybuck, M.; Weinberg, R.A.; Harlow, E. Association between an oncogene and an anti-oncogene: The adenovirus E1A proteins bind to the retinoblastoma gene product. *Nature* **1988**, *334*, 124–129. [CrossRef] [PubMed]

56. Dyson, N.; Howley, P.M.; Munger, K.; Harlow, E. The human papilloma virus-16 E7 oncoprotein is able to bind to the retinoblastoma gene product. *Science* **1989**, *243*, 934–937. [CrossRef] [PubMed]

57. Munakata, T.; Liang, Y.; Kim, S.; McGivern, D.R.; Huibregtse, J.; Nomoto, A.; Lemon, S.M. Hepatitis C Virus induces E6AP-dependent degradation of the retinoblastoma protein. *PLoS Pathog.* **2007**, *3*, 1335–1347. [CrossRef] [PubMed]

58. McGivern, D.R.; Villanueva, R.A.; Chinnaswamy, S.; Kao, C.C.; Lemon, S.M. Impaired replication of hepatitis C Virus containing mutations in a conserved NS5B retinoblastoma protein-binding motif. *J. Virol.* **2009**, *83*, 7422–7433. [CrossRef] [PubMed]

59. Munakata, T.; Nakamura, M.; Liang, Y.; Li, K.; Lemon, S.M. Down-regulation of the retinoblastoma tumor suppressor by the hepatitis C Virus NS5B RNA-dependent RNA polymerase. *Proc. Natl. Acad. Sci. USA* **2005**, *102*, 18159–18164. [CrossRef] [PubMed]

60. Cho, J.; Baek, W.; Yang, S.; Chang, J.; Sung, Y.C.; Suh, M. HCV core protein modulates RB pathway through pRB down-regulation and E2F-1 up-regulation. *Biochim. Biophys. Acta* **2001**, *1538*, 59–66. [CrossRef]

61. Hussain, S.P.; Schwank, J.; Staib, F.; Wang, X.W.; Harris, C.C. Tp53 mutations and hepatocellular carcinoma: Insights into the etiology and pathogenesis of liver cancer. *Oncogene* **2007**, *26*, 2166. [CrossRef] [PubMed]

62. Olivier, M.; Hussain, S.P.; Caron de Fromentel, C.; Hainaut, P.; Harris, C.C. Tp53 mutation spectra and load: A tool for generating hypotheses on the etiology of cancer. *IARC Sci. Publ.* **2004**, 247–270.

63. Sato, Y.; Tsurumi, T. Genome guardian p53 and viral infections. *Rev. Med. Virol.* **2013**, *23*, 213–220. [CrossRef] [PubMed]

64. Lu, W.; Lo, S.Y.; Chen, M.; Wu, K.; Fung, Y.K.; Ou, J.H. Activation of p53 tumor suppressor by hepatitis C Virus core protein. *Virology* **1999**, *264*, 134–141. [CrossRef] [PubMed]

65. Otsuka, M.; Kato, N.; Lan, K.; Yoshida, H.; Kato, J.; Goto, T.; Shiratori, Y.; Omata, M. Hepatitis C Virus core protein enhances p53 function through augmentation of DNA binding affinity and transcriptional ability. *J. Biol. Chem.* **2000**, *275*, 34122–34130. [CrossRef] [PubMed]

66. Ray, R.B.; Steele, R.; Meyer, K.; Ray, R. Hepatitis C Virus core protein represses p21WAF1/CIP1/SID1 promoter activity. *Gene* **1998**, *208*, 331–336. [CrossRef]

67. Kao, C.F.; Chen, S.Y.; Chen, J.Y.; Wu Lee, Y.H. Modulation of p53 transcription regulatory activity and post-translational modification by hepatitis C Virus core protein. *Oncogene* **2004**, *23*, 2472–2483. [CrossRef] [PubMed]

68. Deng, L.; Nagano-Fujii, M.; Tanaka, M.; Nomura-Takigawa, Y.; Ikeda, M.; Kato, N.; Sada, K.; Hotta, H. NS3 protein of hepatitis C Virus associates with the tumour suppressor p53 and inhibits its function in an NS3 sequence-dependent manner. *J. Gen. Virol.* **2006**, *87*, 1703–1713. [CrossRef] [PubMed]

69. Tanaka, M.; Nagano-Fujii, M.; Deng, L.; Ishido, S.; Sada, K.; Hotta, H. Single-point mutations of hepatitis C Virus NS3 that impair p53 interaction and anti-apoptotic activity of NS3. *Biochem. Biophys. Res. Commun.* **2006**, *340*, 792–799. [CrossRef] [PubMed]

70. Lan, K.H.; Sheu, M.L.; Hwang, S.J.; Yen, S.H.; Chen, S.Y.; Wu, J.C.; Wang, Y.J.; Kato, N.; Omata, M.; Chang, F.Y.; et al. Hcv NS5A interacts with p53 and inhibits p53-mediated apoptosis. *Oncogene* **2002**, *21*, 4801–4811. [CrossRef] [PubMed]

71. You, L.R.; Chen, C.M.; Yeh, T.S.; Tsai, T.Y.; Mai, R.T.; Lin, C.H.; Lee, Y.H. Hepatitis C Virus core protein interacts with cellular putative RNA helicase. *J. Virol.* **1999**, *73*, 2841–2853. [PubMed]

72. Mamiya, N.; Worman, H.J. Hepatitis C Virus core protein binds to a dead box RNA helicase. *J. Biol. Chem.* **1999**, *274*, 15751–15756. [CrossRef] [PubMed]

73. Owsianka, A.M.; Patel, A.H. Hepatitis C Virus core protein interacts with a human dead box protein DDX3. *Virology* **1999**, *257*, 330–340. [CrossRef] [PubMed]

74. Chao, C.H.; Chen, C.M.; Cheng, P.L.; Shih, J.W.; Tsou, A.P.; Lee, Y.H. DDX3, a dead box RNA helicase with tumor growth-suppressive property and transcriptional regulation activity of the p21WAF1/CIP1 promoter, is a candidate tumor suppressor. *Cancer Res.* **2006**, *66*, 6579–6588. [CrossRef] [PubMed]

75. Goh, P.Y.; Tan, Y.J.; Lim, S.P.; Tan, Y.H.; Lim, S.G.; Fuller-Pace, F.; Hong, W. Cellular RNA helicase p68 relocalization and interaction with the hepatitis C Virus (HCV) NS5B protein and the potential role of p68 in hcv RNA replication. *J. Virol.* **2004**, *78*, 5288–5298. [CrossRef] [PubMed]

76. Nicol, S.M.; Bray, S.E.; Black, H.D.; Lorimore, S.A.; Wright, E.G.; Lane, D.P.; Meek, D.W.; Coates, P.J.; Fuller-Pace, F.V. The RNA helicase p68 (DDX5) is selectively required for the induction of p53-dependent p21 expression and cell-cycle arrest after DNA damage. *Oncogene* **2013**, *32*, 3461–3469. [CrossRef] [PubMed]

77. Bates, G.J.; Nicol, S.M.; Wilson, B.J.; Jacobs, A.M.; Bourdon, J.C.; Wardrop, J.; Gregory, D.J.; Lane, D.P.; Perkins, N.D.; Fuller-Pace, F.V. The dead box protein p68: A novel transcriptional coactivator of the p53 tumour suppressor. *EMBO J.* **2005**, *24*, 543–553. [CrossRef] [PubMed]

78. Bressac, B.; Galvin, K.M.; Liang, T.J.; Isselbacher, K.J.; Wands, J.R.; Ozturk, M. Abnormal structure and expression of p53 gene in human hepatocellular carcinoma. *Proc. Natl. Acad. Sci. USA* **1990**, *87*, 1973–1977. [CrossRef] [PubMed]

79. Kawabata, M.; Imamura, T.; Miyazono, K. Signal transduction by bone morphogenetic proteins. *Cytokine Growth Factor Rev.* **1998**, *9*, 49–61. [CrossRef]

80. Chaouchi, N.; Arvanitakis, L.; Auffredou, M.T.; Blanchard, D.A.; Vazquez, A.; Sharma, S. Characterization of transforming growth factor-β 1 induced apoptosis in normal human b cells and lymphoma b cell lines. *Oncogene* **1995**, *11*, 1615–1622. [PubMed]

81. Lomo, J.; Blomhoff, H.K.; Beiske, K.; Stokke, T.; Smeland, E.B. TGF-β 1 and cyclic amp promote apoptosis in resting human b lymphocytes. *J. Immunol.* **1995**, *154*, 1634–1643. [PubMed]

82. Wahl, S.M.; Hunt, D.A.; Wong, H.L.; Dougherty, S.; McCartney-Francis, N.; Wahl, L.M.; Ellingsworth, L.; Schmidt, J.A.; Hall, G.; Roberts, A.B.; et al. Transforming growth factor-β is a potent immunosuppressive agent that inhibits Il-1-dependent lymphocyte proliferation. *J. Immunol.* **1988**, *140*, 3026–3032. [PubMed]

83. Roberts, A.B.; Sporn, M.B. Physiological actions and clinical applications of transforming growth factor-β (TGF-β). *Growth Factors* **1993**, *8*, 1–9. [CrossRef] [PubMed]

84. Roberts, A.B. Molecular and cell biology of TGF-β. *Miner. Electrolyte Metab.* **1998**, *24*, 111–119. [CrossRef] [PubMed]

85. Miyazawa, K.; Shinozaki, M.; Hara, T.; Furuya, T.; Miyazono, K. Two major SMAD pathways in TGF-β superfamily signalling. *Genes Cells* **2002**, *7*, 1191–1204. [CrossRef] [PubMed]

86. Meindl-Beinker, N.M.; Matsuzaki, K.; Dooley, S. TGF-β signaling in onset and progression of hepatocellular carcinoma. *Dig. Dis.* **2012**, *30*, 514–523. [CrossRef] [PubMed]

87. Branton, M.H.; Kopp, J.B. TGF-β and fibrosis. *Microbes Infect.* **1999**, *1*, 1349–1365. [CrossRef]

88. Dooley, S.; ten Dijke, P. TGF-β in progression of liver disease. *Cell Tissue Res.* **2012**, *347*, 245–256. [CrossRef] [PubMed]

89. Fabregat, I.; Moreno-Caceres, J.; Sanchez, A.; Dooley, S.; Dewidar, B.; Giannelli, G.; Ten Dijke, P.; Consortium, I.-L. TGF-β signalling and liver disease. *FEBS J.* **2016**, *283*, 2219–2232. [CrossRef] [PubMed]

90. Massague, J. TGFβ in cancer. *Cell* **2008**, *134*, 215–230. [CrossRef] [PubMed]

91. Giannelli, G.; Rani, B.; Dituri, F.; Cao, Y.; Palasciano, G. Moving towards personalised therapy in patients with hepatocellular carcinoma: The role of the microenvironment. *Gut* **2014**, *63*, 1668–1676. [CrossRef] [PubMed]

92. Wrana, J.L.; Attisano, L.; Wieser, R.; Ventura, F.; Massague, J. Mechanism of activation of the TGF-β receptor. *Nature* **1994**, *370*, 341–347. [CrossRef] [PubMed]

93. Heldin, C.H.; Miyazono, K.; ten Dijke, P. TGF-β signalling from cell membrane to nucleus through smad proteins. *Nature* **1997**, *390*, 465–471. [CrossRef] [PubMed]

94. Massaous, J.; Hata, A. TGF-β signalling through the SMAD pathway. *Trends Cell Biol.* **1997**, *7*, 187–192. [CrossRef]

95. Schuster, N.; Krieglstein, K. Mechanisms of TGF-β-mediated apoptosis. *Cell Tissue Res.* **2002**, *307*, 1–14. [CrossRef] [PubMed]

96. HeRNAnda, P.Y.; Chen, K.; Das, A.M.; Sideras, K.; Wang, W.; Li, J.; Cao, W.; Bots, S.J.; Kodach, L.L.; de Man, R.A.; et al. Smad4 exerts a tumor-promoting role in hepatocellular carcinoma. *Oncogene* **2015**, *34*, 5055–5068. [CrossRef] [PubMed]

97. Massague, J. TGFβ signalling in context. *Nat. Rev. Mol. Cell Biol.* **2012**, *13*, 616–630. [CrossRef] [PubMed]

98. Moustakas, A.; Heldin, C.H. Non-smad TGF-β signals. *J. Cell Sci.* **2005**, *118*, 3573–3584. [CrossRef] [PubMed]

99. Zhang, Y.E. Non-smad pathways in TGF-β signaling. *Cell Res.* **2009**, *19*, 128–139. [CrossRef] [PubMed]

100. Battaglia, S.; Benzoubir, N.; Nobilet, S.; Charneau, P.; Samuel, D.; Zignego, A.L.; Atfi, A.; Brechot, C.; Bourgeade, M.F. Liver cancer-derived hepatitis C Virus core proteins shift TGF-β responses from tumor suppression to epithelial-mesenchymal transition. *PLoS ONE* **2009**, *4*, e4355. [CrossRef] [PubMed]

101. Thiery, J.P.; Sleeman, J.P. Complex networks orchestrate epithelial-mesenchymal transitions. *Nat. Rev. Mol. Cell Biol.* **2006**, *7*, 131–142. [CrossRef] [PubMed]

102. Chusri, P.; Kumthip, K.; Hong, J.; Zhu, C.; Duan, X.; Jilg, N.; Fusco, D.N.; Brisac, C.; Schaefer, E.A.; Cai, D.; et al. Hcv induces transforming growth factor β1 through activation of endoplasmic reticulum stress and the unfolded protein response. *Sci. Rep.* **2016**, *6*, 22487. [CrossRef] [PubMed]

103. Asselah, T.; Bieche, I.; Mansouri, A.; Laurendeau, I.; Cazals-Hatem, D.; Feldmann, G.; Bedossa, P.; Paradis, V.; Martinot-Peignoux, M.; Lebrec, D.; et al. In vivo hepatic endoplasmic reticulum stress in patients with chronic hepatitis C. *J. Pathol.* **2010**, *221*, 264–274. [CrossRef] [PubMed]

104. Cheng, P.L.; Chang, M.H.; Chao, C.H.; Lee, Y.H. Hepatitis c viral proteins interact with SMAD3 and differentially regulate TGF-β/SMAD3-mediated transcriptional activation. *Oncogene* **2004**, *23*, 7821–7838. [CrossRef] [PubMed]

105. Pavio, N.; Battaglia, S.; Boucreux, D.; Arnulf, B.; Sobesky, R.; Hermine, O.; Brechot, C. Hepatitis C Virus core variants isolated from liver tumor but not from adjacent non-tumor tissue interact with SMAD3 and inhibit the TGF-β pathway. *Oncogene* **2005**, *24*, 6119–6132. [CrossRef] [PubMed]

106. Kwon, Y.C.; Sasaki, R.; Meyer, K.; Ray, R. Hepatitis C Virus core protein modulates endoglin (CD105) signaling pathway for liver pathogenesis. *J. Virol.* **2017**, *91*, e01235–17. [CrossRef] [PubMed]

107. Lasorella, A.; Benezra, R.; Iavarone, A. The id proteins: Master regulators of cancer stem cells and tumour aggressiveness. *Nat. Rev. Cancer* **2014**, *14*, 77–91. [CrossRef] [PubMed]

108. Yao, Y.; Pan, Y.; Chen, J.; Sun, X.; Qiu, Y.; Ding, Y. Endoglin (CD105) expression in angiogenesis of primary hepatocellular carcinomas: Analysis using tissue microarrays and comparisons with CD34 and VEGF. *Ann. Clin. Lab. Sci.* **2007**, *37*, 39–48. [PubMed]

109. Ho, J.W.; Poon, R.T.; Sun, C.K.; Xue, W.C.; Fan, S.T. Clinicopathological and prognostic implications of endoglin (CD105) expression in hepatocellular carcinoma and its adjacent non-tumorous liver. *World J. Gastroenterol.* **2005**, *11*, 176–181. [CrossRef] [PubMed]

110. Bomken, S.; Fiser, K.; Heidenreich, O.; Vormoor, J. Understanding the cancer stem cell. *Br. J. Cancer* **2010**, *103*, 439–445. [CrossRef] [PubMed]

111. Marquardt, J.U.; Factor, V.M.; Thorgeirsson, S.S. Epigenetic regulation of cancer stem cells in liver cancer: Current concepts and clinical implications. *J. Hepatol.* **2010**, *53*, 568–577. [CrossRef] [PubMed]

112. Shi, G.M.; Xu, Y.; Fan, J.; Zhou, J.; Yang, X.R.; Qiu, S.J.; Liao, Y.; Wu, W.Z.; Ji, Y.; Ke, A.W.; et al. Identification of side population cells in human hepatocellular carcinoma cell lines with stepwise metastatic potentials. *J. Cancer Res. Clin. Oncol.* **2008**, *134*, 1155–1163. [CrossRef] [PubMed]

113. Alison, M.R. Liver stem cells: Implications for hepatocarcinogenesis. *Stem Cell Rev.* **2005**, *1*, 253–260. [CrossRef]

114. Song, K.; Wu, J.; Jiang, C. Dysregulation of signaling pathways and putative biomarkers in liver cancer stem cells (review). *Oncol. Rep.* **2013**, *29*, 3–12. [CrossRef] [PubMed]

115. Nio, K.; Yamashita, T.; Kaneko, S. The evolving concept of liver cancer stem cells. *Mol. Cancer* **2017**, *16*, 4. [CrossRef] [PubMed]

116. Kwon, Y.C.; Bose, S.K.; Steele, R.; Meyer, K.; Di Bisceglie, A.M.; Ray, R.B.; Ray, R. Promotion of cancer stem-like cell properties in hepatitis C Virus-infected hepatocytes. *J. Virol.* **2015**, *89*, 11549–11556. [CrossRef] [PubMed]

117. Ali, N.; Allam, H.; May, R.; Sureban, S.M.; Bronze, M.S.; Bader, T.; Umar, S.; Anant, S.; Houchen, C.W. Hepatitis C Virus-induced cancer stem cell-like signatures in cell culture and murine tumor xenografts. *J. Virol.* **2011**, *85*, 12292–12303. [CrossRef] [PubMed]

118. Ma, S.; Chan, K.W.; Hu, L.; Lee, T.K.; Wo, J.Y.; Ng, I.O.; Zheng, B.J.; Guan, X.Y. Identification and characterization of tumorigenic liver cancer stem/progenitor cells. *Gastroenterology* **2007**, *132*, 2542–2556. [CrossRef] [PubMed]

119. Yin, S.; Li, J.; Hu, C.; Chen, X.; Yao, M.; Yan, M.; Jiang, G.; Ge, C.; Xie, H.; Wan, D.; et al. CD133 positive hepatocellular carcinoma cells possess high capacity for tumorigenicity. *Int. J. Cancer* **2007**, *120*, 1444–1450. [CrossRef] [PubMed]

120. Tang, K.H.; Ma, S.; Lee, T.K.; Chan, Y.P.; Kwan, P.S.; Tong, C.M.; Ng, I.O.; Man, K.; To, K.F.; Lai, P.B.; et al. CD133(+) liver tumor-initiating cells promote tumor angiogenesis, growth, and self-renewal through neurotensin/interleukin-8/CXCl1 signaling. *Hepatology* **2012**, *55*, 807–820. [CrossRef] [PubMed]

121. Yang, Z.F.; Ho, D.W.; Ng, M.N.; Lau, C.K.; Yu, W.C.; Ngai, P.; Chu, P.W.; Lam, C.T.; Poon, R.T.; Fan, S.T. Significance of CD90+ cancer stem cells in human liver cancer. *Cancer Cell* **2008**, *13*, 153–166. [CrossRef] [PubMed]

122. Yang, Z.F.; Ngai, P.; Ho, D.W.; Yu, W.C.; Ng, M.N.; Lau, C.K.; Li, M.L.; Tam, K.H.; Lam, C.T.; Poon, R.T.; et al. Identification of local and circulating cancer stem cells in human liver cancer. *Hepatology* **2008**, *47*, 919–928. [CrossRef] [PubMed]

123. Zhu, Z.; Hao, X.; Yan, M.; Yao, M.; Ge, C.; Gu, J.; Li, J. Cancer stem/progenitor cells are highly enriched in CD133+CD44+ population in hepatocellular carcinoma. *Int. J. Cancer* **2010**, *126*, 2067–2078. [CrossRef] [PubMed]

124. Mima, K.; Okabe, H.; Ishimoto, T.; Hayashi, H.; Nakagawa, S.; Kuroki, H.; Watanabe, M.; Beppu, T.; Tamada, M.; Nagano, O.; et al. CD44s regulates the TGF-β-mediated mesenchymal phenotype and is associated with poor prognosis in patients with hepatocellular carcinoma. *Cancer Res.* **2012**, *72*, 3414–3423. [CrossRef] [PubMed]

125. Yamashita, T.; Honda, M.; Nakamoto, Y.; Baba, M.; Nio, K.; Hara, Y.; Zeng, S.S.; Hayashi, T.; Kondo, M.; Takatori, H.; et al. Discrete nature of epcam+ and CD90+ cancer stem cells in human hepatocellular carcinoma. *Hepatology* **2013**, *57*, 1484–1497. [CrossRef] [PubMed]

126. Yamashita, T.; Budhu, A.; Forgues, M.; Wang, X.W. Activation of hepatic stem cell marker epcam by wnt-β-catenin signaling in hepatocellular carcinoma. *Cancer Res.* **2007**, *67*, 10831–10839. [CrossRef] [PubMed]

127. Yamashita, T.; Ji, J.; Budhu, A.; Forgues, M.; Yang, W.; Wang, H.Y.; Jia, H.; Ye, Q.; Qin, L.X.; Wauthier, E.; et al. Epcam-positive hepatocellular carcinoma cells are tumor-initiating cells with stem/progenitor cell features. *Gastroenterology* **2009**, *136*, 1012–1024. [CrossRef] [PubMed]

128. Roohvand, F.; Maillard, P.; Lavergne, J.P.; Boulant, S.; Walic, M.; Andreo, U.; Goueslain, L.; Helle, F.; Mallet, A.; McLauchlan, J.; et al. Initiation of hepatitis C Virus infection requires the dynamic microtubule network: Role of the viral nucleocapsid protein. *J. Biol. Chem.* **2009**, *284*, 13778–13791. [CrossRef] [PubMed]

129. Wang, W.; Pan, Q.; Fuhler, G.M.; Smits, R.; Peppelenbosch, M.P. Action and function of WNT/β-catenin signaling in the progression from chronic hepatitis C to hepatocellular carcinoma. *J. Gastroenterol.* **2017**, *52*, 419–431. [CrossRef] [PubMed]

130. Lu, J.; Xia, Y.; Chen, K.; Zheng, Y.; Wang, J.; Lu, W.; Yin, Q.; Wang, F.; Zhou, Y.; Guo, C. Oncogenic role of the notch pathway in primary liver cancer. *Oncol. Lett.* **2016**, *12*, 3–10. [CrossRef] [PubMed]

131. Huang, S.; He, J.; Zhang, X.; Bian, Y.; Yang, L.; Xie, G.; Zhang, K.; Tang, W.; Stelter, A.A.; Wang, Q.; et al. Activation of the hedgehog pathway in human hepatocellular carcinomas. *Carcinogenesis* **2006**, *27*, 1334–1340. [CrossRef] [PubMed]

132. Pez, F.; Lopez, A.; Kim, M.; Wands, J.R.; Caron de Fromentel, C.; Merle, P. WNT signaling and hepatocarcinogenesis: Molecular targets for the development of innovative anticancer drugs. *J. Hepatol.* **2013**, *59*, 1107–1117. [CrossRef] [PubMed]

133. Logan, C.Y.; Nusse, R. The WNT signaling pathway in development and disease. *Annu. Rev. Cell Dev. Biol.* **2004**, *20*, 781–810. [CrossRef] [PubMed]

134. Levrero, M. Viral hepatitis and liver cancer: The case of hepatitis C. *Oncogene* **2006**, *25*, 3834–3847. [CrossRef] [PubMed]

135. Xu, L.; Corcoran, R.B.; Welsh, J.W.; Pennica, D.; Levine, A.J. WISP-1 is a WNT-1- and β-catenin-responsive oncogene. *Genes Dev.* **2000**, *14*, 585–595. [PubMed]

136. He, T.C.; Sparks, A.B.; Rago, C.; Hermeking, H.; Zawel, L.; da Costa, L.T.; Morin, P.J.; Vogelstein, B.; Kinzler, K.W. Identification of C-MYC as a target of the APC pathway. *Science* **1998**, *281*, 1509–1512. [CrossRef] [PubMed]

137. Shtutman, M.; Zhurinsky, J.; Simcha, I.; Albanese, C.; D'Amico, M.; Pestell, R.; Ben-Ze'ev, A. The cyclin D1 gene is a target of the β-catenin/LEF-1 pathway. *Proc. Natl. Acad. Sci. USA* **1999**, *96*, 5522–5527. [CrossRef] [PubMed]

138. Muller, M.; Schilling, T.; Sayan, A.E.; Kairat, A.; Lorenz, K.; Schulze-Bergkamen, H.; Oren, M.; Koch, A.; Tannapfel, A.; Stremmel, W.; et al. TAP73/delta NP73 influences apoptotic response, chemosensitivity and prognosis in hepatocellular carcinoma. *Cell Death Differ.* **2005**, *12*, 1564–1577. [CrossRef] [PubMed]

139. Zaika, A.I.; Slade, N.; Erster, S.H.; Sansome, C.; Joseph, T.W.; Pearl, M.; Chalas, E.; Moll, U.M. Deltanp73, a dominant-negative inhibitor of wild-type p53 and tap73, is up-regulated in human tumors. *J. Exp. Med.* **2002**, *196*, 765–780. [CrossRef] [PubMed]

140. Moll, U.M.; Slade, N. P63 and p73: Roles in development and tumor formation. *Mol. Cancer Res.* **2004**, *2*, 371–386. [PubMed]

141. Polakis, P. WNT signaling in cancer. *Cold Spring Harb. Perspect. Biol.* **2012**, *4*, a008052. [CrossRef] [PubMed]

142. Huang, H.; Fujii, H.; Sankila, A.; Mahler-Araujo, B.M.; Matsuda, M.; Cathomas, G.; Ohgaki, H. β-catenin mutations are frequent in human hepatocellular carcinomas associated with hepatitis C Virus infection. *Am. J. Pathol.* **1999**, *155*, 1795–1801. [CrossRef]

143. Street, A.; Macdonald, A.; Crowder, K.; Harris, M. The hepatitis C Virus NS5A protein activates a phosphoinositide 3-kinase-dependent survival signaling cascade. *J. Biol. Chem.* **2004**, *279*, 12232–12241. [CrossRef] [PubMed]

144. Fukutomi, T.; Zhou, Y.; Kawai, S.; Eguchi, H.; Wands, J.R.; Li, J. Hepatitis C Virus core protein stimulates hepatocyte growth: Correlation with upregulation of WNT-1 expression. *Hepatology* **2005**, *41*, 1096–1105. [CrossRef] [PubMed]

145. Pannuti, A.; Foreman, K.; Rizzo, P.; Osipo, C.; Golde, T.; Osborne, B.; Miele, L. Targeting notch to target cancer stem cells. *Clin. Cancer Res.* **2010**, *16*, 3141–3152. [CrossRef] [PubMed]

146. Takebe, N.; Harris, P.J.; Warren, R.Q.; Ivy, S.P. Targeting cancer stem cells by inhibiting WNT, notch, and hedgehog pathways. *Nat. Rev. Clin. Oncol.* **2011**, *8*, 97–106. [CrossRef] [PubMed]

147. Kopan, R.; Ilagan, M.X. The canonical notch signaling pathway: Unfolding the activation mechanism. *Cell* **2009**, *137*, 216–233. [CrossRef] [PubMed]

148. Takebe, N.; Nguyen, D.; Yang, S.X. Targeting notch signaling pathway in cancer: Clinical development advances and challenges. *Pharmacol. Ther.* **2014**, *141*, 140–149. [CrossRef] [PubMed]

149. Kao, H.Y.; Ordentlich, P.; Koyano-Nakagawa, N.; Tang, Z.; Downes, M.; Kintner, C.R.; Evans, R.M.; Kadesch, T. A histone deacetylase corepressor complex regulates the notch signal transduction pathway. *Genes Dev.* **1998**, *12*, 2269–2277. [CrossRef] [PubMed]

150. Lamar, E.; Deblandre, G.; Wettstein, D.; Gawantka, V.; Pollet, N.; Niehrs, C.; Kintner, C. Nrarp is a novel intracellular component of the notch signaling pathway. *Genes Dev.* **2001**, *15*, 1885–1899. [CrossRef] [PubMed]

151. Ronchini, C.; Capobianco, A.J. Induction of cyclin d1 transcription and cdk2 activity by notch(IC): Implication for cell cycle disruption in transformation by notch(IC). *Mol. Cell Biol.* **2001**, *21*, 5925–5934. [CrossRef] [PubMed]

152. Weng, A.P.; Millholland, J.M.; Yashiro-Ohtani, Y.; Arcangeli, M.L.; Lau, A.; Wai, C.; Del Bianco, C.; Rodriguez, C.G.; Sai, H.; Tobias, J.; et al. C-MYC is an important direct target of notch1 in t-cell acute lymphoblastic leukemia/lymphoma. *Genes Dev.* **2006**, *20*, 2096–2109. [CrossRef] [PubMed]

153. Palomero, T.; Lim, W.K.; Odom, D.T.; Sulis, M.L.; Real, P.J.; Margolin, A.; Barnes, K.C.; O'Neil, J.; Neuberg, D.; Weng, A.P.; et al. Notch1 directly regulates c-myc and activates a feed-forward-loop transcriptional network promoting leukemic cell growth. *Proc. Natl. Acad. Sci. USA* **2006**, *103*, 18261–18266. [CrossRef] [PubMed]

154. Sharma, V.M.; Calvo, J.A.; Draheim, K.M.; Cunningham, L.A.; Hermance, N.; Beverly, L.; Krishnamoorthy, V.; Bhasin, M.; Capobianco, A.J.; Kelliher, M.A. Notch1 contributes to mouse t-cell leukemia by directly inducing the expression of C-MYC. *Mol. Cell Biol.* **2006**, *26*, 8022–8031. [CrossRef] [PubMed]

155. Sanchez-Martin, M.; Ferrando, A. The notch1-MYC highway toward t-cell acute lymphoblastic leukemia. *Blood* **2017**, *129*, 1124–1133. [CrossRef] [PubMed]

156. Chen, Y.; Fischer, W.H.; Gill, G.N. Regulation of the ERBB-2 promoter by RBPJκ and notch. *J. Biol. Chem.* **1997**, *272*, 14110–14114. [CrossRef] [PubMed]

157. Cheng, P.; Zlobin, A.; Volgina, V.; Gottipati, S.; Osborne, B.; Simel, E.J.; Miele, L.; Gabrilovich, D.I. Notch-1 regulates NF-κB activity in hemopoietic progenitor cells. *J. Immunol.* **2001**, *167*, 4458–4467. [CrossRef] [PubMed]

158. Iwai, A.; Takegami, T.; Shiozaki, T.; Miyazaki, T. Hepatitis C Virus NS3 protein can activate the notch-signaling pathway through binding to a transcription factor, Srcap. *PLoS ONE* **2011**, *6*, e20718. [CrossRef] [PubMed]

159. Eissenberg, J.C.; Wong, M.; Chrivia, J.C. Human srcap and drosophila melanogaster dom are homologs that function in the notch signaling pathway. *Mol. Cell Biol.* **2005**, *25*, 6559–6569. [CrossRef] [PubMed]

160. Kageyama, R.; Ohtsuka, T.; Kobayashi, T. The hes gene family: Repressors and oscillators that orchestrate embryogenesis. *Development* **2007**, *134*, 1243–1251. [CrossRef] [PubMed]

161. Omenetti, A.; Choi, S.; Michelotti, G.; Diehl, A.M. Hedgehog signaling in the liver. *J. Hepatol.* **2011**, *54*, 366–373. [CrossRef] [PubMed]

162. Briscoe, J.; Therond, P.P. The mechanisms of hedgehog signalling and its roles in development and disease. *Nat. Rev. Mol. Cell Biol.* **2013**, *14*, 416–429. [CrossRef] [PubMed]

163. Machado, M.V.; Diehl, A.M. Hedgehog signalling in liver pathophysiology. *J. Hepatol.* **2018**, *68*, 550–562. [CrossRef] [PubMed]

164. Li, J.; Wu, T.; Lu, J.; Cao, Y.; Song, N.; Yang, T.; Dong, R.; Yang, Y.; Zang, L.; Du, X.; et al. Immunohistochemical evidence of the prognostic value of hedgehog pathway components in primary gallbladder carcinoma. *Surg. Today* **2012**, *42*, 770–775. [CrossRef] [PubMed]

165. Razumilava, N.; Gradilone, S.A.; Smoot, R.L.; Mertens, J.C.; Bronk, S.F.; Sirica, A.E.; Gores, G.J. Non-canonical hedgehog signaling contributes to chemotaxis in cholangiocarcinoma. *J. Hepatol.* **2014**, *60*, 599–605. [CrossRef] [PubMed]

166. Jinawath, A.; Akiyama, Y.; Sripa, B.; Yuasa, Y. Dual blockade of the hedgehog and ERK1/2 pathways coordinately decreases proliferation and survival of cholangiocarcinoma cells. *J. Cancer Res. Clin Oncol.* **2007**, *133*, 271–278. [CrossRef] [PubMed]

167. Fingas, C.D.; Bronk, S.F.; Werneburg, N.W.; Mott, J.L.; Guicciardi, M.E.; Cazanave, S.C.; Mertens, J.C.; Sirica, A.E.; Gores, G.J. Myofibroblast-derived PDGF-BB promotes hedgehog survival signaling in cholangiocarcinoma cells. *Hepatology* **2011**, *54*, 2076–2088. [CrossRef] [PubMed]

168. Eichenmuller, M.; Gruner, I.; Hagl, B.; Haberle, B.; Muller-Hocker, J.; von Schweinitz, D.; Kappler, R. Blocking the hedgehog pathway inhibits hepatoblastoma growth. *Hepatology* **2009**, *49*, 482–490. [CrossRef] [PubMed]

169. Patil, M.A.; Zhang, J.; Ho, C.; Cheung, S.T.; Fan, S.T.; Chen, X. Hedgehog signaling in human hepatocellular carcinoma. *Cancer Biol. Ther.* **2006**, *5*, 111–117. [CrossRef] [PubMed]

170. Omenetti, A.; Yang, L.; Li, Y.X.; McCall, S.J.; Jung, Y.; Sicklick, J.K.; Huang, J.; Choi, S.; Suzuki, A.; Diehl, A.M. Hedgehog-mediated mesenchymal-epithelial interactions modulate hepatic response to bile duct ligation. *Lab. Investig.* **2007**, *87*, 499–514. [CrossRef] [PubMed]

171. Jung, Y.; Brown, K.D.; Witek, R.P.; Omenetti, A.; Yang, L.; Vandongen, M.; Milton, R.J.; Hines, I.N.; Rippe, R.A.; Spahr, L.; et al. Accumulation of hedgehog-responsive progenitors parallels alcoholic liver disease severity in mice and humans. *Gastroenterology* **2008**, *134*, 1532–1543. [CrossRef] [PubMed]

172. Omenetti, A.; Popov, Y.; Jung, Y.; Choi, S.S.; Witek, R.P.; Yang, L.; Brown, K.D.; Schuppan, D.; Diehl, A.M. The hedgehog pathway regulates remodelling responses to biliary obstruction in rats. *Gut* **2008**, *57*, 1275–1282. [CrossRef] [PubMed]

173. Pereira, T.d.A.; Witek, R.P.; Syn, W.-K.; Choi, S.S.; Bradrick, S.; Karaca, G.F.; Agboola, K.M.; Jung, Y.; Omenetti, A.; Moylan, C.A.; et al. Viral factors induce hedgehog pathway activation in humans with viral hepatitis, cirrhosis, and hepatocellular carcinoma. *Lab. Investig.* **2010**, *90*, 1690. [CrossRef] [PubMed]

174. Choi, S.S.; Bradrick, S.; Qiang, G.; Mostafavi, A.; Chaturvedi, G.; Weinman, S.A.; Diehl, A.M.; Jhaveri, R. Up-regulation of hedgehog pathway is associated with cellular permissiveness for hepatitis C Virus replication. *Hepatology* **2011**, *54*, 1580–1590. [CrossRef] [PubMed]

175. Van Renne, N.; Roca Suarez, A.A.; Duong, F.H.T.; Gondeau, C.; Calabrese, D.; Fontaine, N.; Ababsa, A.; Bandiera, S.; Croonenborghs, T.; Pochet, N.; et al. Mir-135a-5p-mediated downregulation of protein tyrosine phosphatase receptor delta is a candidate driver of hcv-associated hepatocarcinogenesis. *Gut* **2018**, *67*, 953–962. [CrossRef] [PubMed]

176. Zhang, J.; Tian, X.J.; Xing, J. Signal transduction pathways of EMT induced by TGF-β, SHH, and WNT and their crosstalks. *J. Clin Med.* **2016**, *5*, 41. [CrossRef] [PubMed]

177. Paternostro, C.; David, E.; Novo, E.; Parola, M. Hypoxia, angiogenesis and liver fibrogenesis in the progression of chronic liver diseases. *World J. Gastroenterol.* **2010**, *16*, 281–288. [CrossRef] [PubMed]

178. Valfre di Bonzo, L.; Novo, E.; Cannito, S.; Busletta, C.; Paternostro, C.; Povero, D.; Parola, M. Angiogenesis and liver fibrogenesis. *Histol. Histopathol.* **2009**, *24*, 1323–1341. [PubMed]

179. Semela, D.; Dufour, J.F. Angiogenesis and hepatocellular carcinoma. *J. Hepatol.* **2004**, *41*, 864–880. [CrossRef] [PubMed]

180. Nishida, N.; Yano, H.; Nishida, T.; Kamura, T.; Kojiro, M. Angiogenesis in cancer. *Vasc. Health Risk Manag.* **2006**, *2*, 213–219. [CrossRef] [PubMed]

181. Whittaker, S.; Marais, R.; Zhu, A.X. The role of signaling pathways in the development and treatment of hepatocellular carcinoma. *Oncogene* **2010**, *29*, 4989–5005. [CrossRef] [PubMed]

182. Mas, V.R.; Maluf, D.G.; Archer, K.J.; Yanek, K.C.; Fisher, R.A. Angiogenesis soluble factors as hepatocellular carcinoma noninvasive markers for monitoring hepatitis C Virus cirrhotic patients awaiting liver transplantation. *Transplantation* **2007**, *84*, 1262–1271. [CrossRef] [PubMed]

183. Hassan, M.; Selimovic, D.; El-Khattouti, A.; Soell, M.; Ghozlan, H.; Haikel, Y.; Abdelkader, O.; Megahed, M. Hepatitis C Virus-mediated angiogenesis: Molecular mechanisms and therapeutic strategies. *World J. Gastroenterol.* **2014**, *20*, 15467–15475. [CrossRef] [PubMed]

184. Salcedo Mora, X.; Sanz-Cameno, P.; Medina, J.; Martin-Vilchez, S.; Garcia-Buey, L.; Borque, M.J.; Moreno-Otero, R. Association between angiogenesis soluble factors and disease progression markers in chronic hepatitis c patients. *Rev. Esp. Enferm. Dig.* **2005**, *97*, 699–706. [CrossRef] [PubMed]

185. Hassan, M.; Selimovic, D.; Ghozlan, H.; Abdel-kader, O. Hepatitis C Virus core protein triggers hepatic angiogenesis by a mechanism including multiple pathways. *Hepatology* **2009**, *49*, 1469–1482. [CrossRef] [PubMed]

186. Folkman, J. What is the evidence that tumors are angiogenesis dependent? *J. Natl. Cancer Inst.* **1990**, *82*, 4–6. [CrossRef] [PubMed]

187. Nasimuzzaman, M.; Waris, G.; Mikolon, D.; Stupack, D.G.; Siddiqui, A. Hepatitis C Virus stabilizes hypoxia-inducible factor 1α and stimulates the synthesis of vascular endothelial growth factor. *J. Virol.* **2007**, *81*, 10249–10257. [CrossRef] [PubMed]

188. Hussain, S.P.; Hofseth, L.J.; Harris, C.C. Radical causes of cancer. *Nat. Rev. Cancer* **2003**, *3*, 276–285. [CrossRef] [PubMed]

189. He, G.; Karin, M. NF-κB and STAT3-key players in liver inflammation and cancer. *Cell Res.* **2011**, *21*, 159–168. [CrossRef] [PubMed]

190. Disis, M.L. Immune regulation of cancer. *J. Clin. Oncol.* **2010**, *28*, 4531–4538. [CrossRef] [PubMed]

191. Kondylis, V.; Kumari, S.; Vlantis, K.; Pasparakis, M. The interplay of IKK, NF-κB and RIPK1 signaling in the regulation of cell death, tissue homeostasis and inflammation. *Immunol. Rev.* **2017**, *277*, 113–127. [CrossRef] [PubMed]

192. Xia, Y.; Shen, S.; Verma, I.M. NF-κB, an active player in human cancers. *Cancer Immunol. Res.* **2014**, *2*, 823–830. [CrossRef] [PubMed]

193. Karin, M. NF-κB as a critical link between inflammation and cancer. *Cold Spring Harb. Perspect. Biol.* **2009**, *1*, a000141. [CrossRef] [PubMed]

194. Grivennikov, S.I.; Greten, F.R.; Karin, M. Immunity, inflammation, and cancer. *Cell* **2010**, *140*, 883–899. [CrossRef] [PubMed]

195. Oeckinghaus, A.; Ghosh, S. The NF-κB family of transcription factors and its regulation. *Cold Spring Harb. Perspect. Biol.* **2009**, *1*, a000034. [CrossRef] [PubMed]

196. Tieri, P.; Termanini, A.; Bellavista, E.; Salvioli, S.; Capri, M.; Franceschi, C. Charting the NF-kappab pathway interactome map. *PLoS ONE* **2012**, *7*, e32678. [CrossRef] [PubMed]

197. Bohm, F.; Kohler, U.A.; Speicher, T.; Werner, S. Regulation of liver regeneration by growth factors and cytokines. *EMBO Mol. Med.* **2010**, *2*, 294–305. [CrossRef] [PubMed]

198. Akira, S.; Nishio, Y.; Inoue, M.; Wang, X.J.; Wei, S.; Matsusaka, T.; Yoshida, K.; Sudo, T.; Naruto, M.; Kishimoto, T. Molecular cloning of aprf, a novel ifn-stimulated gene factor 3 p91-related transcription factor involved in the GP130-mediated signaling pathway. *Cell* **1994**, *77*, 63–71. [CrossRef]

199. Mackey-Lawrence, N.M.; Petri, W.A., Jr. Leptin and mucosal immunity. *Mucosal. Immunol.* **2012**, *5*, 472–479. [CrossRef] [PubMed]

200. McCartney, E.M.; Helbig, K.J.; Narayana, S.K.; Eyre, N.S.; Aloia, A.L.; Beard, M.R. Signal transducer and activator of transcription 3 is a proviral host factor for hepatitis C Virus. *Hepatology* **2013**, *58*, 1558–1568. [CrossRef] [PubMed]

201. Wilks, A.F.; Harpur, A.G.; Kurban, R.R.; Ralph, S.J.; Zurcher, G.; Ziemiecki, A. Two novel protein-tyrosine kinases, each with a second phosphotransferase-related catalytic domain, define a new class of protein kinase. *Mol. Cell Biol.* **1991**, *11*, 2057–2065. [CrossRef] [PubMed]

202. Firmbach-Kraft, I.; Byers, M.; Shows, T.; Dalla-Favera, R.; Krolewski, J.J. Tyk2, prototype of a novel class of non-receptor tyrosine kinase genes. *Oncogene* **1990**, *5*, 1329–1336. [PubMed]

203. Kaptein, A.; Paillard, V.; Saunders, M. Dominant negative STAT3 mutant inhibits interleukin-6-induced JAK-STAT signal transduction. *J. Biol. Chem.* **1996**, *271*, 5961–5964. [CrossRef] [PubMed]

204. Yang, J.; Chatterjee-Kishore, M.; Staugaitis, S.M.; Nguyen, H.; Schlessinger, K.; Levy, D.E.; Stark, G.R. Novel roles of unphosphorylated STAT3 in oncogenesis and transcriptional regulation. *Cancer Res.* **2005**, *65*, 939–947. [PubMed]

205. Yang, J.; Liao, X.; Agarwal, M.K.; Barnes, L.; Auron, P.E.; Stark, G.R. Unphosphorylated STAT3 accumulates in response to Il-6 and activates transcription by binding to nfkappab. *Genes Dev.* **2007**, *21*, 1396–1408. [CrossRef] [PubMed]

206. Maeda, S.; Kamata, H.; Luo, J.L.; Leffert, H.; Karin, M. Ikkβ couples hepatocyte death to cytokine-driven compensatory proliferation that promotes chemical hepatocarcinogenesis. *Cell* **2005**, *121*, 977–990. [CrossRef] [PubMed]

207. Lee, H.; Herrmann, A.; Deng, J.H.; Kujawski, M.; Niu, G.; Li, Z.; Forman, S.; Jove, R.; Pardoll, D.M.; Yu, H. Persistently activated STAT3 maintains constitutive NF-kappab activity in tumors. *Cancer Cell* **2009**, *15*, 283–293. [CrossRef] [PubMed]

208. Chen, L.; Fischle, W.; Verdin, E.; Greene, W.C. Duration of nuclear NF-κB action regulated by reversible acetylation. *Science* **2001**, *293*, 1653–1657. [CrossRef] [PubMed]

209. Pham, C.G.; Bubici, C.; Zazzeroni, F.; Papa, S.; Jones, J.; Alvarez, K.; Jayawardena, S.; de Smaele, E.; Cong, R.; Beaumont, C.; et al. Ferritin heavy chain upregulation by NF-kappab inhibits TNFα-induced apoptosis by suppressing reactive oxygen species. *Cell* **2004**, *119*, 529–542. [CrossRef] [PubMed]

210. He, G.; Yu, G.Y.; Temkin, V.; Ogata, H.; Kuntzen, C.; Sakurai, T.; Sieghart, W.; Peck-Radosavljevic, M.; Leffert, H.L.; Karin, M. Hepatocyte Ikkβ/NF-κB inhibits tumor promotion and progression by preventing oxidative stress-driven STAT3 activation. *Cancer Cell* **2010**, *17*, 286–297. [CrossRef] [PubMed]

211. Seeff, L.B. Introduction: The burden of hepatocellular carcinoma. *Gastroenterology* **2004**, *127*, S1–S4. [CrossRef] [PubMed]

212. Lindenbach, B.D.; Rice, C.M. Unravelling hepatitis C Virus replication from genome to function. *Nature* **2005**, *436*, 933–938. [CrossRef] [PubMed]

213. Zampino, R.; Marrone, A.; Restivo, L.; Guerrera, B.; Sellitto, A.; Rinaldi, L.; Romano, C.; Adinolfi, L.E. Chronic hcv infection and inflammation: Clinical impact on hepatic and extra-hepatic manifestations. *World J. Hepatol.* **2013**, *5*, 528–540. [CrossRef] [PubMed]

214. Roca Suarez, A.A.; van Renne, N.; Baumert, T.F.; Lupberger, J. Viral manipulation of STAT3: Evade, exploit, and injure. *PLoS Pathog.* **2018**, *14*, e1006839. [CrossRef] [PubMed]

215. Park, J.; Kang, W.; Ryu, S.W.; Kim, W.I.; Chang, D.Y.; Lee, D.H.; Park, D.Y.; Choi, Y.H.; Choi, K.; Shin, E.C.; et al. Hepatitis C Virus infection enhances TNFα-induced cell death via suppression of NF-κB. *Hepatology* **2012**, *56*, 831–840. [CrossRef] [PubMed]

216. Machida, K.; Cheng, K.T.; Lai, C.K.; Jeng, K.S.; Sung, V.M.; Lai, M.M. Hepatitis C Virus triggers mitochondrial permeability transition with production of reactive oxygen species, leading to DNA damage and stat3 activation. *J. Virol.* **2006**, *80*, 7199–7207. [CrossRef] [PubMed]

217. Devhare, P.B.; Sasaki, R.; Shrivastava, S.; Di Bisceglie, A.M.; Ray, R.; Ray, R.B. Exosome-mediated intercellular communication between hepatitis C Virus-infected hepatocytes and hepatic stellate cells. *J. Virol.* **2017**, *91*, JVI–02225. [CrossRef] [PubMed]

218. Wan, S.; Kuo, N.; Kryczek, I.; Zou, W.; Welling, T.H. Myeloid cells in hepatocellular carcinoma. *Hepatology* **2015**, *62*, 1304–1312. [CrossRef] [PubMed]

219. Yoshida, T.; Hanada, T.; Tokuhisa, T.; Kosai, K.; Sata, M.; Kohara, M.; Yoshimura, A. Activation of STAT3 by the hepatitis C Virus core protein leads to cellular transformation. *J. Exp. Med.* **2002**, *196*, 641–653. [CrossRef] [PubMed]

220. Baumert, T.F.; Juhling, F.; Ono, A.; Hoshida, Y. Hepatitis C-related hepatocellular carcinoma in the era of new generation antivirals. *BMC Med.* **2017**, *15*, 52. [CrossRef] [PubMed]

221. Van der Meer, A.J.; Feld, J.J.; Hofer, H.; Almasio, P.L.; Calvaruso, V.; FeRNAndez-Rodriguez, C.M.; Aleman, S.; Ganne-Carrie, N.; D'Ambrosio, R.; Pol, S.; et al. Risk of cirrhosis-related complications in patients with advanced fibrosis following hepatitis C Virus eradication. *J. Hepatol.* **2017**, *66*, 485–493. [CrossRef] [PubMed]

222. Heckley, G.A.; Jarl, J.; Asamoah, B.O.; U, G.G. How the risk of liver cancer changes after alcohol cessation: A review and meta-analysis of the current literature. *BMC Cancer* **2011**, *11*, 446. [CrossRef] [PubMed]

223. Seitz, H.K.; Bataller, R.; Cortez-Pinto, H.; Gao, B.; Gual, A.; Lackner, C.; Mathurin, P.; Mueller, S.; Szabo, G.; Tsukamoto, H. Alcoholic liver disease. *Nat. Rev. Dis. Primers* **2018**, *4*, 16. [CrossRef] [PubMed]

224. Seitz, H.K.; Bataller, R.; Cortez-Pinto, H.; Gao, B.; Gual, A.; Lackner, C.; Mathurin, P.; Mueller, S.; Szabo, G.; Tsukamoto, H. Publisher correction: Alcoholic liver disease. *Nat. Rev. Dis. Primers* **2018**, *4*, 18. [CrossRef] [PubMed]

225. Conti, F.; Buonfiglioli, F.; Scuteri, A.; Crespi, C.; Bolondi, L.; Caraceni, P.; Foschi, F.G.; Lenzi, M.; Mazzella, G.; Verucchi, G.; et al. Early occurrence and recurrence of hepatocellular carcinoma in hcv-related cirrhosis treated with direct-acting antivirals. *J. Hepatol.* **2016**, *65*, 727–733. [CrossRef] [PubMed]

226. Reig, M.; Marino, Z.; Perello, C.; Inarrairaegui, M.; Ribeiro, A.; Lens, S.; Diaz, A.; Vilana, R.; Darnell, A.; Varela, M.; et al. Unexpected high rate of early tumor recurrence in patients with hcv-related hcc undergoing interferon-free therapy. *J. Hepatol.* **2016**, *65*, 719–726. [CrossRef] [PubMed]

227. Cheung, M.C.M.; Walker, A.J.; Hudson, B.E.; Verma, S.; McLauchlan, J.; Mutimer, D.J.; Brown, A.; Gelson, W.T.H.; MacDonald, D.C.; Agarwal, K.; et al. Outcomes after successful direct-acting antiviral therapy for patients with chronic hepatitis c and decompensated cirrhosis. *J. Hepatol.* **2016**, *65*, 741–747. [CrossRef] [PubMed]

228. Mettke, F.; Schlevogt, B.; Deterding, K.; Wranke, A.; Smith, A.; Port, K.; Manns, M.P.; Vogel, A.; Cornberg, M.; Wedemeyer, H. Interferon-free therapy of chronic hepatitis C with direct-acting antivirals does not change the short-term risk for de novo hepatocellular carcinoma in patients with liver cirrhosis. *Aliment. Pharmacol. Ther.* **2018**, *47*, 516–525. [CrossRef] [PubMed]

229. European Association for the Study of the Liver. European Association for the Study of the, L. Easl recommendations on treatment of hepatitis c 2018. *J. Hepatol.* **2018**, *69*, 461–511. [CrossRef] [PubMed]

230. Pinter, M.; Trauner, M.; Peck-Radosavljevic, M.; Sieghart, W. Cancer and liver cirrhosis: Implications on prognosis and management. *ESMO Open* **2016**, *1*, e000042. [CrossRef] [PubMed]

231. Bandiera, S.; Billie Bian, C.; Hoshida, Y.; Baumert, T.F.; Zeisel, M.B. Chronic hepatitis C Virus infection and pathogenesis of hepatocellular carcinoma. *Curr. Opin. Virol.* **2016**, *20*, 99–105. [CrossRef] [PubMed]

232. Hanahan, D.; Weinberg, R.A. Hallmarks of cancer: The next generation. *Cell* **2011**, *144*, 646–674. [CrossRef] [PubMed]

233. Keating, G.M. Sorafenib: A review in hepatocellular carcinoma. *Target. Oncol.* **2017**, *12*, 243–253. [CrossRef] [PubMed]

234. Cainap, C.; Qin, S.; Huang, W.T.; Chung, I.J.; Pan, H.; Cheng, Y.; Kudo, M.; Kang, Y.K.; Chen, P.J.; Toh, H.C.; et al. Linifanib versus sorafenib in patients with advanced hepatocellular carcinoma: Results of a randomized phase iii trial. *J. Clin. Oncol.* **2015**, *33*, 172–179. [CrossRef] [PubMed]

235. Zhu, A.X.; Rosmorduc, O.; Evans, T.R.; Ross, P.J.; Santoro, A.; Carrilho, F.J.; Bruix, J.; Qin, S.; Thuluvath, P.J.; Llovet, J.M.; et al. Search: A phase III, randomized, double-blind, placebo-controlled trial of sorafenib plus erlotinib in patients with advanced hepatocellular carcinoma. *J. Clin. Oncol.* **2015**, *33*, 559–566. [CrossRef] [PubMed]

236. Llovet, J.M.; HeRNAndez-Gea, V. Hepatocellular carcinoma: Reasons for phase III failure and novel perspectives on trial design. *Clin. Cancer Res.* **2014**, *20*, 2072–2079. [CrossRef] [PubMed]

237. Guichard, C.; Amaddeo, G.; Imbeaud, S.; Ladeiro, Y.; Pelletier, L.; Maad, I.B.; Calderaro, J.; Bioulac-Sage, P.; Letexier, M.; Degos, F.; et al. Integrated analysis of somatic mutations and focal copy-number changes identifies key genes and pathways in hepatocellular carcinoma. *Nat. Genet.* **2012**, *44*, 694–698. [CrossRef] [PubMed]

238. Nakagawa, S.; Wei, L.; Song, W.M.; Higashi, T.; Ghoshal, S.; Kim, R.S.; Bian, C.B.; Yamada, S.; Sun, X.; Venkatesh, A.; et al. Molecular liver cancer prevention in cirrhosis by organ transcriptome analysis and lysophosphatidic acid pathway inhibition. *Cancer Cell* **2016**, *30*, 879–890. [CrossRef] [PubMed]

Annex VII: Stromal and Immune Drivers of Hepatocarcinogenesis

Book chapter

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Chapter 15
Stromal and Immune Drivers of Hepatocarcinogenesis

Antonio Saviano, Natascha Roehlen, Alessia Virzì, Armando Andres Roca Suarez, Yujin Hoshida, Joachim Lupberger, and Thomas F. Baumert

Introduction

The liver is a multifunctional organ that plays a key role in metabolism and detoxification as well as in regulation of immune response and tolerance. The liver is physiologically exposed to many pathogens and toxic substances derived from the gut and has the largest population of resident macrophages (i.e., Kupffer cells, KCs) in the body and a high prevalence of natural killer cells (NK), natural killer T cells (NKT), and T cells. In normal conditions, the liver removes a large amount of microbes and pathogen-associated and damage-associated molecular patterns (PAMPs and DAMPs) and maintains an immunosuppressive environment [1].

Following chronic hepatocyte damage, immune and stromal cells modify a liver environment, which triggers chronic inflammation and ultimately promotes hepatocellular carcinoma (HCC) [2]. Indeed, independently from the etiology, chronic liver disease is characterized by a deregulation in the liver immune network

* Antonio Saviano and Natascha Roehlen are co-first authors of this chapter

A. Saviano · T. F. Baumert (✉)
Inserm U1110, Institut de Recherche sur les Maladies Virales et Hépatiques, Université de Strasbourg, Strasbourg, France

Pôle Hépato-digestif, Institut Hopitalo-Universitaire, Hôpitaux Universitaires, Strasbourg, France
e-mail: thomas.baumert@unistra.fr

N. Roehlen · A. Virzì · A. A. R. Suarez · J. Lupberger
Inserm U1110, Institut de Recherche sur les Maladies Virales et Hépatiques, Université de Strasbourg, Strasbourg, France

Y. Hoshida
Liver Tumor Translational Research Program, Simmons Comprehensive Cancer Center, Division of Digestive and Liver Diseases, Department of Internal Medicine, University of Texas Southwestern Medical Center, Dallas, TX, USA

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that stimulates cellular stress and death favoring liver fibrosis, hepatocyte proliferation, and epithelial-to-mesenchymal transition (EMT) [2]. A combination of EMT, genetic mutations, and epigenetic alterations that accumulate during cell proliferation is the most important driver of hepatocarcinogenesis [3].

Once HCC has developed, liver microenvironment greatly affects tumor progression and response to therapy [4]. This is the reason why gene expression signatures in liver tissues adjacent to the HCC—and the not in tumor itself—highly correlate with long-term survival of patients with liver fibrosis [5]. Similarly, HCC infiltration by non-parenchymal cells (e.g., regulatory T cells, T_{reg}) has been associated with tumor progression [5–8]. New therapies targeting liver microenvironment are recently developed or under clinical investigation for both chronic liver disease (e.g., nonalcoholic steatohepatitis, NASH) and HCC.

Hence, liver microenvironment plays an essential role in both hepatocarcinogenesis and tumor progression and it is an important therapeutic target for HCC prevention and treatment.

From Chronic Inflammation to Hepatocellular Carcinoma

HCC almost universally evolves on the background of chronic liver inflammation and liver fibrosis [9]. Chronic hepatocyte cell injury induces activation of the immune system that initiates and supports chronic inflammation by generation of proinflammatory cytokines and chemokines and activation of hepatic stellate cells (HSCs), finally resulting in liver fibrosis, cirrhosis, and cancer [10] (Fig. 15.1).

During chronic infections (e.g., hepatitis B virus, HBV, or hepatitis C virus, HCV) as well as metabolic (e.g., NASH) or toxic diseases (e.g., alcoholic steatohepatitis, ASH), immune cells—first of all KCs—are activated by the release of PAMPs and DAMPs produced by hepatocyte apoptosis and death. Activated KCs present viral antigens to T cells and/or secrete cytokines and chemokines that recruit circulating monocytes, lymphocytes, and neutrophils [11]. Proinflammatory signals are mainly mediated by the accumulation of tumor necrosis factor alpha (TNF-α); interleukins (IL) such as IL-6, IL-1β, IL-2, IL-7, IL-15, IL-17; C-C motif chemokine ligand 2 (CCL2); and interferon gamma (IFN-ɣ).

Following activation by antigen-presenting cells, T cells and especially T-helper 17 (Th17) cells and the mucosal-associated invariant T (MAIT) cells are major promoters of liver inflammation primarily by secretion of IL-17 [12, 13]. IL-17 secreted by T cells as well as transforming growth factor beta 1 (TGF-β1) and platelet-derived growth factor subunit B (PDGF-B) secreted by KCs and monocyte-derived macrophages are able to activate and differentiate HSC into collagen-producing myofibroblasts [12, 13]. Finally, also DAMPs can directly activate HSC and participate in fibrosis [7, 14]. HSC-derived myofibroblasts account for abnormal production of collagen in the liver and are main components of the hepatic precancerous microenvironment [15].

The inflammatory microenvironment causes hepatocellular stress, accompanied by epigenetic modifications, mitochondrial alterations, DNA damage, and
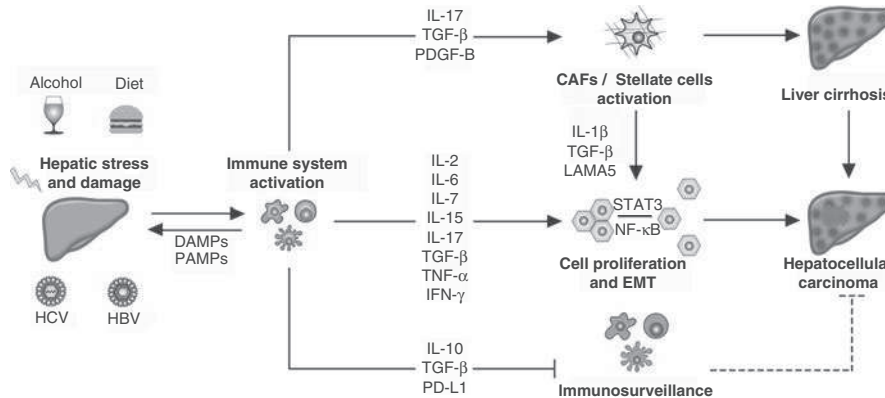
Fig. 15.1 Chronic inflammation is a pan-etiological driver of hepatocarcinogenesis. Hepatocarcinogenesis can be induced by multiple etiological and environmental conditions. Chronic HBV and HCV infections, as well as chronic alcohol abuse and metabolic syndrome trigger the activation of the innate immune system via release of Damage-Associated Molecular Patterns (DAMPs) and Pathogen Associated Molecular Patterns (PAMPs). The persistent dysregulation of the immunological network of the liver, promoted by the secretion of pro-inflammatory cytokines/chemokines (e.g. IL-2, IL-6, IL-7, IL-15, IL-17, TGF-β, TNF-α, IFN-γ), leads to cells death, compensatory hepatocellular proliferation, activation of cancer-associated fibroblasts (CAFs) and hepatic stellate cells (HSCs) as well as epithelial-tomesenchymal transition (EMT). Moreover, sustained necro-inflammatory status attenuates immune-surveillance and anti-tumor immune response, by secretion of anti-inflammatory molecules (e.g. IL-10, TGF-β, PD-L1). In addition, the activation of HSCs contributes significantly to cell proliferation (by the release of IL-1β, TGF-β and LAMA5) and cirrhosis. In conclusion, cellular proliferation and EMT, further sustained by STAT3/NF-κB pathway activation, cirrhosis and impaired immunosurveillance activity collectively contribute to HCC development

chromosomal alterations that determine cell transformations [7]. Inflammation has been shown to upregulate nuclear factor kappa B (NF-κB) and signal transducer and activator of transcription 3 (STAT3) thereby affecting cell proliferation, survival, angiogenesis, and chemotaxis [16–18]. STAT3 is further induced by several other cytokines and growth factors that are known to be upregulated under conditions of chronic liver inflammation [19]. Regarding chronic HBV and HCV infection, upregulation of the cytokines lymphotoxin beta and TNF-α in CD4+ and CD8+ T cells has been shown to promote hepatocarcinogenesis [20, 21].

Collectively, persistence of infection by hepatotropic viruses or toxic condition may cause a chronic inflammatory state, accompanied by continual cell death and promotion of compensatory tissue repair mechanisms, finally resulting in liver cirrhosis and cell transformation. Since chronic inflammation induces impaired immune surveillance due to exhausted T cells, chronic inflammatory liver status not only provokes cell transformation but also attenuates physiological antitumor defense mechanisms by the immune system. Thus, tumor cell attack by cytolytic T cells is weakened in chronic inflammatory liver tissue and HCC microenvironment [22–24].

Moreover, upregulation of immunosuppressive T_{reg} cells has been related to chronic inflammation associated with attenuated immune surveillance contributing to risk of HCC development [25, 26]. The inducible type 1 T regulatory (Tr1) cells

possess many immunosuppressive functions by secretion of the cytokines IL-10 and TGF-β, as well as by expression of the checkpoint inhibitors cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and programmed death 1 (PD1) on the cell surface [27–29]. T_{reg} or KC-secreted IL-10 was reported to reduce immune surveillance by suppressing macrophage activation, T-cell proliferation, and IFN-ɣ production, hereby inhibiting antitumor response mediated by the immune system [30–32]. Moreover, TGF-β is known to inhibit IL-2-dependent T-cell proliferation as well as production of proinflammatory cytokines and performance of cytolytic functions by effector cells [33–35]. Suggesting its involvement in chronic inflammatory liver disease and contribution to hepatocarcinogenesis, levels of the immunoregulatory cytokine IL-10 and TGF-β have been reported to be elevated in patients with chronic liver disease and related to disease progression and patients' survival [30, 36, 37].

Immune Cells in HCC Microenvironment

Leukocytes are one of the main drivers in chronic inflammation. They are highly enriched in both the precancerous state of liver cirrhosis and in malignant tissue of HCC. Indeed, liver carcinoma is characterized by an immunogenic micro-environment, consisting of high amounts of lymphocytes, including NK cells, NKT cells, B cells, and T cells [38]. T-cell exhaustion due to chronic inflammation hereby shapes an immunogenic microenvironment that is characterized by an enhanced immunotolerance. Thus, the endogenous antitumor function of cytotoxic lymphocytes can be restored by antigen-presenting cells, which are typically reduced in the HCC microenvironment [39]. Indeed, decreased activity of NK cells, one of the most important antigen-presenting cells, correlates with an increased incidence of HCC in patients with liver cirrhosis [40]. Moreover, infiltration and density of T cells in human HCCs correlate with better patient prognosis, whereas tumor-infiltrating B cells reduce tumor viability [41].

Macrophages perpetuate chronic inflammation following liver injury and promote fibrogenesis via HSC activation. This therefore represents a significant component of HCC microenvironment. Of note, tumor-associated macrophages (TAMs) are considered to promote tumor development and favor angiogenesis and tumor cell migration [42, 43]. Moreover, TAMs may stimulate tumor growth by suppression of the adaptive immune system. They express high levels of cell death-ligand 1 (PD-L1), thereby suppressing the antitumor cytotoxic T-cell responses [44]. TAMs provide cytokines and growth factors that enhance tumor cell proliferation and NF-κB-mediated protection from cancer cell apoptosis and angiogenesis [45]. Accordingly, TAM infiltration correlates with HCC progression and poor survival [46, 47].

Dendritic cells (DCs) are a heterogeneous cell population and one of the most powerful antigen-presenting cells which regulate the primary immune response and the immune homeostasis in the liver [48]. By forming a bridge between the innate and the adaptive immune system [49], DCs are regarded as key players in immune

regulation [50, 51]. An impaired DC function has frequently been suggested as an important factor contributing to an immunosuppressive microenvironment in chronic liver disease, which is favoring tumor development. Accordingly, several studies report lower DC numbers in both the peripheral blood and liver tissue of patients with HCC [52, 53]. A reduced IL-12 secretion by DCs is hereby attributed to an attenuated stimulation of T cells [54]. Moreover, DC inhibition and its effects on downstream effector cells have further been identified as immune escape mechanisms of HCC [55, 56].

Stromal Cells Participate in HCC Development and Progression

Liver cirrhosis is one of the main risk factors for hepatocarcinogenesis and therefore regarded as a precancerous liver state [57]. Thus, the lifetime risk of HCC development in patients with advanced liver cirrhosis is approximately 30%, and 80–90% of HCCs evolve in cirrhotic liver tissue [58, 59]. Considering HSCs as the most important progenitor cells of myofibroblasts that account for enhanced production of the extracellular matrix in liver fibrosis and liver cirrhosis, HSC-derived myofibroblasts are the main components of the hepatic precancerous microenvironment as well as the HCC tumor environment. Indeed, differentiation of HSCs from pericyte-like cells to collagen-producing myofibroblasts provides 85–95% of the myofibroblasts in liver fibrosis and liver cirrhosis, independent of the underlying trigger [15]. Hence, together with bone marrow (BM)-derived fibroblasts and portal fibroblasts (PF), HSC-derived myofibroblasts compose the stromal population of cancer-associated myofibroblasts (CAFs) that contribute actively to HCC development and progression [60]. Of note, CAFs show a markedly altered phenotype compared to normal fibroblasts [61, 62]. Normal fibroblasts may suppress tumor growth by contact inhibition [62], whereas CAFs promote an immune-tolerant tumor environment by interaction with monocytes and lymphocytes [63]. Indeed, CAFs inhibit lymphocyte tumor infiltration, increase the activity of immunosuppressive regulatory T cells, and induce apoptosis in monocytes [64, 65]. Furthermore, CAFs were reported to impair antitumor functions of T cells via activation of neutrophils [66]. CAFs may further promote hepatocarcinogenesis by downregulation of tumor-suppressive microRNAs [67, 68]. CAF activity has also been associated with tumor angiogenesis. CAFs have been shown to secrete vascular endothelial growth factor (VEGF) and angiopoietin 1 or 2 [69–71]. The cross talk between CAFs and cancer cells is crucial for HCC biology. The secretion of laminin 5 (LAMA5) [72] and IL-1β [73] by CAFs has been shown to promote HCC migration, and on the other hand, highly metastatic HCC cells were found to be able to convert normal fibroblasts to CAFs, which in turn promote cancer progression by secretion of proinflammatory cytokines [74]. Several studies further suggest an association of CAFs and CSCs that are thought to promote tumor development and to mediate therapeutic resistance. CAFs have been reported to recruit CSCs and to

drive their self-renewal [75, 76]. Moreover, CAFs have been observed to increase expression of keratin 19 by paracrine interactions [77], a marker for hepatic stem cells that has been observed to be correlated with poor prognosis [78]. In summary, CAFs are key drivers in hepatic carcinogenesis by increasing angiogenesis, inflammation, and proliferation and attenuating immune surveillance [60] (Fig. 15.2). CAFs correlate with HCC tumor stage and progression, tumor recurrence after surgery, as well as overall prognosis [79–81].

Lymphatic vessels function as a tissue drainage and immunological control system. They are highly enriched in the liver, carrying approximately 25–50% of the thoracic duct's lymph flow [82]. For a long time, lymphatic vessels were considered to affect carcinogenesis only by providing the structural pathway for metastatic spread of tumor cells. However, recent observations indicate a functional role of the lymphatic endothelium also in the hepatocytes' immunogenic microenvironment, which is affecting the development of chronic liver disease and hepatocarcinogenesis [83]. Thus, lymphatic endothelial cells (LECs) guide immune cell migration by lining the inner surface of lymphatic capillaries and regulate the expression of adhesion molecules and cytokines [84, 85]. Moreover, by secretion of immunosuppressive cytokines (i.e., TGF-β) and the overexpression of co-inhibitory checkpoint
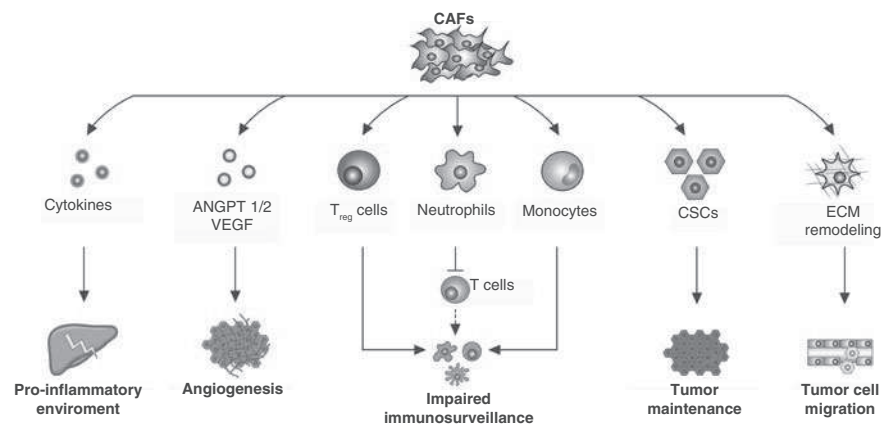

Fig. 15.2 Cancer-associated fibroblasts (CAFs) characterize the stromal tumor microenvironment and promote hepatocarcinogenesis, tumor progression and treatment resistance. Tumor microenvironment in HCC is predominantly characterized by cancer-associated fibroblasts (CAFs) that contribute actively to tumor development, progression and metastatic spread. Interacting with the immune cells and secreting angiogenic factors, these cells reduce immune surveillance and drive tumor angiogenesis. Moreover, CAFs promote cancer cell proliferation by paracrine interactions as well as production of prooncogenic cytokines (e.g. TGF-β). CAFs are also reported to recruit cancer stem cells, hereby affecting tumor maintenance, heterogeneity and treatment resistance. Finally, CAFs are responsible for the alteration of liver extracellular matrix by production and secretion of Laminin 5 and Integrin β1 that further promote HCC cell invasion and migration

proteins (i.e., PD-L1), LECs suppress a maturation and proliferation of circulating immune cells [84–86]. LECs further mediate CD4$^+$ and CD8$^+$ T-cell tolerance by expression of self-antigens in the presence of inhibitory ligands [87].

Lymphangiogenesis is increased in liver fibrosis and cirrhosis and positively correlate with portal venous pressure and disease severity [88–90]. The enhanced interstitial flow and increased number of LECs is accompanied by increased cytokine production and immune cell recruitment to the inflammatory environment present in almost all chronic liver diseases [91]. The primarily immunosuppressive functions of LECs hereby contribute to an immunotolerant microenvironment favoring HCC development [83, 92]. Moreover, expression of chemokines by LECs may facilitate lymphogenic metastatic tumor spread [84]. Vascular endothelial growth factor C (VEGF-C) is an important stimulator of LEC growth and lymphangiogenesis. VEGF-C is enhanced in liver cirrhosis and HCC, and its expression in HCCs correlates with metastasis and poor patients' outcome [93, 94].

Epithelial-to-Mesenchymal Transition in HCC

Epithelial-to-mesenchymal transition (EMT) describes a reversible process, by which epithelial cell types gradually develop mesenchymal characteristics leading to higher motility and invasive properties that are essential in embryogenic development and wound healing but also implicated in hepatic fibrogenesis and carcinogenesis [95, 96]. Thus, while epithelial cells are characterized by polarity and stable morphology, mesenchymal cells lack polarity, show a loose arrangement, and exhibit the capacity of migration [97]. EMT can be divided in three different biological subtypes [98]. While type 1 EMT determines embryonal development and organogenesis, types 2 and 3 EMT affect liver disease progression and can be activated by several proinflammatory cytokines and growth factors present in the inflammatory state of the liver [99].

Type 2 EMT occurs in response to cell injury as a mechanism of tissue repair and may cause fibrosis due to generation of collagen-producing fibroblasts. TGF-β, a cytokine increased under condition of chronic inflammation, has been shown to be one of the strongest activators of type 2 EMT that can affect hepatocytes, cholangiocytes, and hepatic stellate cells (HSC) [100]. Quiescent HSCs, the most frequent progenitor cells of collagen-producing fibroblasts [15], are actually regarded as transitional cells that have undergone partial EMT from epithelial cells and may complete transition upon inflammatory signals [101]. Hence, EMT is regarded as one of the most important promoters of liver fibrogenesis in response to chronic inflammation [101].

Type 3 EMT may occur due to genetic and epigenetic changes during malignant transformation of epithelial cells and is implicated in HCC growth and progression [3]. Cells generated by type 3 EMT differ significantly from types 1 and 2 EMT cells and develop properties of invasion and migration as well as escape from apop-

tosis. Weakened or loss of E-cadherin expression, characteristic for development of the mesenchymal unpolarized phenotype, could be revealed in 58% of human HCC patients and correlated with the presence of metastases and patients' survival [102]. Besides proinflammatory cytokines and growth factors, several studies further indicate induction of type 3 EMT by core proteins of HCV itself [103]. Given not only the correlation of EMT with tumor stage but also response to therapy [104], therapeutic targeting of molecular key players in EMT is highly clinically relevant.

Clinical Perspectives

Considering the implication of stromal and immunogenic cell compounds in HCC development and progression, medical treatments targeting these factors represent promising tools for future medical treatment of advanced HCC. Presently, sorafenib, an oral multikinase inhibitor targeting vascular endothelial growth factor receptor (VEGFR-2/VEGFR-3) and platelet-derived growth factor receptor (PDGFR), produced by the stromal HCC microenvironment already represents the standard of care treatment for patients with advanced HCC [105]. Lenvatinib, another tyrosine kinase inhibitor with multiple targets, has recently been revealed to be noninferior compared to sorafenib according to the REFLECT trial and has lately been approved by the FDA as first-line treatment for unresectable HCC [106]. Moreover, recently therapeutic strategies targeting the immunogenic tumor microenvironment have been demonstrated to be effective as systemic therapy for several cancer types. Consequently, drugs targeting exhausted lymphocytes expressing PD1 and infiltrating the tumor are able to activate T-cell-driven immune response against cancer cells and were approved for melanoma and non-small cell lung cancer treatment [107, 108]. Preliminary results from open-label trials of these drugs in HCC treatment are encouraging. Indeed, nivolumab and pembrolizumab, anti-PD1 monoclonal antibodies, have been demonstrated to be more effective than placebo in patients with advanced unresectable HCC previously treated with sorafenib [109, 110]. For that reason, these compounds were recently approved by FDA as a second-line treatment for advanced HCC. Moreover, currently several randomized controlled trials investigate the effects of other drugs targeting the HCC immunogenic and stromal microenvironment. Thus, aiming to activate tumor-targeting cytotoxic T lymphocytes, a growing number of studies recently worked on ex vivo tumor-antigen-loaded dendritic cells as an approach of cancer immunotherapy by DC vaccination [111–113]. Several other studies are focused on immunotherapy targeting TAMs, aiming to decrease TAM population present in the HCC by elimination, blocking recruitment, or functional reprogramming of TAM polarization [43]. The results of current ongoing clinical studies are expected in the next few years and may revolutionize future HCC medical treatment.

References

1. Jenne CN, Kubes P. Immune surveillance by the liver. Nat Immunol. 2013;14:996. https://doi.org/10.1038/ni.2691. https://www.nature.com/articles/ni.2691#supplementary-information.

2. Marrone G, Shah VH, Gracia-Sancho J. Sinusoidal communication in liver fibrosis and regeneration. J Hepatol. 2016;65(3):608–17. https://doi.org/10.1016/j.jhep.2016.04.018.

3. van Zijl F, Zulehner G, Petz M, Schneller D, Kornauth C, Hau M, et al. Epithelial-mesenchymal transition in hepatocellular carcinoma. Future Oncol. 2009;5(8):1169–79. https://doi.org/10.2217/fon.09.91.

4. Nishida N, Kudo M. Oncogenic signal and tumor microenvironment in hepatocellular carcinoma. Oncology. 2017;93(Suppl 1):160–4. https://doi.org/10.1159/000481246.

5. Hoshida Y, Villanueva A, Kobayashi M, Peix J, Chiang DY, Camargo A, et al. Gene expression in fixed tissues and outcome in hepatocellular carcinoma. N Engl J Med. 2008;359(19):1995–2004. https://doi.org/10.1056/NEJMoa0804525.

6. Caja L, Dituri F, Mancarella S, Caballero-Diaz D, Moustakas A, Giannelli G, et al. TGF-beta and the tissue microenvironment: relevance in fibrosis and cancer. Int J Mol Sci. 2018;19(5) https://doi.org/10.3390/ijms19051294.

7. Ringelhan M, Pfister D, O'Connor T, Pikarsky E, Heikenwalder M. The immunology of hepatocellular carcinoma. Nat Immunol. 2018;19(3):222–32. https://doi.org/10.1038/s41590-018-0044-z.

8. Robinson MW, Harmon C, O'Farrelly C. Liver immunology and its role in inflammation and homeostasis. Cell Mol Immunol. 2016;13(3):267–76. https://doi.org/10.1038/cmi.2016.3.

9. Llovet JM, Zucman-Rossi J, Pikarsky E, Sangro B, Schwartz M, Sherman M, et al. Hepatocellular carcinoma. Nat Rev Dis Primers. 2016;2:16018. https://doi.org/10.1038/nrdp.2016.18.

10. Pellicoro A, Ramachandran P, Iredale JP, Fallowfield JA. Liver fibrosis and repair: immune regulation of wound healing in a solid organ. Nat Rev Immunol. 2014;14(3):181–94. https://doi.org/10.1038/nri3623.

11. Mossanen JC, Krenkel O, Ergen C, Govaere O, Liepelt A, Puengel T, et al. Chemokine (C-C motif) receptor 2-positive monocytes aggravate the early phase of acetaminophen-induced acute liver injury. Hepatology. 2016;64(5):1667–82. https://doi.org/10.1002/hep.28682.

12. Lemmers A, Moreno C, Gustot T, Marechal R, Degre D, Demetter P, et al. The interleukin-17 pathway is involved in human alcoholic liver disease. Hepatology. 2009;49(2):646–57. https://doi.org/10.1002/hep.22680.

13. Meng F, Wang K, Aoyama T, Grivennikov SI, Paik Y, Scholten D, et al. Interleukin-17 signaling in inflammatory, Kupffer cells, and hepatic stellate cells exacerbates liver fibrosis in mice. Gastroenterology. 2012;143(3):765–76 e3. https://doi.org/10.1053/j.gastro.2012.05.049.

14. Tu T, Calabro SR, Lee A, Maczurek AE, Budzinska MA, Warner FJ, et al. Hepatocytes in liver injury: victim, bystander, or accomplice in progressive fibrosis? J Gastroenterol Hepatol. 2015;30(12):1696–704. https://doi.org/10.1111/jgh.13065.

15. Mederacke I, Hsu CC, Troeger JS, Huebener P, Mu X, Dapito DH, et al. Fate tracing reveals hepatic stellate cells as dominant contributors to liver fibrosis independent of its aetiology. Nat Commun. 2013;4:2823. https://doi.org/10.1038/ncomms3823.

16. Maeda S, Kamata H, Luo JL, Leffert H, Karin M. IKKbeta couples hepatocyte death to cytokine-driven compensatory proliferation that promotes chemical hepatocarcinogenesis. Cell. 2005;121(7):977–90. https://doi.org/10.1016/j.cell.2005.04.014.

17. Akira S, Nishio Y, Inoue M, Wang XJ, Wei S, Matsusaka T, et al. Molecular cloning of APRF, a novel IFN-stimulated gene factor 3 p91-related transcription factor involved in the gp130-mediated signaling pathway. Cell. 1994;77(1):63–71.

18. Mackey-Lawrence NM, Petri WA Jr. Leptin and mucosal immunity. Mucosal Immunol. 2012;5(5):472–9. https://doi.org/10.1038/mi.2012.40.

19. McCartney EM, Helbig KJ, Narayana SK, Eyre NS, Aloia AL, Beard MR. Signal transducer and activator of transcription 3 is a proviral host factor for hepatitis C virus. Hepatology. 2013;58(5):1558–68. https://doi.org/10.1002/hep.26496.

20. Haybaeck J, Zeller N, Wolf MJ, Weber A, Wagner U, Kurrer MO, et al. A lymphotoxin-driven pathway to hepatocellular carcinoma. Cancer Cell. 2009;16(4):295–308. https://doi.org/10.1016/j.ccr.2009.08.021.

21. Wolf MJ, Adili A, Piotrowitz K, Abdullah Z, Boege Y, Stemmer K, et al. Metabolic activation of intrahepatic CD8+ T cells and NKT cells causes nonalcoholic steatohepatitis and liver cancer via cross-talk with hepatocytes. Cancer Cell. 2014;26(4):549–64. https://doi.org/10.1016/j.ccell.2014.09.003.

22. Kang TW, Yevsa T, Woller N, Hoenicke L, Wuestefeld T, Dauch D, et al. Senescence surveillance of pre-malignant hepatocytes limits liver cancer development. Nature. 2011;479(7374):547–51. https://doi.org/10.1038/nature10599.

23. Ma C, Kesarwala AH, Eggert T, Medina-Echeverz J, Kleiner DE, Jin P, et al. NAFLD causes selective CD4(+) T lymphocyte loss and promotes hepatocarcinogenesis. Nature. 2016;531(7593):253–7. https://doi.org/10.1038/nature16969.

24. van der Windt DJ, Sud V, Zhang H, Varley PR, Goswami J, Yazdani HO, et al. Neutrophil extracellular traps promote inflammation and development of hepatocellular carcinoma in nonalcoholic steatohepatitis. Hepatology. 2018;68(4):1347–60. https://doi.org/10.1002/hep.29914.

25. Jiang R, Tang J, Chen Y, Deng L, Ji J, Xie Y, et al. The long noncoding RNA lnc-EGFR stimulates T-regulatory cells differentiation thus promoting hepatocellular carcinoma immune evasion. Nat Commun. 2017;8:15129. https://doi.org/10.1038/ncomms15129.

26. Li K, Liu H, Guo T. Th17/Treg imbalance is an indicator of liver cirrhosis process and a risk factor for HCC occurrence in HBV patients. Clin Res Hepatol Gastroenterol. 2017;41(4):399–407. https://doi.org/10.1016/j.clinre.2016.12.004.

27. Read S, Malmstrom V, Powrie F. Cytotoxic T lymphocyte-associated antigen 4 plays an essential role in the function of CD25(+)CD4(+) regulatory cells that control intestinal inflammation. J Exp Med. 2000;192(2):295–302.

28. Nishimura H, Nose M, Hiai H, Minato N, Honjo T. Development of lupus-like autoimmune diseases by disruption of the PD-1 gene encoding an ITIM motif-carrying immunoreceptor. Immunity. 1999;11(2):141–51.

29. Carter L, Fouser LA, Jussif J, Fitz L, Deng B, Wood CR, et al. PD-1:PD-L inhibitory pathway affects both CD4(+) and CD8(+) T cells and is overcome by IL-2. Eur J Immunol. 2002;32(3):634–43. https://doi.org/10.1002/1521-4141(200203)32:3<634::AID-IMMU634>3.0.CO;2-9.

30. Knolle P, Schlaak J, Uhrig A, Kempf P, Meyer zum Buschenfelde KH, Gerken G. Human Kupffer cells secrete IL-10 in response to lipopolysaccharide (LPS) challenge. J Hepatol. 1995;22(2):226–9.

31. Hattori E, Okumoto K, Adachi T, Takeda T, Ito J, Sugahara K, et al. Possible contribution of circulating interleukin-10 (IL-10) to anti-tumor immunity and prognosis in patients with unresectable hepatocellular carcinoma. Hepatol Res. 2003;27(4):309–14.

32. Moore KW, de Waal Malefyt R, Coffman RL, O'Garra A. Interleukin-10 and the interleukin-10 receptor. Annu Rev Immunol. 2001;19:683–765. https://doi.org/10.1146/annurev.immunol.19.1.683.

33. Kehrl JH, Wakefield LM, Roberts AB, Jakowlew S, Alvarez-Mon M, Derynck R, et al. Production of transforming growth factor beta by human T lymphocytes and its potential role in the regulation of T cell growth. J Exp Med. 1986;163(5):1037–50.

34. Espevik T, Waage A, Faxvaag A, Shalaby MR. Regulation of interleukin-2 and interleukin-6 production from T-cells: involvement of interleukin-1 beta and transforming growth factor-beta. Cell Immunol. 1990;126(1):47–56.

35. Smyth MJ, Strobl SL, Young HA, Ortaldo JR, Ochoa AC. Regulation of lymphokine-activated killer activity and pore-forming protein gene expression in human peripheral blood CD8+ T lymphocytes. Inhibition by transforming growth factor-beta. J Immunol. 1991;146(10):3289–97.

36. Othman MS, Aref AM, Mohamed AA, Ibrahim WA. Serum levels of Interleukin-6 and Interleukin-10 as biomarkers for hepatocellular carcinoma in Egyptian patients. ISRN Hepatol. 2013;2013:412317. https://doi.org/10.1155/2013/412317.

37. El-Emshaty HM, Nasif WA, Mohamed IE. Serum cytokine of IL-10 and IL-12 in chronic liver disease: the immune and inflammatory response. Dis Markers. 2015;2015:707254. https://doi.org/10.1155/2015/707254.

38. Thomson AW, Knolle PA. Antigen-presenting cell function in the tolerogenic liver environment. Nat Rev Immunol. 2010;10(11):753–66. https://doi.org/10.1038/nri2858.

39. Chen DS, Mellman I. Oncology meets immunology: the cancer-immunity cycle. Immunity. 2013;39(1):1–10. https://doi.org/10.1016/j.immuni.2013.07.012.

40. Nakajima T, Mizushima N, Kanai K. Relationship between natural killer activity and development of hepatocellular carcinoma in patients with cirrhosis of the liver. Jpn J Clin Oncol. 1987;17(4):327–32.

41. Garnelo M, Tan A, Her Z, Yeong J, Lim CJ, Chen J, et al. Interaction between tumour-infiltrating B cells and T cells controls the progression of hepatocellular carcinoma. Gut. 2017;66(2):342–51. https://doi.org/10.1136/gutjnl-2015-310814.

42. Condeelis J, Pollard JW. Macrophages: obligate partners for tumor cell migration, invasion, and metastasis. Cell. 2006;124(2):263–6. https://doi.org/10.1016/j.cell.2006.01.007.

43. Degroote H, Van Dierendonck A, Geerts A, Van Vlierberghe H, Devisscher L. Preclinical and clinical therapeutic strategies affecting tumor-associated macrophages in hepatocellular carcinoma. J Immunol Res. 2018;2018:7819520. https://doi.org/10.1155/2018/7819520.

44. Wu K, Kryczek I, Chen L, Zou W, Welling TH. Kupffer cell suppression of CD8+ T cells in human hepatocellular carcinoma is mediated by B7-H1/programmed death-1 interactions. Cancer Res. 2009;69(20):8067–75. https://doi.org/10.1158/0008-5472.CAN-09-0901.

45. Tacke F. Targeting hepatic macrophages to treat liver diseases. J Hepatol. 2017;66(6):1300–12. https://doi.org/10.1016/j.jhep.2017.02.026.

46. Yeung OW, Lo CM, Ling CC, Qi X, Geng W, Li CX, et al. Alternatively activated (M2) macrophages promote tumour growth and invasiveness in hepatocellular carcinoma. J Hepatol. 2015;62(3):607–16. https://doi.org/10.1016/j.jhep.2014.10.029.

47. Ding T, Xu J, Wang F, Shi M, Zhang Y, Li SP, et al. High tumor-infiltrating macrophage density predicts poor prognosis in patients with primary hepatocellular carcinoma after resection. Hum Pathol. 2009;40(3):381–9. https://doi.org/10.1016/j.humpath.2008.08.011.

48. Banchereau J, Steinman RM. Dendritic cells and the control of immunity. Nature. 1998;392(6673):245–52. https://doi.org/10.1038/32588.

49. Steinman RM, Hemmi H. Dendritic cells: translating innate to adaptive immunity. Curr Top Microbiol Immunol. 2006;311:17–58.

50. Almand B, Resser JR, Lindman B, Nadaf S, Clark JI, Kwon ED, et al. Clinical significance of defective dendritic cell differentiation in cancer. Clin Cancer Res. 2000;6(5):1755–66.

51. Steinman RM. Lasker Basic Medical Research Award. Dendritic cells: versatile controllers of the immune system. Nat Med. 2007;13(10):1155–9. https://doi.org/10.1038/nm1643.

52. Kakumu S, Ito S, Ishikawa T, Mita Y, Tagaya T, Fukuzawa Y, et al. Decreased function of peripheral blood dendritic cells in patients with hepatocellular carcinoma with hepatitis B and C virus infection. J Gastroenterol Hepatol. 2000;15(4):431–6.

53. Chen S, Akbar SM, Tanimoto K, Ninomiya T, Iuchi H, Michitaka K, et al. Absence of CD83-positive mature and activated dendritic cells at cancer nodules from patients with hepatocellular carcinoma: relevance to hepatocarcinogenesis. Cancer Lett. 2000;148(1):49–57.

54. Ormandy LA, Farber A, Cantz T, Petrykowska S, Wedemeyer H, Horning M, et al. Direct ex vivo analysis of dendritic cells in patients with hepatocellular carcinoma. World J Gastroenterol. 2006;12(20):3275–82.

55. Gabrilovich DI, Chen HL, Girgis KR, Cunningham HT, Meny GM, Nadaf S, et al. Production of vascular endothelial growth factor by human tumors inhibits the functional maturation of dendritic cells. Nat Med. 1996;2(10):1096–103.

56. Beckebaum S, Zhang X, Chen X, Yu Z, Frilling A, Dworacki G, et al. Increased levels of interleukin-10 in serum from patients with hepatocellular carcinoma correlate with profound

numerical deficiencies and immature phenotype of circulating dendritic cell subsets. Clin Cancer Res. 2004;10(21):7260–9. https://doi.org/10.1158/1078-0432.CCR-04-0872.

57. Maier KP. Cirrhosis of the liver as a precancerous condition. Praxis (Bern 1994). 1998;87(44):1462–5.

58. Singal AG, El-Serag HB. Hepatocellular carcinoma from epidemiology to prevention: translating knowledge into practice. Clin Gastroenterol Hepatol. 2015;13(12):2140–51. https://doi.org/10.1016/j.cgh.2015.08.014.

59. El-Serag HB. Hepatocellular carcinoma. N Engl J Med. 2011;365(12):1118–27. https://doi.org/10.1056/NEJMra1001683.

60. Affo S, Yu LX, Schwabe RF. The role of cancer-associated fibroblasts and fibrosis in liver cancer. Annu Rev Pathol. 2017;12:153–86. https://doi.org/10.1146/annurev-pathol-052016-100322.

61. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell. 2011;144(5):646–74. https://doi.org/10.1016/j.cell.2011.02.013.

62. Bissell MJ, Hines WC. Why don't we get more cancer? A proposed role of the microenvironment in restraining cancer progression. Nat Med. 2011;17(3):320–9. https://doi.org/10.1038/nm.2328.

63. Ji J, Eggert T, Budhu A, Forgues M, Takai A, Dang H, et al. Hepatic stellate cell and monocyte interaction contributes to poor prognosis in hepatocellular carcinoma. Hepatology. 2015;62(2):481–95. https://doi.org/10.1002/hep.27822.

64. Zhao W, Su W, Kuang P, Zhang L, Liu J, Yin Z, et al. The role of hepatic stellate cells in the regulation of T-cell function and the promotion of hepatocellular carcinoma. Int J Oncol. 2012;41(2):457–64. https://doi.org/10.3892/ijo.2012.1497.

65. Zhao W, Zhang L, Yin Z, Su W, Ren G, Zhou C, et al. Activated hepatic stellate cells promote hepatocellular carcinoma development in immunocompetent mice. Int J Cancer. 2011;129(11):2651–61. https://doi.org/10.1002/ijc.25920.

66. Cheng Y, Li H, Deng Y, Tai Y, Zeng K, Zhang Y, et al. Cancer-associated fibroblasts induce PDL1+ neutrophils through the IL6-STAT3 pathway that foster immune suppression in hepatocellular carcinoma. Cell Death Dis. 2018;9(4):422. https://doi.org/10.1038/s41419-018-0458-4.

67. Zhang Z, Li X, Sun W, Yue S, Yang J, Li J, et al. Loss of exosomal miR-320a from cancer-associated fibroblasts contributes to HCC proliferation and metastasis. Cancer Lett. 2017;397:33–42. https://doi.org/10.1016/j.canlet.2017.03.004.

68. Wang F, Li L, Piontek K, Sakaguchi M, Selaru FM. Exosome miR-335 as a novel therapeutic strategy in hepatocellular carcinoma. Hepatology. 2018;67(3):940–54. https://doi.org/10.1002/hep.29586.

69. Torimura T, Ueno T, Kin M, Harada R, Taniguchi E, Nakamura T, et al. Overexpression of angiopoietin-1 and angiopoietin-2 in hepatocellular carcinoma. J Hepatol. 2004;40(5):799–807. https://doi.org/10.1016/j.jhep.2004.01.027.

70. Taura K, De Minicis S, Seki E, Hatano E, Iwaisako K, Osterreicher CH, et al. Hepatic stellate cells secrete angiopoietin 1 that induces angiogenesis in liver fibrosis. Gastroenterology. 2008;135(5):1729–38. https://doi.org/10.1053/j.gastro.2008.07.065.

71. Lin N, Chen Z, Lu Y, Li Y, Hu K, Xu R. Role of activated hepatic stellate cells in proliferation and metastasis of hepatocellular carcinoma. Hepatol Res. 2015;45(3):326–36. https://doi.org/10.1111/hepr.12356.

72. Santamato A, Fransvea E, Dituri F, Caligiuri A, Quaranta M, Niimi T, et al. Hepatic stellate cells stimulate HCC cell migration via laminin-5 production. Clin Sci (Lond). 2011;121(4):159–68. https://doi.org/10.1042/CS20110002.

73. Okabe H, Beppu T, Ueda M, Hayashi H, Ishiko T, Masuda T, et al. Identification of CXCL5/ENA-78 as a factor involved in the interaction between cholangiocarcinoma cells and cancer-associated fibroblasts. Int J Cancer. 2012;131(10):2234–41. https://doi.org/10.1002/ijc.27496.

74. Fang T, Lv H, Lv G, Li T, Wang C, Han Q, et al. Tumor-derived exosomal miR-1247-3p induces cancer-associated fibroblast activation to foster lung metastasis of liver cancer. Nat Commun. 2018;9(1):191. https://doi.org/10.1038/s41467-017-02583-0.

75. Jiang J, Ye F, Yang X, Zong C, Gao L, Yang Y, et al. Peri-tumor associated fibroblasts promote intrahepatic metastasis of hepatocellular carcinoma by recruiting cancer stem cells. Cancer Lett. 2017;404:19–28. https://doi.org/10.1016/j.canlet.2017.07.006.

76. Liu C, Liu L, Chen X, Cheng J, Zhang H, Zhang C, et al. LSD1 stimulates cancer-associated fibroblasts to drive Notch3-dependent self-renewal of liver cancer stem-like cells. Cancer Res. 2018;78(4):938–49. https://doi.org/10.1158/0008-5472.CAN-17-1236.

77. Rhee H, Kim HY, Choi JH, Woo HG, Yoo JE, Nahm JH, et al. Keratin 19 expression in hepatocellular carcinoma is regulated by fibroblast-derived HGF via a MET-ERK1/2-AP1 and SP1 Axis. Cancer Res. 2018;78(7):1619–31. https://doi.org/10.1158/0008-5472.CAN-17-0988.

78. Kim H, Choi GH, Na DC, Ahn EY, Kim GI, Lee JE, et al. Human hepatocellular carcinomas with "Stemness"-related marker expression: keratin 19 expression and a poor prognosis. Hepatology. 2011;54(5):1707–17. https://doi.org/10.1002/hep.24559.

79. Coulouarn C, Corlu A, Glaise D, Guenon I, Thorgeirsson SS, Clement B. Hepatocyte-stellate cell cross-talk in the liver engenders a permissive inflammatory microenvironment that drives progression in hepatocellular carcinoma. Cancer Res. 2012;72(10):2533–42. https://doi.org/10.1158/0008-5472.CAN-11-3317.

80. Ju MJ, Qiu SJ, Fan J, Xiao YS, Gao Q, Zhou J, et al. Peritumoral activated hepatic stellate cells predict poor clinical outcome in hepatocellular carcinoma after curative resection. Am J Clin Pathol. 2009;131(4):498–510. https://doi.org/10.1309/AJCP86PPBNGOHNNL.

81. Zhang DY, Goossens N, Guo J, Tsai MC, Chou HI, Altunkaynak C, et al. A hepatic stellate cell gene expression signature associated with outcomes in hepatitis C cirrhosis and hepatocellular carcinoma after curative resection. Gut. 2016;65(10):1754–64. https://doi.org/10.1136/gutjnl-2015-309655.

82. Pupulim LF, Vilgrain V, Ronot M, Becker CD, Breguet R, Terraz S. Hepatic lymphatics: anatomy and related diseases. Abdom Imaging. 2015;40(6):1997–2011. https://doi.org/10.1007/s00261-015-0350-y.

83. Lund AW, Wagner M, Fankhauser M, Steinskog ES, Broggi MA, Spranger S, et al. Lymphatic vessels regulate immune microenvironments in human and murine melanoma. J Clin Invest. 2016;126(9):3389–402. https://doi.org/10.1172/JCI79434.

84. Swartz MA. Immunomodulatory roles of lymphatic vessels in cancer progression. Cancer Immunol Res. 2014;2(8):701–7. https://doi.org/10.1158/2326-6066.CIR-14-0115.

85. Lukacs-Kornek V, Malhotra D, Fletcher AL, Acton SE, Elpek KG, Tayalia P, et al. Regulated release of nitric oxide by nonhematopoietic stroma controls expansion of the activated T cell pool in lymph nodes. Nat Immunol. 2011;12(11):1096–104. https://doi.org/10.1038/ni.2112.

86. Fletcher AL, Lukacs-Kornek V, Reynoso ED, Pinner SE, Bellemare-Pelletier A, Curry MS, et al. Lymph node fibroblastic reticular cells directly present peripheral tissue antigen under steady-state and inflammatory conditions. J Exp Med. 2010;207(4):689–97. https://doi.org/10.1084/jem.20092642.

87. Rouhani SJ, Eccles JD, Riccardi P, Peske JD, Tewalt EF, Cohen JN, et al. Roles of lymphatic endothelial cells expressing peripheral tissue antigens in CD4 T-cell tolerance induction. Nat Commun. 2015;6:6771. https://doi.org/10.1038/ncomms7771.

88. Vollmar B, Wolf B, Siegmund S, Katsen AD, Menger MD. Lymph vessel expansion and function in the development of hepatic fibrosis and cirrhosis. Am J Pathol. 1997;151(1):169–75.

89. Yamauchi Y, Michitaka K, Onji M. Morphometric analysis of lymphatic and blood vessels in human chronic viral liver diseases. Am J Pathol. 1998;153(4):1131–7. https://doi.org/10.1016/S0002-9440(10)65657-X.

90. Yokomori H, Oda M, Kaneko F, Kawachi S, Tanabe M, Yoshimura K, et al. Lymphatic marker podoplanin/D2-40 in human advanced cirrhotic liver--re-evaluations of microlymphatic abnormalities. BMC Gastroenterol. 2010;10:131. https://doi.org/10.1186/1471-230X-10-131.

91. Limatola E, Filosa S. Exogenous vitellogenesis and micropinocytosis in the lizard, Podarcis sicula, treated with follicle-stimulating hormone. Gen Comp Endocrinol. 1989;75(2):165–76.

92. Shields JD, Kourtis IC, Tomei AA, Roberts JM, Swartz MA. Induction of lymphoid-like stroma and immune escape by tumors that express the chemokine CCL21. Science. 2010;328(5979):749–52. https://doi.org/10.1126/science.1185837.

93. Xiang Z, Zeng Z, Tang Z, Fan J, Sun H, Wu W, et al. Increased expression of vascular endothelial growth factor-C and nuclear CXCR4 in hepatocellular carcinoma is correlated with lymph node metastasis and poor outcome. Cancer J. 2009;15(6):519–25. https://doi.org/10.1097/PPO.0b013e3181c6aa6b.

94. Yamaguchi R, Yano H, Nakashima O, Akiba J, Nishida N, Kurogi M, et al. Expression of vascular endothelial growth factor-C in human hepatocellular carcinoma. J Gastroenterol Hepatol. 2006;21(1 Pt 1):152–60. https://doi.org/10.1111/j.1440-1746.2005.04217.x.

95. Nieto MA, Huang RY, Jackson RA, Thiery JP. Emt: 2016. Cell. 2016;166(1):21–45. https://doi.org/10.1016/j.cell.2016.06.028.

96. Dongre A, Weinberg RA. New insights into the mechanisms of epithelial-mesenchymal transition and implications for cancer. Nat Rev Mol Cell Biol. 2018; https://doi.org/10.1038/s41580-018-0080-4.

97. Polyak K, Weinberg RA. Transitions between epithelial and mesenchymal states: acquisition of malignant and stem cell traits. Nat Rev Cancer. 2009;9(4):265–73. https://doi.org/10.1038/nrc2620.

98. Acloque H, Adams MS, Fishwick K, Bronner-Fraser M, Nieto MA. Epithelial-mesenchymal transitions: the importance of changing cell state in development and disease. J Clin Invest. 2009;119(6):1438–49. https://doi.org/10.1172/JCI38019.

99. Yan L, Xu F, Dai CL. Relationship between epithelial-to-mesenchymal transition and the inflammatory microenvironment of hepatocellular carcinoma. J Exp Clin Cancer Res. 2018;37(1):203. https://doi.org/10.1186/s13046-018-0887-z.

100. Zavadil J, Bottinger EP. TGF-beta and epithelial-to-mesenchymal transitions. Oncogene. 2005;24(37):5764–74. https://doi.org/10.1038/sj.onc.1208927.

101. Choi SS, Diehl AM. Epithelial-to-mesenchymal transitions in the liver. Hepatology. 2009;50(6):2007–13. https://doi.org/10.1002/hep.23196.

102. Zhai B, Yan HX, Liu SQ, Chen L, Wu MC, Wang HY. Reduced expression of E-cadherin/catenin complex in hepatocellular carcinomas. World J Gastroenterol. 2008;14(37):5665–73.

103. Battaglia S, Benzoubir N, Nobilet S, Charneau P, Samuel D, Zignego AL, et al. Liver cancer-derived hepatitis C virus core proteins shift TGF-beta responses from tumor suppression to epithelial-mesenchymal transition. PLoS One. 2009;4(2):e4355. https://doi.org/10.1371/journal.pone.0004355.

104. Fuchs BC, Fujii T, Dorfman JD, Goodwin JM, Zhu AX, Lanuti M, et al. Epithelial-to-mesenchymal transition and integrin-linked kinase mediate sensitivity to epidermal growth factor receptor inhibition in human hepatoma cells. Cancer Res. 2008;68(7):2391–9. https://doi.org/10.1158/0008-5472.CAN-07-2460.

105. Llovet JM, Ricci S, Mazzaferro V, Hilgard P, Gane E, Blanc JF, et al. Sorafenib in advanced hepatocellular carcinoma. N Engl J Med. 2008;359(4):378–90. https://doi.org/10.1056/NEJMoa0708857.

106. Kudo M, Finn RS, Qin S, Han KH, Ikeda K, Piscaglia F, et al. Lenvatinib versus sorafenib in first-line treatment of patients with unresectable hepatocellular carcinoma: a randomised phase 3 non-inferiority trial. Lancet. 2018;391(10126):1163–73. https://doi.org/10.1016/S0140-6736(18)30207-1.

107. Sui H, Ma N, Wang Y, Li H, Liu X, Su Y, et al. Anti-PD-1/PD-L1 therapy for non-small-cell lung cancer: toward personalized medicine and combination strategies. J Immunol Res. 2018;2018:6984948. https://doi.org/10.1155/2018/6984948.

108. Herzberg B, Fisher DE. Metastatic melanoma and immunotherapy. Clin Immunol. 2016;172:105–10. https://doi.org/10.1016/j.clim.2016.07.006.

109. Killock D. Immunotherapy: Nivolumab keeps HCC in check and opens avenues for checkmate. Nat Rev Clin Oncol. 2017;14(7):392. https://doi.org/10.1038/nrclinonc.2017.70.

110. Zhu AX, Finn RS, Edeline J, Cattan S, Ogasawara S, Palmer D, et al. Pembrolizumab in patients with advanced hepatocellular carcinoma previously treated with sorafenib (KEYNOTE-224): a non-randomised, open-label phase 2 trial. Lancet Oncol. 2018;19(7):940–52. https://doi.org/10.1016/S1470-2045(18)30351-6.
111. Shang N, Figini M, Shangguan J, Wang B, Sun C, Pan L, et al. Dendritic cells based immunotherapy. Am J Cancer Res. 2017;7(10):2091–102.
112. Palmer DH, Midgley RS, Mirza N, Torr EE, Ahmed F, Steele JC, et al. A phase II study of adoptive immunotherapy using dendritic cells pulsed with tumor lysate in patients with hepatocellular carcinoma. Hepatology. 2009;49(1):124–32. https://doi.org/10.1002/hep.22626.
113. El Ansary M, Mogawer S, Elhamid SA, Alwakil S, Aboelkasem F, Sabaawy HE, et al. Immunotherapy by autologous dendritic cell vaccine in patients with advanced HCC. J Cancer Res Clin Oncol. 2013;139(1):39–48. https://doi.org/10.1007/s00432-012-1298-8.

Annex VIII: Rewiring Host Signaling: Hepatitis C Virus in Liver Pathogenesis

Book chapter

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My main contribution was to conceptualize and write the book chapter.

Rewiring Host Signaling: Hepatitis C Virus in Liver Pathogenesis

Alessia Virzì,[1,2] Armando Andres Roca Suarez,[1,2] Thomas F. Baumert,[1,2,3,4] and Joachim Lupberger[1,2]

[1]Inserm, U1110, Institut de Recherche sur les Maladies Virales et Hépatiques, 67000 Strasbourg, France

[2]Université de Strasbourg, 67000 Strasbourg, France

[3]Institut Hospitalo-Universitaire, Pôle Hépato-digestif, Nouvel Hôpital Civil, 67000 Strasbourg, France

[4]Institut Universitaire de France (IUF), 75231 Paris, France

Correspondence: joachim.lupberger@unistra.fr

Hepatitis C virus (HCV) is a major cause of liver disease including metabolic disease, fibrosis, cirrhosis, and hepatocellular carcinoma (HCC). HCV induces and promotes liver disease progression by perturbing a range of survival, proliferative, and metabolic pathways within the proinflammatory cellular microenvironment. The recent breakthrough in antiviral therapy using direct-acting antivirals (DAAs) can cure >90% of HCV patients. However, viral cure cannot fully eliminate the HCC risk, especially in patients with advanced liver disease or comorbidities. HCV induces an epigenetic viral footprint that promotes a pro-oncogenic hepatic signature, which persists after DAA cure. In this review, we summarize the main signaling pathways deregulated by HCV infection, with potential impact on liver pathogenesis. HCV-induced persistent signaling patterns may serve as biomarkers for the stratification of HCV-cured patients at high risk of developing HCC. Moreover, these signaling pathways are potential targets for novel chemopreventive strategies.

Hepatitis C virus (HCV) is a main cause of chronic liver disease worldwide. Chronic HCV infection causes chronic hepatic inflammation, steatosis, and fibrosis, which progresses to cirrhosis and hepatocellular carcinoma (HCC) (Polaris Observatory HCV Collaborators 2017). HCC is the most common type of liver cancer and the second leading cause of cancer-related death on the globe (Baumert and Hoshida 2019). The liver is an extraordinarily resistant organ with a unique regeneration capacity, but the persistent stress induced by chronic inflammation and deregulation of signaling and metabolism culminate in a >10-fold increased HCC risk in HCV-infected patients compared with HCV-negative subjects in cross-sectional and case-control studies (El-Serag 2012). The rate of HCC among HCV-infected persons ranges from 1% to 3% and the interval from infection to HCC has been estimated to be ~30 years (Thrift et al. 2017). It is believed that a combination of direct (viral proteins) and indirect (chronic inflammation, deregulated signaling) factors are responsible for HCV-induced

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liver disease development and progression. Because of the absence of a latent phase in the viral life cycle or any DNA integration event, HCV must ensure an optimal condition to maintain its replication (Lupberger et al. 2019) and to escape from the host innate immune response (Gale and Foy 2005). In this review, we summarize the main pathways that are deregulated during chronic HCV infection, which are relevant for the development and progression of HCV-induced liver disease and HCC. Some of these pathways remain deregulated in HCV-cured patients, serving as potential biomarkers for the identification of risk patients and novel drug targets for chemopreventive clinical strategies.

HCV-INDUCED CHRONIC INFLAMMATION, FIBROSIS, AND CIRRHOSIS

Inflammation is a life-preserving process to maintain cellular homeostasis. It is mostly activated in response to pathogens or tissue injury and is part of a physiological recovery response. The liver harbors a large spectrum of immune cells distributed within the hepatic compartments (Freitas-Lopes et al. 2017). This organ is constantly exposed to external signaling from commensal molecules and produces a series of neo-antigens derived by its metabolic activities. This leads to the development of a constant and physiological immunotolerance state in the organ (Jenne and Kubes 2013), which was first recognized by Calne and coworkers in 1969 (Calne et al. 1969). The relative immunotolerance in the liver is necessary to avoid overactivation of the immune system but it also facilitates the adaptation and persistence of different liver pathogens, such as malaria, hepatitis B virus (HBV), and HCV (Horst et al. 2016). HCV has developed several strategies to evade the innate and adaptive antiviral responses to infection (Gale and Foy 2005; Rosen 2013). Consequently, failure of viral clearance promotes a chronically inflamed liver that leads to scarification (fibrosis), cirrhosis, and ultimately provokes the development of HCC. According to the World Health Organization (see who.int), most of the HCV-infected patients do not achieve viral clearance

and 60%–80% develop chronic hepatic inflammation. In these patients, the risk of developing cirrhosis is ∼15%–35% after 20–30 years of infection (Thrift et al. 2017). The virus directly accelerates the inflammatory response through a large range of interconnected mechanisms, including pathogen pattern recognition, host–viral protein interactions, activation of inflammasomes, and reactive oxygen species (ROS) production (Gale and Foy 2005; Horner and Gale 2013; Negash et al. 2019). Liver diseases and fibrosis associated with HCV infection evolve in the context of a strong oxidative microenvironment. HCV core, E1, E2, NS3, NS4B, and NS5A are known to encourage the production of ROS (Bureau et al. 2001; Pal et al. 2010; Ivanov et al. 2011). The antioxidant defense machine involves different ROS scavenging enzymes and their synthesis depends on many genes commonly regulated by the transcription factor NF-E2-related factor 2 (Nrf2) (Bureau et al. 2001). Nrf2 expression is inversely correlated with the severity of liver injury in chronic HCV patients and is impaired in end-stage liver disease (Kurzawski et al. 2012; Jiang et al. 2015). In HCV-positive cells, free Nrf2 is trapped at the replicon complexes and is therefore prevented from its entry into the nucleus (Medvedev et al. 2017). This observation is in line with impaired expression levels of antioxidative enzymes like catalase (Lupberger et al. 2019) and superoxide dismutase SOD1 (Levent et al. 2006; Diamond et al. 2012) in infected hepatocytes, which further promote oxidative stress damaging host proteins, lipids, and DNA. This coincides with a perturbed endogenous DNA repair by HCV infection (Nguyen et al. 2018; Lupberger et al. 2019) further contributing to the development of HCC in HCV patients. Because ROS-induced lipid peroxidation hampers viral membrane fusion, HCV has developed strategies to divert oxidative stress, for example, by the modulation of phospholipid hydroperoxide glutathione peroxidase (GPx4) (Brault et al. 2016). Importantly, ROS levels strongly promote liver fibrosis, characterized by an excessive production of extracellular matrix (ECM) and scarring of the tissue (Luangmonkong et al. 2018). At the same time, ROS stimulates pro-oncogenic

signaling pathways, promoting cell survival, proliferation, and angiogenesis (Zhang et al. 2016). Chronic inflammation is accompanied by elevated plasma levels of proinflammatory cytokines, such as tumor necrosis factor α (TNF-α), which are further induced by HCV proteins NS3, NS4, and NS5 (Hosomura et al. 2011; Alhetheel et al. 2016). The levels of liver and blood cytokines are associated with HCV microenvironment and liver fibrosis (de Souza-Cruz et al. 2016). In particular, interleukin (IL)-1α is increased in HCV patients and correlates with liver cirrhosis and HCC (Tawfik et al. 2018). Therefore, HCV-induced cytokine signaling increases the oncogenic pressure within the host cell and contributes to a recalibration of hepatocyte functions (Fig. 1).
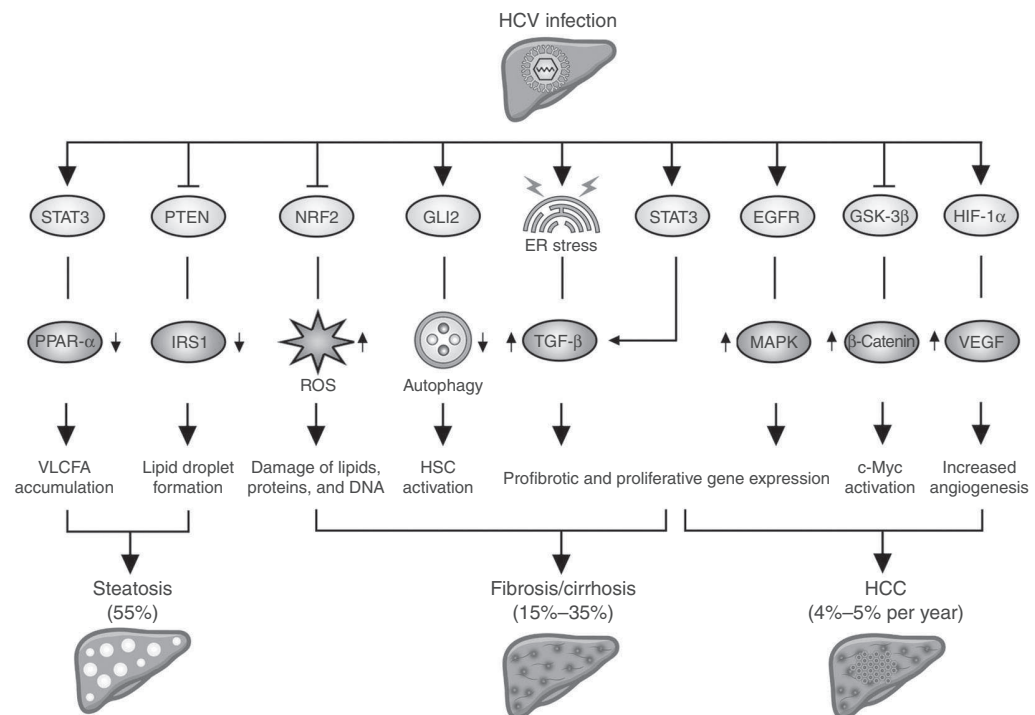

Figure 1. Hepatitis C virus (HCV) infection alters signaling pathways relevant for liver disease. HCV-mediated activation of signal transducer and activator of transcription 3 (STAT3) causes very long-chain fatty acid (VLCFA) accumulation in the infected hepatocytes via down-regulation of peroxisome proliferator-activated receptor α (PPAR-α) expression. STAT3 activation sustains profibrotic gene expression via up-regulation of transforming growth factor β (TGF-β). Down-regulation of phosphatase and tensin homolog (PTEN) by HCV decreases insulin receptor substrate 1 (IRS1) expression and the formation of large lipid droplets favoring hepatic steatosis. HCV impairs NF-E2-related factor 2 (NRF2) activity and enhances the accumulation of reactive oxygen species (ROS). Activation of the Hedgehog (Hh) pathway via GLI family zinc finger 2 (GLI2) inhibits autophagy in hepatic stellate cells (HSCs), favoring their conversion into myofibroblasts and the development of fibrosis. HCV infection induces endoplasmic reticulum (ER) stress triggering TGF-β expression. Epithelial growth factor receptor (EGFR) is activated by several mechanisms and induces mitogen-activated protein kinase (MAPK) signaling and the expression of genes related to fibrosis and hepatocyte proliferation. Following HCV infection, the Wnt pathway is activated and inhibits the β-catenin destruction complex. As a consequence, β-catenin migrates to the nucleus and activates c-Myc oncogene. HCV sustains vascular endothelial growth factor (VEGF) via the stabilization of hypoxia inducible factor 1 subunit α (HIF1-α), which consequently up-regulates VEGF signaling and increases angiogenesis. The percentage of infected patients developing steatosis, cirrhosis, or the cumulative incidence of hepatocellular carcinoma (HCC) is indicated. GSK-3β, Glycogen synthase kinase 3β.

HCV Sustains Hedgehog Signaling Pathway and Promotes Fibrogenesis

The Hedgehog (Hh) pathway regulates liver development and differentiation and is a critical modulator of adult liver repair (Ingham and McMahon 2001; Machado and Diehl 2018). Interestingly, stimulation of the Hh pathway results in increased permissiveness for HCV replication in cell culture (Choi et al. 2011). HCV activates Hh signaling during fibrogenic repair of liver damage and increases the production of Hh ligands in HCV-infected cells (de Almeida Pereira et al. 2010). Complementary studies confirm that HCV derived from the sera of HCV-infected patients stimulates Hh signaling in human primary fibroblasts via activation of zinc finger protein GLI2 transcription factor. Especially, GLI2 inhibits autophagy in fibroblasts, thus forcing their conversion into myofibroblasts, which promotes fibrogenesis (Granato et al. 2016). The increase in Hh ligands may additionally be sustained by the accumulation of liver damage markers, such as epithelial growth factor (EGF), transforming growth factor β (TGF-β), and platelet-derived growth factor (PDGF) (Stepan et al. 2005; Jung et al. 2008; Omenetti et al. 2008), creating a persistent proliferative and antiapoptotic environment in the infected liver.

HCV Modulates Activation of the TGF-β Pathway

TGF-β has a key role in fibrogenesis and it is involved in all stages of liver disease progression (Dooley and ten Dijke 2012; Fabregat et al. 2016). The TGF-β superfamily includes pleiotropic growth factors that are essential for embryonic development and organ homeostasis. TGF-β is responsible for cell proliferation, differentiation, and migration during embryogenesis, while it is involved in tissue regeneration, cell growth control, and remodeling throughout adulthood. Under certain conditions, TGF-β1 is also involved in the induction of apoptotic cell death in the liver (Oberhammer et al. 1992). The TGF-β cytokine is physiologically sequestered in the ECM as part of latent complexes and it is released in response to different environmental perturbations (Xu et al. 2018). This cytokine triggers downstream signaling through the activation of canonical and noncanonical pathways. First, TGF-β mediates the formation of a heterotrimeric complex of type I and type II serine/threonine kinase receptors, which phosphorylate receptor-associated SMAD (R-SMADs) proteins. The trimeric complex formed by R-SMADs (Smad2 and Smad3) and Smad4 enters the nucleus and regulates gene expression (Miyazawa et al. 2002). Second, TGF-β triggers other signaling pathways, such as mitogen-activated protein kinase (MAPK) and transforming protein RhoA cascades, even in absence of SMADs activation (Yu et al. 2002; Derynck and Zhang 2003). In addition, both canonical and noncanonical signaling pathways can be modulated by TGF-β to tightly control epithelial-to-mesenchymal transition (EMT) (Bhowmick et al. 2001; Katsuno et al. 2019), which is a physiopathological program implicated in liver disease progression (Thiery and Sleeman 2006). TGF-β1 triggers hepatic fibrosis and cirrhosis in both animal models and human hepatic disorders (Castilla et al. 1991; Bedossa et al. 1995; Sanderson et al. 1995), and thus most evidently also plays an important role during HCV pathogenesis. Several studies and clinical observations highlighted a clear correlation between TGF-β and chronic HCV infection (Nelson et al. 1997; Grüngreiff et al. 1999; Ray et al. 2003; Chen et al. 2017). TGF-β plasma levels are associated with a high degree of hepatic fibrosis in patients with chronic HCV (Tsushima et al. 1999; Flisiak et al. 2002). Notably, HCV core protein seems to up-regulate the transcription of TGF-β (Taniguchi et al. 2004). HCV induces TGF-β1 via endoplasmic reticulum stress activation and the unfolded protein response (UPR) (Chusri et al. 2016). Additionally, in vitro studies show that HCV-induced oxidative stress indirectly regulates TGF-β1 expression through p38 MAPK, c-jun amino-terminal kinase (JNK), and extracellular signal-regulated kinase (ERK) via nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB) signaling (Erhardt et al. 2002; Lin et al. 2010). More recent studies observed decreased TGF-β1 levels in the serum of chronic HCV-infected patients that achieved sustained virologic response (SVR) after antiviral treatment (Janczewska-Kazek et al. 2006; Kotsiri

et al. 2016). Therefore, uncovering the role of HCV proteins in TGF-β signaling pathways may contribute to understanding the mechanisms involved in HCV-induced HCC. Indeed, HCV core and NS3 have been shown to interact with Smad3 in vitro and in vivo (Cheng et al. 2004). Interestingly, some HCV core variants isolated from HCC tissue interact with Smad3 and inhibit TGF-β signaling. According to this study, a possible selection of viral variants during chronic HCV infection gradually promotes antiapoptotic effects in the liver that overcome the initial antiproliferative functions of TGF-β (Cheng et al. 2004). Hence, although TGF-β may have proapoptotic effects during the early stages of chronic liver disease, it probably acquires pro-cancerogenic responses after HCV core variants selection (Pavio et al. 2005; Battaglia et al. 2009).

HCV-Induced IL-6/STAT3 Signaling

Signal transducer and activator of transcription 3 (STAT3) is involved in tissue repair mechanisms by the regulation of proliferative and prosurvival cellular programs. In this context, activation of STAT3 can be induced by a vast number of different cytokines, including IL-6, which sensitizes hepatocytes to regenerative signals (Michalopoulos 2007). Beyond its physiological role, persistent activation of STAT3 induces chronic inflammation and fibrosis, increasing the risk to develop severe pathological conditions (Yu et al. 2014; Kasembeli et al. 2018). HCV requires IL-6/STAT3 signaling to maintain infection (Lupberger et al. 2013; McCartney et al. 2013); therefore, it induces its activation by several mechanisms. HCV core directly binds and sustains STAT3 activation (Yoshida et al. 2002), whereas the expression of NS5A, E1, and NS3 promotes STAT3 signaling indirectly via ROS production (Gong et al. 2001; Machida et al. 2006). The activation of STAT3 is not limited to HCV-infected hepatocytes. miR19a secreted in endosomes from HCV-infected hepatocytes impairs suppressor of cytokine signaling 3 (SOCS3) in hepatic stellate cells (HSCs). As a negative regulator of STAT3, impaired SOCS3 levels cause a subsequent activation of TGF-β in HSCs (Devhare

et al. 2017). Therefore, considering the profibrotic role of STAT3 signaling and its strong cooperation with the TGF-β pathway, it has been suggested as a potential target for antifibrotic therapies (Chakraborty et al. 2017).

HCV INCREASES CANCER RISK BY DEREGULATION OF ONCOGENIC SIGNALING PATHWAYS

The liver is a key organ for the detoxification and metabolism of a wide range of potentially harmful substances. Therefore, liver regeneration is a tightly controlled process (Cordero-Espinoza and Huch 2018) that converges in the reconstruction of hepatocyte parenchyma in response to damage. The replacement of the damaged tissue occurs mainly through hepatocyte proliferation and to a lesser extent via an activation of ductal progenitor cells. During regeneration, the HSCs differentiate in myofibroblasts that release ECM within the space of Disse. Under normal conditions, the excess of ECM is promptly degraded by matrix metalloproteinases (MMPs), which restore the original architecture and function of the tissue without scar formation (Kholodenko and Yarygin 2017). During chronic inflammation this balance is perturbed, which leads to a progressive deposition of ECM and the development of liver fibrosis. HCV infection causes oxidative stress, steatohepatitis, and fibrosis, which create a hepatic pro-oncogenic environment. The oncogenic pressure on the diseased liver is further promoted by virus-induced growth factors and signaling pathways such as EGF, vascular endothelial growth factor (VEGF), Wnt/β-catenin, which are strongly implicated in the cirrhotic remodeling of the tissue and hepatocarcinogenesis (Fuchs et al. 2014; Wang et al. 2018a; Moon et al. 2019). As a consequence, patients affected with HCV-associated cirrhosis present a 4% to 5% cumulative annual incidence of HCC (El-Serag 2012).

HCV Up-Regulates EGFR and Stimulates MAPK Signaling

The growing knowledge on the interplay between HCV and epithelial growth factor re-

ceptor (EGFR) cascade has markedly contributed to explain the pathologic consequences of the viral infection, such as fibrosis development and HCC (Lupberger et al. 2011, 2013; Fuchs et al. 2014; Roca Suarez et al. 2018). It has been shown that EGFR signaling promotes the formation of the cluster of differentiation 81 (CD81)/claudin1 (CLDN1) coreceptor complex, which is required for HCV entry (Harris et al. 2010; Krieger et al. 2010; Lupberger et al. 2011; Zona et al. 2013). Inhibition of EGFR kinase hampers the CD81/CLDN1 coreceptor association and thus prevents HCV particle entry (Lupberger et al. 2011). The physical link between EGFR kinase and CD81/CLDN1 interaction is mediated by GTPase HRas, activated downstream from the EGFR signaling (Zona et al. 2013). HCV has an interest in maintaining EGFR signaling and elevated EGFR signaling is observed in liver biopsies of HCV patients (Mailly et al. 2015). EGFR signaling is further prolonged by a NS5A-induced retention of activated EGFR in the early endosomal compartment (Mankouri et al. 2008) and by an increasing level of Netrin-1 that impedes EGFR recycling (Plissonnier et al. 2016). Furthermore, NS3/4A protease mediates the down-regulation of T-cell protein tyrosine phosphatase (TC-PTP), which is negative regulator of EGFR and MAPK signaling (Brenndörfer et al. 2009; Stanford et al. 2012). The activation of EGFR during HCV infection induces MAPK signaling (Hayashi et al. 2000; Bürckstümmer et al. 2006; Mankouri et al. 2008; Diao et al. 2012), an evolutionarily conserved mechanism of cellular transduction that regulates many vital cellular functions, such as proliferation, differentiation, survival, and apoptosis (Zhang and Liu 2002; Dhillon et al. 2007). EGFR is overexpressed in ∼50% of patients with chronic HCV and in most patients with cirrhosis and HCC. The extent of EGFR expression is even higher in the advanced stages of HCV-related fibrosis (Badawy et al. 2015). These observations have a potential clinical application because EGF is a major driver of liver disease progression, and inhibition of EGFR signaling using clinical compounds in animal models attenuates the development of liver fibrosis and HCC nodules (Fuchs et al. 2014).

HCV Up-Regulates VEGF and Promotes Angiogenesis

Angiogenesis is a growth factor-dependent program responsible of the formation of new vessels from preexisting ones. It is commonly induced in response to hypoxia-related and inflammatory mechanisms (Paternostro et al. 2010). Hepatic angiogenesis is triggered by HCV via the deregulation of multiple pathways (Hassan et al. 2009). Several studies have shown an up-regulation of VEGF in HCV-related HCC patient tissues (Llovet et al. 2012; Mukozu et al. 2013). The HCV core protein seems to sustain VEGF signaling by several mechanisms. It can lead to hypoxia inducible factor 1 (HIF-1α) stabilization, which consequently up-regulates VEGF expression (Shimoda et al. 1999; Abe et al. 2012; Zhu et al. 2014). Additionally, HCV-mediated VEGF expression seems to also engage Janus kinase (JAK)/STAT signaling. Indeed, the inhibition of the JAK/STAT pathway in cell culture blocks the HCV core protein-mediated activation of the androgen receptor (AR), causing a down-regulation of VEGF (Kanda et al. 2008). HCV core protein potentiates VEGF expression by the activation of activator protein 1 (AP-1) transcription factor, which is binding to the VEGF promoter region (Shao et al. 2017).

HCV Induces β-Catenin Accumulation and Wnt Pathway Activation

Wnt pathway is crucial for embryonic development and cellular differentiation (Kielman et al. 2002; Reya and Clevers 2005; Grigoryan et al. 2008; Bone et al. 2011). When Wnt signaling is active, β-catenin phosphorylation is reduced via the inhibition of the β-catenin destruction complex (Behrens et al. 1998; Amit et al. 2002; Liu et al. 2002). The augmented unphosphorylated β-catenin migrates from the cytoplasm to the nucleus, where it binds to T-cell factor (TCF) and promotes transcription of genes such as *Cyclin D1* (Tetsu and McCormick 1999), c-*MYC* (He et al. 1998), *Axin-2* (Jho et al. 2002), and *c-Jun* (Mann et al. 1999). In cell culture, NS5A triggers the serine/threonine-protein kinase Akt, by interacting with phosphoinositide

3-kinases (PI3K). Consequently, this leads to an inhibition of glycogen synthase kinase (GSK)-3β, which is a key component of the destruction complex (Street et al. 2005). Moreover, NS5A stabilizes β-catenin in the cytoplasm and therefore promotes β-catenin signaling, which is also reflected in elevated β-catenin levels in livers of HCV patients (Park et al. 2009). This is very relevant for liver pathogenesis because β-catenin is most frequently activated in HCC pathogenesis (Khalaf et al. 2018). NS5A-induced stabilization of β-catenin transcription factor stimulates c-Myc expression in cell lines, human liver tissues, and livers from FL-N/35 transgenic mice (Colman et al. 2013; Higgs et al. 2013). c-Myc is an essential regulator of liver regeneration and its perturbation is considered as an early event during HCC development (Colman et al. 2013). Moreover, HCV-induced c-Myc expression drives the metabolic shift from glucose to glutamine dependence, which is a hallmark of cancer cells (Lévy et al. 2017).

HCV INFECTION ALTERS LIVER METABOLISM

The liver plays an essential role in the metabolic regulation during both the postprandial period and fasting state. The energetic balance of the organism is finely maintained by a series of biochemical reactions involved in metabolism, storing, and redistribution of carbohydrates, proteins, and lipids (Bechmann et al. 2012). HCV circulates in the serum of patients as lipo-viro-particles and interacts with very low-density lipoprotein (VLDL) components of the host. The striking association between the HCV life cycle and the VLDL pathway is not only crucial for HCV entry, maturation, and morphogenesis, but has also an impact on the immune escape capacity of the virus (Miyanari et al. 2007; Gondar et al. 2015). Importantly, the interplay between the virus and metabolic pathways contributes to the pathogenesis of liver disease via deregulation of the host lipid metabolism (Syed et al. 2010). HCV infection is strongly associated with hepatic steatosis and dysmetabolic syndromes, such as hypocholesterolemia, altered body fat distribution, insulin

resistance (IR), and hyperuricemia (Kralj et al. 2016). Estimates suggest that ~55% of HCV-infected patients develop hepatic steatosis, which is defined as an excessive accumulation of triglycerides (TGs) within the hepatocyte cytoplasm (Lonardo et al. 2006; Vilgrain et al. 2013). Although this has been observed for several HCV genotypes, steatosis is most frequent and severe in patients infected with genotype 3 (Leandro et al. 2006), which correlates with the viral load (Rubbia-Brandt et al. 2001). HCV-induced steatosis is triggered by the interaction between HCV proteins and host factors and its development does not require the presence of visceral obesity (Adinolfi et al. 2001). HCV infection deregulates metabolic pathways via miR146a5p expression, probably dependent on NF-κB signaling (Bandiera et al. 2016). In addition, it has been suggested that HCV core protein expression may be sufficient to induce liver fat accumulation and steatosis (Moriya et al. 1997). In particular, core protein 3a induces the activation of miR-21-5p, thereby promoting HCV replication and steatosis (Clément et al. 2019). An important factor in lipid homeostasis is the β-oxidation of fatty acids in mitochondria and the peroxisomal compartment. HCV infection suppresses peroxisomal β-oxidation, which leads to the accumulation of very long-chain fatty acids (VLCFAs) in the infected hepatocytes (Lupberger et al. 2019). This is partially mediated by HCV-induced STAT3 signaling (Van Renne et al. 2018), suppressing the peroxisome proliferator-activated receptor α (PPAR-α) expression (Lupberger et al. 2019). These results are consistent with decreased hepatic PPAR-α levels in HCV-infected patients (Dharancy et al. 2005). Importantly, HCV antiviral therapy can restore lipidic levels in serum (Batsaikhan et al. 2018; Doyle et al. 2019) and attenuate hepatic steatosis after viral clearance (Shimizu et al. 2018). However, many genes relevant for metabolism remain deregulated even after viral cure (Hamdane et al. 2019), including peroxisomal genes. Restoration of peroxisomal function may be therefore a clinical strategy to improve liver function in HCC risk patients. Notably, HCV genotype 3 infection is associated with the down-regulation of phosphatase and tensin

homolog deleted on chromosome 10 (PTEN) leading to decreased levels of insulin receptor substrate 1 (IRS1) and the formation of large lipid droplets (Clément et al. 2011). This is relevant for the viral life cycle and liver disease progression because PTEN overexpression has been shown to reduce HCV viral particle secretion (Peyrou et al. 2013), and it is one of the most important tumor suppressors frequently mutated in many tumors, including HCC (Schulze et al. 2015). PTEN is also an important regulator of the insulin pathway and HCV infection perturbs the glucose homeostasis in the liver. Epidemiological studies suggest a link between chronic HCV infection and diabetes type 2 (Shintani et al. 2004; Gastaldi et al. 2017) and HCV core transgenic mice develop IR (Shintani et al. 2004). This is accompanied by a marked reduction in insulin-stimulated Akt phosphorylation without any alterations in MAPK activity in HCV-infected subjects (Aytug et al. 2003). HCV proteins up-regulate the protein phosphatase 2α (PP2A) catalytic subunit and alter signaling pathways controlling hepatic glucose homeostasis by inhibiting Akt and dephosphorylation of FoxO1 (Bernsmeier et al. 2008, 2014). Importantly, DAA treatment improves glycemic control and IR in livers, muscles, and adipose tissues of HCV cured patients (Adinolfi et al. 2018; Lim et al. 2019).

HCV-INDUCED LIVER DISEASE—IS THERE A POINT OF NO RETURN?

Since the discovery of HCV in 1989, there has been a remarkable breakthrough in antiviral therapy using DAAs. Meanwhile, >90% of patients can be cured by interferon-free treatments (Chung and Baumert 2014; Arends et al. 2016). However, in patients with advanced liver disease the risk of mortality and HCC development cannot be fully eliminated (Carrat et al. 2019). It has been estimated that HCV-induced HCC will remain one of the major health burdens for the next decades (Harris et al. 2014; Sievert et al. 2014; Petrick et al. 2016; Baumert et al. 2017). This also raises the question of whether some of the HCV-induced pro-oncogenic signaling pathways remain deregulated after viral cure.

Indeed, HCV infection causes epigenetic alterations, which act as genetic circuits that influence gene expression patterns in the long term. DNA hypermethylation has been observed in livers of patients with chronic HCV infection, leading to a silencing of tumor suppressor gene expression (Wijetunga et al. 2017). In addition, HCV induces histone modifications, which also result in persistently altered gene expression patterns (Hamdane et al. 2019; Perez and Gal-Tanamy 2019). Importantly, this epigenetic footprint is still detectable in livers of HCV-cured chimeric mice and patients (Hamdane et al. 2019; Perez and Gal-Tanamy 2019). Associated with this viral footprint, the transcriptional signature reflecting many of the earlier mentioned HCV-induced pro-oncogenic signaling pathways remains deregulated after viral cure (Hamdane et al. 2019). This may partially account for the observed elevated HCC risk. Therefore, a detailed knowledge of these pathways will be potentially useful as biomarkers to identify patients at risk and highlight potential targets for future chemopreventive strategies.

Clinical methods to predict HCV-related fibrosis and cirrhosis and its associated HCC risk are still limited. The clinical outcome also very much depends on comorbidities like human immunodeficiency virus (HIV)/HBV coinfection or alcohol. Hoshida et al. (2008) developed a prognostic liver signature (PLS) from genome-wide transcriptomics of nontumor liver tissues adjacent from HCCs, which correlates to the clinical outcome of the patients. This has been later extended to a composite prognostic model for HCC recurrence (Villanueva et al. 2011). The PLS consists of 186 genes representing a powerful tool to predict the risk for patients to progress to cirrhosis and HCC and help prioritizing those for regular follow-up and HCC surveillance. Importantly, the PLS is induced also by HCV infection (Hoshida et al. 2013; King et al. 2015). PLS components are cytokines and signaling mediators that may be useful as targets for chemoprevention of their biological impact on liver disease development.

Small molecule inhibitors targeting signaling pathways arrived in clinical practice a long time ago, especially in cancer therapy. Some of

these inhibitors target pathways that are potentially involved in an HCV-induced signaling pattern and have been tested or are currently in clinical trials for the treatment of liver disease progression. Human fibrosis and HSC activation are regulated by Wnt/β-catenin signaling (Berg et al. 2010; Ye et al. 2013; Lam et al. 2014), which therefore represents a promising target for the treatment of liver fibrosis (Cheng et al. 2008). Proof-of-concept has been provided targeting the interaction of CREB-binding protein (CBP) and β-catenin using the small molecule inhibitor PRI-724. This compound hampers HSC activation and accelerated fibrosis resolution, which seems to be accompanied by an increased expression of MMP2, MMP8, and MMP9 in intrahepatic leukocytes (Osawa et al. 2015). Currently, the safety and tolerability of PRI724 is being evaluated in patients with HCV or HBV-associated cirrhosis (NCT03620474). The Hh pathway is involved in the development of cirrhosis and HCC. Sonidegib (LDE225), a specific inhibitor of Hh is currently being tested in a phase I clinical trial for toxicity in patients with cirrhosis and advanced/metastatic HCC, who are intolerant to sorafenib (NCT02151864). In the last few years, a large number of nonspecific and specific TGF-β inhibitors have been developed (Giannelli et al. 2011; de Gramont et al. 2017). Despite that, galunisertib (LY2157299), a selective ATP-mimetic inhibitor of TGFβRI/ALK5, is the only inhibitor of TGF-β signaling currently under clinical trials in HCC patients (NCT01246986). Moreover, it seems to down-regulate the expression of stemness-related genes (such as *CD44* and *THY1*) in HCC patients (Rani et al. 2018). Receptor tyrosine kinases (RTKs), such as EGFR and vascular endothelial growth factor receptor (VEGFR), have been shown to play crucial roles in fibrogenesis, cirrhosis, and HCC development, highlighting the importance of their therapeutic inhibition (Kömüves et al. 2000; Yoshiji et al. 2003; Fuchs et al. 2014; Badawy et al. 2015). Ramucirumab, a VEGFR-2 inhibitor, was recently evaluated as a second-line treatment for HCC patients previously treated with sorafenib, showing an improved overall survival compared with placebo (Zhu et al. 2019) (NCT02435433). STAT3 signaling pathway has shown to be up-regulated during HCV infection (Yoshida et al. 2002; McCartney et al. 2013; Van Renne et al. 2018) and strong data reveal its role in fibrosis development (Chakraborty et al. 2017). A large spectrum of clinical and preclinical data supports STAT3 as a pharmacological target for different typologies of cancers (Laudisi et al. 2018). This has prompted substantial efforts to design and test different types of STAT3 inhibitors. Some of the potential therapeutic opportunities to target STAT3 pathway are to be found upstream of its activation, at STAT3 SH2 domain and at STAT3 DNA-binding domain levels. AZD1480 (NCT01219543) and AG490 inhibitors belong to the first category and inhibit JAK2 kinase (Meydan et al. 1996; Hedvat et al. 2009). The safety and tolerability of AZD1480 have been tested in a phase I study in patients with solid tumors (including HCC). However, the unusual dose limit toxicity and the lack of clinical activity brought its discontinuation in clinical development (Plimack et al. 2013). OPB-31121, a potent SH2 domain inhibitor exerting also JAK inhibitory activity (Kim et al. 2013; Brambilla et al. 2015), has shown insufficient antitumoral activity and toxicity in patients with advanced HCC (Okusaka et al. 2015). S3I-201 (NSC 74859), discovered by structure-based virtual screening (Siddiquee et al. 2007), seems to suppress HSC activation and proliferation, as well as angiogenesis and fibrogenesis in fibrotic livers (Wang et al. 2018b). A promising therapeutic agent for liver fibrosis can be represented by HJC0123, which inhibits human HSC proliferation and STAT3 dimerization (Chen et al. 2013; Nunez Lopez et al. 2016). Recently, OPB-111077 (NCT01942083) has been shown to be well tolerated in patients with advanced HCC after failure of sorafenib therapy (Yoo et al. 2019). However, the preliminary outcomes of OPB-111077 treatment are still very limited (Yoo et al. 2019), and further investigation of the role of the STAT3 signaling pathway in fibrosis and HCC are required.

CONCLUSION

Studying HCV–host interactions is not only important for the understanding of the viral life

cycle but also to answer how the virus manages to tweak its host cell to ensure persistence with all its consequences for liver pathogenesis. The molecular circuits exploited and triggered by HCV strikingly resemble other liver disease etiologies like nonalcoholic fatty liver disease (NAFLD) following a very similar path of disease progression. Studying HCV with all the experimental tools that have been developed during the last 30 years serves here as a powerful model to understand the specific and common mechanisms of liver disease development. This is essential to develop new diagnostic biomarkers and chemopreventive strategies to help HCV cured patients with advanced liver disease to tackle the epigenetic turnouts set by decades of chronic HCV infection. These tools will be potentially very useful also for other liver disease etiologies.

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REFERENCES

Abe M, Koga H, Yoshida T, Masuda H, Iwamoto H, Sakata M, Hanada S, Nakamura T, Taniguchi E, Kawaguchi T, et al. 2012. Hepatitis C virus core protein upregulates the expression of vascular endothelial growth factor via the nuclear factor-κB/hypoxia-inducible factor-1 α axis under hypoxic conditions. *Hepatol Res* **42:** 591–600. doi:10.1111/j.1872-034X.2011.00953.x

Adinolfi LE, Gambardella M, Andreana A, Tripodi MF, Utili R, Ruggiero G. 2001. Steatosis accelerates the progression of liver damage of chronic hepatitis C patients and correlates with specific HCV genotype and visceral obesity. *Hepatology* **33:** 1358–1364. doi:10.1053/jhep.2001.24432

Adinolfi LE, Nevola R, Guerrera B, D'Alterio G, Marrone A, Giordano M, Rinaldi L. 2018. Hepatitis C virus clearance by direct-acting antiviral treatments and impact on insulin resistance in chronic hepatitis C patients. *J Gastroenterol Hepatol* **33:** 1379–1382. doi:10.1111/jgh.14067

Alhetheel A, Albarrag A, Shakoor Z, Alswat K, Abdo A, Al-Hamoudi W. 2016. Assessment of pro-inflammatory cytokines in sera of patients with hepatitis C virus infection before and after anti-viral therapy. *J Infect Dev Ctries* **10:** 1093–1098. doi:10.3855/jidc.7595

Amit S, Hatzubai A, Birman Y, Andersen JS, Ben-Shushan E, Mann M, Ben-Neriah Y, Alkalay I. 2002. Axin-mediated CKI phosphorylation of β-catenin at Ser 45: a molecular switch for the Wnt pathway. *Genes Dev* **16:** 1066–1076. doi:10.1101/gad.230302

Arends JE, Kracht PA, Hoepelman AI; European Study Group for Viral Hepatitits (ESGVH). 2016. Performance of hepatitis C virus (HCV) direct-acting antivirals in clinical trials and daily practice. *Clin Microbiol Infect* **22:** 846–852. doi:10.1016/j.cmi.2016.05.027

Aytug S, Reich D, Sapiro LE, Bernstein D, Begum N. 2003. Impaired IRS-1/PI3-kinase signaling in patients with HCV: a mechanism for increased prevalence of type 2 diabetes. *Hepatology* **38:** 1384–1392. doi:10.1053/jhep.2003.09012

Badawy AA, El-Hindawi A, Hammam O, Moussa M, Gabal S, Said N. 2015. Impact of epidermal growth factor receptor and transforming growth factor-α on hepatitis C virus-induced hepatocarcinogenesis. *APMIS* **123:** 823–831. doi:10.1111/apm.12431

Bandiera S, Pernot S, El Saghire H, Durand SC, Thumann C, Crouchet E, Ye T, Fofana I, Oudot MA, Barths J, et al. 2016. Hepatitis C virus-induced upregulation of micro-RNA miR-146a-5p in hepatocytes promotes viral infection and deregulates metabolic pathways associated with liver disease pathogenesis. *J Virol* **90:** 6387–6400.

Batsaikhan B, Huang CI, Yeh ML, Huang CF, Hou NJ, Lin ZY, Chen SC, Huang JF, Yu ML, Chuang WL, et al. 2018. The effect of antiviral therapy on serum lipid profiles in chronic hepatitis C. *Oncotarget* **9:** 21313–21321. doi:10.18632/oncotarget.25092

Battaglia S, Benzoubir N, Nobilet S, Charneau P, Samuel D, Zignego AL, Atfi A, Brechot C, Bourgeade MF. 2009. Liver cancer-derived hepatitis C virus core proteins shift TGF-β responses from tumor suppression to epithelial–mesenchymal transition. *PLoS ONE* **4:** e4355. doi:10.1371/journal.pone.0004355

Baumert TF, Hoshida Y. 2019. Addressing the challenges of hepatitis C cure and persistent risk of hepatocellular carcinoma. *Viruses* **11:** 441. doi:10.3390/v11050441

Baumert TF, Jühling F, Ono A, Hoshida Y. 2017. Hepatitis C-related hepatocellular carcinoma in the era of new gener-

ation antivirals. *BMC Med* **15:** 52. doi:10.1186/s12916-017-0815-7

Bechmann LP, Hannivoort RA, Gerken G, Hotamisligil GS, Trauner M, Canbay A. 2012. The interaction of hepatic lipid and glucose metabolism in liver diseases. *J Hepatol* **56:** 952–964. doi:10.1016/j.jhep.2011.08.025

Bedossa P, Peltier E, Terris B, Franco D, Poynard T. 1995. Transforming growth factor-β1 (TGFβ1) and TGF-β1 receptors in normal, cirrhotic, and neoplastic human livers. *Hepatology* **21:** 760–766.

Behrens J, Jerchow BA, Wurtele M, Grimm J, Asbrand C, Wirtz R, Kuhl M, Wedlich D, Birchmeier W. 1998. Functional interaction of an axin homolog, conductin, with β-catenin, APC, and GSK3β. *Science* **280:** 596–599. doi:10.1126/science.280.5363.596

Berg T, DeLanghe S, Al Alam D, Utley S, Estrada J, Wang KS. 2010. β-Catenin regulates mesenchymal progenitor cell differentiation during hepatogenesis. *J Surg Res* **164:** 276–285.

Bernsmeier C, Duong FH, Christen V, Pugnale P, Negro F, Terracciano L, Heim MH. 2008. Virus-induced over-expression of protein phosphatase 2A inhibits insulin signalling in chronic hepatitis C. *J Hepatol* **49:** 429–440. doi:10.1016/j.jhep.2008.04.007

Bernsmeier C, Calabrese D, Heim MH, Duong HT. 2014. Hepatitis C virus dysregulates glucose homeostasis by a dual mechanism involving induction of PGC1α and dephosphorylation of FoxO1. *J Viral Hepat* **21:** 9–18. doi:10.1111/jvh.12208

Bhowmick NA, Ghiassi M, Bakin A, Aakre M, Lundquist CA, Engel ME, Arteaga CL, Moses HL. 2001. Transforming growth factor-β1 mediates epithelial to mesenchymal transdifferentiation through a RhoA-dependent mechanism. *Mol Biol Cell* **12:** 27–36. doi:10.1091/mbc.12.1.27

Bone HK, Nelson AS, Goldring CE, Tosh D, Welham MJ. 2011. A novel chemically directed route for the generation of definitive endoderm from human embryonic stem cells based on inhibition of GSK-3. *J Cell Sci* **124:** 1992–2000. doi:10.1242/jcs.081679

Brambilla L, Genini D, Laurini E, Merulla J, Perez L, Fermeglia M, Carbone GM, Pricl S, Catapano CV. 2015. Hitting the right spot: Mechanism of action of OPB-31121, a novel and potent inhibitor of the signal transducer and activator of transcription 3 (STAT3). *Mol Oncol* **9:** 1194–1206. doi:10.1016/j.molonc.2015.02.012

Brault C, Lévy P, Duponchel S, Michelet M, Sallé A, Pécheur EI, Plissonnier ML, Parent R, Véricel E, Ivanov AV, et al. 2016. Glutathione peroxidase 4 is reversibly induced by HCV to control lipid peroxidation and to increase virion infectivity. *Gut* **65:** 144–154. doi:10.1136/gutjnl-2014-307904

Brenndörfer ED, Karthe J, Frelin L, Cebula P, Erhardt A, Schulte am Esch J, Hengel H, Bartenschlager R, Sällberg M, Häussinger D, et al. 2009. Nonstructural 3/4A protease of hepatitis C virus activates epithelial growth factor-induced signal transduction by cleavage of the T-cell protein tyrosine phosphatase. *Hepatology* **49:** 1810–1820. doi:10.1002/hep.22857

Bürckstümmer T, Kriegs M, Lupberger J, Pauli EK, Schmittel S, Hildt E. 2006. Raf-1 kinase associates with hepatitis C virus NS5A and regulates viral replication. *FEBS Lett* **580:** 575–580. doi:10.1016/j.febslet.2005.12.071

Bureau C, Bérnad J, Chaouche N, Orfila C, Béraud M, Gonindard C, Alric L, Vinel JP, Pipy B. 2001. Nonstructural 3 protein of hepatitis C virus triggers an oxidative burst in human monocytes via activation of NADPH oxidase. *J Biol Chem* **276:** 23077–23083. doi:10.1074/jbc.M100698200

Calne RY, Sells RA, Pena JR, Davis DR, Millard PR, Herbertson BM, Binns RM, Davies DA. 1969. Induction of immunological tolerance by porcine liver allografts. *Nature* **223:** 472–476. doi:10.1038/223472a0

Carrat F, Fontaine H, Dorival C, Simony M, Diallo A, Hezode C, De Ledinghen V, Larrey D, Haour G, Bronowicki JP, et al. 2019. Clinical outcomes in patients with chronic hepatitis C after direct-acting antiviral treatment: a prospective cohort study. *Lancet* **393:** 1453–1464. doi:10.1016/S0140-6736(18)32111-1

Castilla A, Prieto J, Fausto N. 1991. Transforming growth factors β1 and α in chronic liver disease. Effects of interferon alfa therapy. *N Engl J Med* **324:** 933–940. doi:10.1056/NEJM199104043241401

Chakraborty D, Šumová B, Mallano T, Chen CW, Distler A, Bergmann C, Ludolph I, Horch RE, Gelse K, Ramming A, et al. 2017. Activation of STAT3 integrates common profibrotic pathways to promote fibroblast activation and tissue fibrosis. *Nat Commun* **8:** 1130. doi:10.1038/s41467-017-01236-6

Chen H, Yang Z, Ding C, Chu L, Zhang Y, Terry K, Liu H, Shen Q, Zhou J. 2013. Fragment-based drug design and identification of HJC0123, a novel orally bioavailable STAT3 inhibitor for cancer therapy. *Eur J Med Chem* **62:** 498–507. doi:10.1016/j.ejmech.2013.01.023

Chen Q, Yang W, Wang X, Li X, Qi S, Zhang Y, Gao MQ. 2017. TGF-β1 induces EMT in bovine mammary epithelial cells through the TGFβ1/Smad signaling pathway. *Cell Physiol Biochem* **43:** 82–93. doi:10.1159/000480321

Cheng PL, Chang MH, Chao CH, Lee YH. 2004. Hepatitis C viral proteins interact with Smad3 and differentially regulate TGF-β/Smad3-mediated transcriptional activation. *Oncogene* **23:** 7821–7838. doi:10.1038/sj.onc.1208066

Cheng JH, She H, Han YP, Wang J, Xiong S, Asahina K, Tsukamoto H. 2008. Wnt antagonism inhibits hepatic stellate cell activation and liver fibrosis. *Am J Physiol Gastrointest Liver Physiol* **294:** G39–G49.

Choi SS, Bradrick S, Qiang G, Mostafavi A, Chaturvedi G, Weinman SA, Diehl AM, Jhaveri R. 2011. Up-regulation of Hedgehog pathway is associated with cellular permissiveness for hepatitis C virus replication. *Hepatology* **54:** 1580–1590. doi:10.1002/hep.24576

Chung RT, Baumert TF. 2014. Curing chronic hepatitis C—the arc of a medical triumph. *N Engl J Med* **370:** 1576–1578. doi:10.1056/NEJMp1400986

Chusri P, Kumthip K, Hong J, Zhu C, Duan X, Jilg N, Fusco DN, Brisac C, Schaefer EA, Cai D, et al. 2016. HCV induces transforming growth factor β1 through activation of endoplasmic reticulum stress and the unfolded protein response. *Sci Rep* **6:** 22487. doi:10.1038/srep22487

Clément S, Peyrou M, Sanchez-Pareja A, Bourgoin L, Ramadori P, Suter D, Vinciguerra M, Guilloux K, Pascarella S, Rubbia-Brandt L, et al. 2011. Down-regulation of phosphatase and tensin homolog by hepatitis C virus core 3a in hepatocytes triggers the formation of large lipid droplets. *Hepatology* **54:** 38–49. doi:10.1002/hep.24340

Clément S, Sobolewski C, Gomes D, Rojas A, Goossens N, Conzelmann S, Calo N, Negro F, Foti M. 2019. Activation of the oncogenic miR-21-5p promotes HCV replication and steatosis induced by the viral core 3a protein. *Liver Int* doi:10.1111/liv.14112.

Colman H, Le Berre-Scoul C, Hernandez C, Pierredon S, Bihouee A, Houlgatte R, Vagner S, Rosenberg AR, Feray C. 2013. Genome-wide analysis of host mRNA translation during hepatitis C virus infection. *J Virol* **87:** 6668–6677. doi:10.1128/JVI.00538-13

Cordero-Espinoza L, Huch M. 2018. The balancing act of the liver: tissue regeneration versus fibrosis. *J Clin Invest* **128:** 85–96. doi:10.1172/JCI93562

de Almeida Pereira T, Witek RP, Syn WK, Choi SS, Bradrick S, Karaca GF, Agboola KM, Jung Y, Omenetti A, Moylan CA, et al. 2010. Viral factors induce Hedgehog pathway activation in humans with viral hepatitis, cirrhosis, and hepatocellular carcinoma. *Lab Invest* **90:** 1690–1703. doi:10.1038/labinvest.2010.147

de Gramont A, Faivre S, Raymond E. 2017. Novel TGF-β inhibitors ready for prime time in onco-immunology. *Oncoimmunology* **6:** e1257453. doi:10.1080/2162402X.2016.1257453

Derynck R, Zhang YE. 2003. Smad-dependent and Smad-independent pathways in TGF-β family signalling. *Nature* **425:** 577–584. doi:10.1038/nature02006

de Souza-Cruz S, Victória MB, Tarragô AM, da Costa AG, Pimentel JP, Pires EF, Araújo Lde P, Coelho-dos-Reis JG, Gomes Mde S, Amaral LR, et al. 2016. Liver and blood cytokine microenvironment in HCV patients is associated to liver fibrosis score: a proinflammatory cytokine ensemble orchestrated by TNF and tuned by IL-10. *BMC Microbiol* **16:** 3. doi:10.1186/s12866-015-0610-6

Devhare PB, Sasaki R, Shrivastava S, Di Bisceglie AM, Ray R, Ray RB. 2017. Exosome-mediated intercellular communication between hepatitis C virus-infected hepatocytes and hepatic stellate cells. *J Virol* **91:** e02225-16. doi:10.1128/JVI.02225-16

Dharancy S, Malapel M, Perlemuter G, Roskams T, Cheng Y, Dubuquoy L, Podevin P, Conti F, Canva V, Philippe D, et al. 2005. Impaired expression of the peroxisome proliferator-activated receptor α during hepatitis C virus infection. *Gastroenterology* **128:** 334–342. doi:10.1053/j.gastro.2004.11.016

Dhillon AS, Hagan S, Rath O, Kolch W. 2007. MAP kinase signalling pathways in cancer. *Oncogene* **26:** 3279–3290. doi:10.1038/sj.onc.1210421

Diamond DL, Krasnoselsky AL, Burnum KE, Monroe ME, Webb-Robertson BJ, McDermott JE, Yeh MM, Dzib JF, Susnow N, Strom S, et al. 2012. Proteome and computational analyses reveal new insights into the mechanisms of hepatitis C virus-mediated liver disease posttransplantation. *Hepatology* **56:** 28–38. doi:10.1002/hep.25649

Diao J, Pantua H, Ngu H, Komuves L, Diehl L, Schaefer G, Kapadia SB. 2012. Hepatitis C virus induces epidermal growth factor receptor activation via CD81 binding for viral internalization and entry. *J Virol* **86:** 10935–10949. doi:10.1128/JVI.00750-12

Dooley S, ten Dijke P. 2012. TGF-β in progression of liver disease. *Cell Tissue Res* **347:** 245–256. doi:10.1007/s00441-011-1246-y

Doyle MA, Galanakis C, Mulvihill E, Crawley A, Cooper CL. 2019. Hepatitis C direct acting antivirals and ribavirin modify lipid but not glucose parameters. *Cells* **8:** 252. doi:10.3390/cells8030252

El-Serag HB. 2012. Epidemiology of viral hepatitis and hepatocellular carcinoma. *Gastroenterology* **142:** 1264–1273e1. doi:10.1053/j.gastro.2011.12.061

Erhardt A, Hassan M, Heintges T, Häussinger D. 2002. Hepatitis C virus core protein induces cell proliferation and activates ERK, JNK, and p38 MAP kinases together with the MAP kinase phosphatase MKP-1 in a HepG2 Tet-Off cell line. *Virology* **292:** 272–284. doi:10.1006/viro.2001.1227

Fabregat I, Moreno-Càceres J, Sánchez A, Dooley S, Dewidar B, Giannelli G, Ten Dijke P, Consortium I-L. 2016. TGF-β signalling and liver disease. *FEBS J* **283:** 2219–2232. doi:10.1111/febs.13665

Flisiak R, Maxwell P, Prokopowicz D, Timms PM, Panasiuk A. 2002. Plasma tissue inhibitor of metalloproteinases-1 and transforming growth factor β1—possible non-invasive biomarkers of hepatic fibrosis in patients with chronic B and C hepatitis. *Hepatogastroenterology* **49:** 1369–1372.

Freitas-Lopes MA, Mafra K, David BA, Carvalho-Gontijo R, Menezes GB. 2017. Differential location and distribution of hepatic immune cells. *Cells* **6:** 48. doi:10.3390/cells6040048

Fuchs BC, Hoshida Y, Fujii T, Wei L, Yamada S, Lauwers GY, McGinn CM, DePeralta DK, Chen X, Kuroda T, et al. 2014. Epidermal growth factor receptor inhibition attenuates liver fibrosis and development of hepatocellular carcinoma. *Hepatology* **59:** 1577–1590. doi:10.1002/hep.26898

Gale M Jr, Foy EM. 2005. Evasion of intracellular host defence by hepatitis C virus. *Nature* **436:** 939–945. doi:10.1038/nature04078

Gastaldi G, Goossens N, Clément S, Negro F. 2017. Current level of evidence on causal association between hepatitis C virus and type 2 diabetes: a review. *J Adv Res* **8:** 149–159. doi:10.1016/j.jare.2016.11.003

Giannelli G, Mazzocca A, Fransvea E, Lahn M, Antonaci S. 2011. Inhibiting TGF-β signaling in hepatocellular carcinoma. *Biochim Biophys Acta* **1815:** 214–223. doi:10.1016/j.bbcan.2010.11.004

Gondar V, Molina-Jiménez F, Hishiki T, García-Buey L, Koutsoudakis G, Shimotohno K, Benedicto I, Majano PL. 2015. Apolipoprotein E, but not apolipoprotein B, is essential for efficient cell-to-cell transmission of hepatitis C virus. *J Virol* **89:** 9962–9973. doi:10.1128/JVI.00577-15

Gong G, Waris G, Tanveer R, Siddiqui A. 2001. Human hepatitis C virus NS5A protein alters intracellular calcium levels, induces oxidative stress, and activates STAT-3 and NF-κB. *Proc Natl Acad Sci* **98:** 9599–9604. doi:10.1073/pnas.171311298

Granato M, Zompetta C, Vescarelli E, Rizzello C, Cardi A, Valia S, Antonelli G, Marchese C, Torrisi MR, Faggioni A, et al. 2016. HCV derived from sera of HCV-infected patients induces pro-fibrotic effects in human primary fibroblasts by activating GLI2. *Sci Rep* **6:** 30649. doi:10.1038/srep30649

Grigoryan T, Wend P, Klaus A, Birchmeier W. 2008. Deciphering the function of canonical Wnt signals in devel-

opment and disease: conditional loss- and gain-of-function mutations of β-catenin in mice. *Genes Dev* **22:** 2308–2341. doi:10.1101/gad.1686208

Grüngreiff K, Reinhold D, Ansorge S. 1999. Serum concentrations of sIL-2R, IL-6, TGF- β1, neopterin, and zinc in chronic hepatitis C patients treated with interferon-α. *Cytokine* **11:** 1076–1080. doi:10.1006/cyto.1999.0504

Hamdane N, Jühling F, Crouchet E, El Saghire H, Thumann C, Oudot MA, Bandiera S, Saviano A, Ponsolles C, Roca Suarez AA, et al. 2019. HCV-induced epigenetic changes associated with liver cancer risk persist after sustained virologic response. *Gastroenterology* **156:** 2313–2329.e7. doi:10.1053/j.gastro.2019.02.038

Harris HJ, Davis C, Mullins JG, Hu K, Goodall M, Farquhar MJ, Mee CJ, McCaffrey K, Young S, Drummer H, et al. 2010. Claudin association with CD81 defines hepatitis C virus entry. *J Biol Chem* **285:** 21092–21102. doi:10.1074/jbc.M110.104836

Harris RJ, Thomas B, Griffiths J, Costella A, Chapman R, Ramsay M, De Angelis D, Harris HE. 2014. Increased uptake and new therapies are needed to avert rising hepatitis C-related end stage liver disease in England: modelling the predicted impact of treatment under different scenarios. *J Hepatol* **61:** 530–537. doi:10.1016/j.jhep.2014.05.008

Hassan M, Selimovic D, Ghozlan H, Abdel-kader O. 2009. Hepatitis C virus core protein triggers hepatic angiogenesis by a mechanism including multiple pathways. *Hepatology* **49:** 1469–1482. doi:10.1002/hep.22849

Hayashi J, Aoki H, Kajino K, Moriyama M, Arakawa Y, Hino O. 2000. Hepatitis C virus core protein activates the MAPK/ERK cascade synergistically with tumor promoter TPA, but not with epidermal growth factor or transforming growth factor α. *Hepatology* **32:** 958–961. doi:10.1053/jhep.2000.19343

He TC, Sparks AB, Rago C, Hermeking H, Zawel L, da Costa LT, Morin PJ, Vogelstein B, Kinzler KW. 1998. Identification of c-MYC as a target of the APC pathway. *Science* **281:** 1509–1512. doi:10.1126/science.281.5382.1509

Hedvat M, Huszar D, Herrmann A, Gozgit JM, Schroeder A, Sheehy A, Buettner R, Proia D, Kowolik CM, Xin H, et al. 2009. The JAK2 inhibitor AZD1480 potently blocks Stat3 signaling and oncogenesis in solid tumors. *Cancer Cell* **16:** 487–497. doi:10.1016/j.ccr.2009.10.015

Higgs MR, Lerat H, Pawlotsky JM. 2013. Hepatitis C virus-induced activation of β-catenin promotes c-Myc expression and a cascade of pro-carcinogenetic events. *Oncogene* **32:** 4683–4693. doi:10.1038/onc.2012.484

Horner SM, Gale M Jr. 2013. Regulation of hepatic innate immunity by hepatitis C virus. *Nat Med* **19:** 879–888. doi:10.1038/nm.3253

Horst AK, Neumann K, Diehl L, Tiegs G. 2016. Modulation of liver tolerance by conventional and nonconventional antigen-presenting cells and regulatory immune cells. *Cell Mol Immunol* **13:** 277–292. doi:10.1038/cmi.2015.112

Hoshida Y, Villanueva A, Kobayashi M, Peix J, Chiang DY, Camargo A, Gupta S, Moore J, Wrobel MJ, Lerner J, et al. 2008. Gene expression in fixed tissues and outcome in hepatocellular carcinoma. *N Engl J Med* **359:** 1995–2004. doi:10.1056/NEJMoa0804525

Hoshida Y, Villanueva A, Sangiovanni A, Sole M, Hur C, Andersson KL, Chung RT, Gould J, Kojima K, Gupta S,

et al. 2013. Prognostic gene expression signature for patients with hepatitis C-related early-stage cirrhosis. *Gastroenterology* **144:** 1024–1030. doi:10.1053/j.gastro.2013.01.021

Hosomura N, Kono H, Tsuchiya M, Ishii K, Ogiku M, Matsuda M, Fujii H. 2011. HCV-related proteins activate Kupffer cells isolated from human liver tissues. *Dig Dis Sci* **56:** 1057–1064. doi:10.1007/s10620-010-1395-y

Ingham PW, McMahon AP. 2001. Hedgehog signaling in animal development: paradigms and principles. *Genes Dev* **15:** 3059–3087. doi:10.1101/gad.938601

Ivanov AV, Smirnova OA, Ivanova ON, Masalova OV, Kochetkov SN, Isaguliants MG. 2011. Hepatitis C virus proteins activate NRF2/ARE pathway by distinct ROS-dependent and independent mechanisms in HUH7 cells. *PLoS ONE* **6:** e24957. doi:10.1371/journal.pone.0024957

Janczewska-Kazek E, Marek B, Kajdaniuk D, Borgiel-Marek H. 2006. Effect of interferon α and ribavirin treatment on serum levels of transforming growth factor-β1, vascular endothelial growth factor, and basic fibroblast growth factor in patients with chronic hepatitis C. *World J Gastroenterol* **12:** 961–965. doi:10.3748/wjg.v12.i6.961

Jenne CN, Kubes P. 2013. Immune surveillance by the liver. *Nat Immunol* **14:** 996–1006. doi:10.1038/ni.2691

Jho EH, Zhang T, Domon C, Joo CK, Freund JN, Costantini F. 2002. Wnt/β-catenin/Tcf signaling induces the transcription of Axin2, a negative regulator of the signaling pathway. *Mol Cell Biol* **22:** 1172–1183. doi:10.1128/MCB.22.4.1172-1183.2002

Jiang Y, Bao H, Ge Y, Tang W, Cheng D, Luo K, Gong G, Gong R. 2015. Therapeutic targeting of GSK3β enhances the Nrf2 antioxidant response and confers hepatic cytoprotection in hepatitis C. *Gut* **64:** 168–179. doi:10.1136/gutjnl-2013-306043

Jung Y, Brown KD, Witek RP, Omenetti A, Yang L, Vandongen M, Milton RJ, Hines IN, Rippe RA, Spahr L, et al. 2008. Accumulation of hedgehog-responsive progenitors parallels alcoholic liver disease severity in mice and humans. *Gastroenterology* **134:** 1532–1543.e3. doi:10.1053/j.gastro.2008.02.022

Kanda T, Steele R, Ray R, Ray RB. 2008. Hepatitis C virus core protein augments androgen receptor-mediated signaling. *J Virol* **82:** 11066–11072. doi:10.1128/JVI.01300-08

Kasembeli MM, Bharadwaj U, Robinson P, Tweardy DJ. 2018. Contribution of STAT3 to inflammatory and fibrotic diseases and prospects for its targeting for treatment. *Int J Mol Sci* **19:** 2299. doi:10.3390/ijms19082299

Katsuno Y, Meyer DS, Zhang Z, Shokat KM, Akhurst RJ, Miyazono K, Derynck R. 2019. Chronic TGF-β exposure drives stabilized EMT, tumor stemness, and cancer drug resistance with vulnerability to bitopic mTOR inhibition. *Sci Signal* **12:** eaau8544. doi:10.1126/scisignal.aau8544

Khalaf AM, Fuentes D, Morshid AI, Burke MR, Kaseb AO, Hassan M, Hazle JD, Elsayes KM. 2018. Role of Wnt/β-catenin signaling in hepatocellular carcinoma, pathogenesis, and clinical significance. *J Hepatocell Carcinoma* **5:** 61–73. doi:10.2147/JHC.S156701

Kholodenko IV, Yarygin KN. 2017. Cellular mechanisms of liver regeneration and cell-based therapies of liver

diseases. *Biomed Res Int* **2017:** 8910821. doi:10.1155/2017/8910821

Kielman MF, Rindapää M, Gaspar C, van Poppel N, Breukel C, van Leeuwen S, Taketo MM, Roberts S, Smits R, Fodde R. 2002. Apc modulates embryonic stem-cell differentiation by controlling the dosage of β-catenin signaling. *Nat Genet* **32:** 594–605. doi:10.1038/ng1045

Kim MJ, Nam HJ, Kim HP, Han SW, Im SA, Kim TY, Oh DY, Bang YJ. 2013. OPB-31121, a novel small molecular inhibitor, disrupts the JAK2/STAT3 pathway and exhibits an antitumor activity in gastric cancer cells. *Cancer Lett* **335:** 145–152. doi:10.1016/j.canlet.2013.02.010

King LY, Canasto-Chibuque C, Johnson KB, Yip S, Chen X, Kojima K, Deshmukh M, Venkatesh A, Tan PS, Sun X, et al. 2015. A genomic and clinical prognostic index for hepatitis C-related early-stage cirrhosis that predicts clinical deterioration. *Gut* **64:** 1296–1302. doi:10.1136/gutjnl-2014-307862

Kömüves LG, Feren A, Jones AL, Fodor E. 2000. Expression of epidermal growth factor and its receptor in cirrhotic liver disease. *J Histochem Cytochem* **48:** 821–830. doi:10.1177/002215540004800610

Kotsiri I, Hadziyannis E, Georgiou A, Papageorgiou MV, Vlachogiannakos I, Papatheodoridis G. 2016. Changes in serum transforming growth factor-β1 levels in chronic hepatitis C patients under antiviral therapy. *Ann Gastroenterol* **29:** 79–84.

Kralj D, Virovic Jukic L, Stojsavljevic S, Duvnjak M, Smolic M, Curcic IB. 2016. Hepatitis C virus, insulin resistance, and steatosis. *J Clin Transl Hepatol* **4:** 66–75. doi:10.14218/JCTH.2015.00051

Krieger SE, Zeisel MB, Davis C, Thumann C, Harris HJ, Schnober EK, Mee C, Soulier E, Royer C, Lambotin M, et al. 2010. Inhibition of hepatitis C virus infection by anti-claudin-1 antibodies is mediated by neutralization of E2-CD81-claudin-1 associations. *Hepatology* **51:** 1144–1157. doi:10.1002/hep.23445

Kurzawski M, Dziedziejko V, Urasińska E, Post M, Wójcicki M, Miętkiewski J, Droździk M. 2012. Nuclear factor erythroid 2-like 2 (Nrf2) expression in end-stage liver disease. *Environ Toxicol Pharmacol* **34:** 87–95. doi:10.1016/j.etap.2012.03.001

Lam AP, Herazo-Maya JD, Sennello JA, Flozak AS, Russell S, Mutlu GM, Budinger GR, DasGupta R, Varga J, Kaminski N, et al. 2014. Wnt coreceptor Lrp5 is a driver of idiopathic pulmonary fibrosis. *Am J Respir Crit Care Med* **190:** 185–195.

Laudisi F, Cherubini F, Monteleone G, Stolfi C. 2018. STAT3 interactors as potential therapeutic targets for cancer treatment. *Int J Mol Sci* **19:** 1797. doi:10.3390/ijms19061787

Leandro G, Mangia A, Hui J, Fabris P, Rubbia-Brandt L, Colloredo G, Adinolfi LE, Asselah T, Jonsson JR, Smedile A, et al. 2006. Relationship between steatosis, inflammation, and fibrosis in chronic hepatitis C: a meta-analysis of individual patient data. *Gastroenterology* **130:** 1636–1642. doi:10.1053/j.gastro.2006.03.014

Levent G, Ali A, Ahmet A, Polat EC, Aytaç C, Ayşe E, Ahmet S. 2006. Oxidative stress and antioxidant defense in patients with chronic hepatitis C patients before and after pegylated interferon α-2b plus ribavirin therapy. *J Transl Med* **4**. doi:10.1186/1479-5876-4-25

Lévy PL, Duponchel S, Eischeid H, Molle J, Michelet M, Diserens G, Vermathen M, Vermathen P, Dufour JF, Dienes HP, et al. 2017. Hepatitis C virus infection triggers a tumor-like glutamine metabolism. *Hepatology* **65:** 789–803. doi:10.1002/hep.28949

Lim TR, Hazlehurst JM, Oprescu AI, Armstrong MJ, Abdullah SF, Davies NP, Flintham R, Balfe P, Mutimer DJ, McKeating JA, et al. 2019. Hepatitis C virus infection is associated with hepatic and adipose tissue insulin resistance that improves after viral cure. *Clin Endocrinol (Oxf)* **90:** 440–448. doi:10.1111/cen.13924

Lin W, Tsai WL, Shao RX, Wu G, Peng LF, Barlow LL, Chung WJ, Zhang L, Zhao H, Jang JY, et al. 2010. Hepatitis C virus regulates transforming growth factor β1 production through the generation of reactive oxygen species in a nuclear factor κB-dependent manner. *Gastroenterology* **138:** 2509–2518.e1. 2518 e2501. doi:10.1053/j.gastro.2010.03.008

Liu C, Li Y, Semenov M, Han C, Baeg GH, Tan Y, Zhang Z, Lin X, He X. 2002. Control of β-catenin phosphorylation/degradation by a dual-kinase mechanism. *Cell* **108:** 837–847. doi:10.1016/S0092-8674(02)00685-2

Llovet JM, Pena CE, Lathia CD, Shan M, Meinhardt G, Bruix J, Group SIS. 2012. Plasma biomarkers as predictors of outcome in patients with advanced hepatocellular carcinoma. *Clin Cancer Res* **18:** 2290–2300. doi:10.1158/1078-0432.CCR-11-2175

Lonardo A, Loria P, Adinolfi LE, Carulli N, Ruggiero G. 2006. Hepatitis C and steatosis: a reappraisal. *J Viral Hepat* **13:** 73–80. doi:10.1111/j.1365-2893.2005.00669.x

Luangmonkong T, Suriguga S, Mutsaers HAM, Groothuis GMM, Olinga P, Boersema M. 2018. Targeting oxidative stress for the treatment of liver fibrosis. *Rev Physiol Biochem Pharmacol* **175:** 71–102. doi:10.1007/112_2018_10

Lupberger J, Zeisel MB, Xiao F, Thumann C, Fofana I, Zona L, Davis C, Mee CJ, Turek M, Gorke S, et al. 2011. EGFR and EphA2 are host factors for hepatitis C virus entry and possible targets for antiviral therapy. *Nat Med* **17:** 589–595. doi:10.1038/nm.2341

Lupberger J, Duong FH, Fofana I, Zona L, Xiao F, Thumann C, Durand SC, Pessaux P, Zeisel MB, Heim MH, et al. 2013. Epidermal growth factor receptor signaling impairs the antiviral activity of interferon-α. *Hepatology* **58:** 1225–1235. doi:10.1002/hep.26404

Lupberger J, Croonenborghs T, Roca Suarez AA, Van Renne N, Juhling F, Oudot MA, Virzi A, Bandiera S, Jamey C, Meszaros G, et al. 2019. Combined analysis of metabolomes, proteomes, and transcriptomes of hepatitis C virus-infected cells and liver to identify pathways associated with disease development. *Gastroenterology* doi:10.1053/j.gastro.2019.04.003.

Machado MV, Diehl AM. 2018. Hedgehog signalling in liver pathophysiology. *J Hepatol* **68:** 550–562. doi:10.1016/j.jhep.2017.10.017

Machida K, Cheng KT, Lai CK, Jeng KS, Sung VM, Lai MM. 2006. Hepatitis C virus triggers mitochondrial permeability transition with production of reactive oxygen species, leading to DNA damage and STAT3 activation. *J Virol* **80:** 7199–7207. doi:10.1128/JVI.00321-06

Mailly L, Xiao F, Lupberger J, Wilson GK, Aubert P, Duong FHT, Calabrese D, Leboeuf C, Fofana I, Thumann C, et al. 2015. Clearance of persistent hepatitis C virus infection in

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humanized mice using a claudin-1-targeting monoclonal antibody. *Nat Biotechnol* **33:** 549–554. doi:10.1038/nbt .3179

Mankouri J, Griffin S, Harris M. 2008. The hepatitis C virus non-structural protein NS5A alters the trafficking profile of the epidermal growth factor receptor. *Traffic* **9:** 1497–1509. doi:10.1111/j.1600-0854.2008.00779.x

Mann B, Gelos M, Siedow A, Hanski ML, Gratchev A, Ilyas M, Bodmer WF, Moyer MP, Riecken EO, Buhr HJ, et al. 1999. Target genes of β-catenin-T cell-factor/lymphoid-enhancer-factor signaling in human colorectal carcinomas. *Proc Natl Acad Sci* **96:** 1603–1608. doi:10.1073/pnas.96.4.1603

McCartney EM, Helbig KJ, Narayana SK, Eyre NS, Aloia AL, Beard MR. 2013. Signal transducer and activator of transcription 3 is a proviral host factor for hepatitis C virus. *Hepatology* **58:** 1558–1568. doi:10.1002/hep.26496

Medvedev R, Ploen D, Spengler C, Elgner F, Ren H, Bunten S, Hildt E. 2017. HCV-induced oxidative stress by inhibition of Nrf2 triggers autophagy and favors release of viral particles. *Free Radic Biol Med* **110:** 300–315. doi:10 .1016/j.freeradbiomed.2017.06.021

Meydan N, Grunberger T, Dadi H, Shahar M, Arpaia E, Lapidot Z, Leeder JS, Freedman M, Cohen A, Gazit A, et al. 1996. Inhibition of acute lymphoblastic leukaemia by a Jak-2 inhibitor. *Nature* **379:** 645–648. doi:10.1038/379645a0

Michalopoulos GK. 2007. Liver regeneration. *J Cell Physiol* **213:** 286–300. doi:10.1002/jcp.21172

Miyanari Y, Atsuzawa K, Usuda N, Watashi K, Hishiki T, Zayas M, Bartenschlager R, Wakita T, Hijikata M, Shimotohno K. 2007. The lipid droplet is an important organelle for hepatitis C virus production. *Nat Cell Biol* **9:** 1089–1097. doi:10.1038/ncb1631

Miyazawa K, Shinozaki M, Hara T, Furuya T, Miyazono K. 2002. Two major Smad pathways in TGF-β superfamily signalling. *Genes Cells* **7:** 1191–1204. doi:10.1046/j.1365-2443.2002.00599.x

Moon H, Cho K, Shin S, Kim DY, Han KH, Ro SW. 2019. High risk of hepatocellular carcinoma development in fibrotic liver: role of the Hippo-YAP/TAZ signaling pathway. *Int J Mol Sci* **20:** 581. doi:10.3390/ijms20030581

Moriya K, Yotsuyanagi H, Shintani Y, Fujie H, Ishibashi K, Matsuura Y, Miyamura T, Koike K. 1997. Hepatitis C virus core protein induces hepatic steatosis in transgenic mice. *J Gen Virol* **78:** 1527–1531. doi:10.1099/0022-1317-78-7-1527

Mukozu T, Nagai H, Matsui D, Kanekawa T, Sumino Y. 2013. Serum VEGF as a tumor marker in patients with HCV-related liver cirrhosis and hepatocellular carcinoma. *Anticancer Res* **33:** 1013–1021.

Negash AA, Olson RM, Griffin S, Gale M Jr. 2019. Modulation of calcium signaling pathway by hepatitis C virus core protein stimulates NLRP3 inflammasome activation. *PLoS Pathog* **15:** e1007593. doi:10.1371/journal.ppat .1007593

Nelson DR, Gonzalez-Peralta RP, Qian K, Xu Y, Marousis CG, Davis GL, Lau JY. 1997. Transforming growth factor-β1 in chronic hepatitis C. *J Viral Hepat* **4:** 29–35. doi:10 .1046/j.1365-2893.1997.00124.x

Nguyen TTT, Park EM, Lim YS, Hwang SB. 2018. Nonstructural protein 5A impairs DNA damage repair: implica-

tions for hepatitis C virus-mediated hepatocarcinogenesis. *J Virol* **92:** e00178-18. doi:10.1128/JVI.00178-18

Nunez Lopez O, Bohanon FJ, Wang X, Ye N, Corsello T, Rojas-Khalil Y, Chen H, Chen H, Zhou J, Radhakrishnan RS. 2016. STAT3 inhibition suppresses hepatic stellate cell fibrogenesis: HJC0123, a potential therapeutic agent for liver fibrosis. *RSC Adv* **6:** 100652–100663. doi:10.1039/C6RA17459K

Oberhammer FA, Pavelka M, Sharma S, Tiefenbacher R, Purchio AF, Bursch W, Schulte-Hermann R. 1992. Induction of apoptosis in cultured hepatocytes and in regressing liver by transforming growth factor β1. *Proc Natl Acad Sci* **89:** 5408–5412. doi:10.1073/pnas.89.12.5408

Okusaka T, Ueno H, Ikeda M, Mitsunaga S, Ozaka M, Ishii H, Yokosuka O, Ooka Y, Yoshimoto R, Yanagihara Y, et al. 2015. Phase 1 and pharmacological trial of OPB-31121, a signal transducer and activator of transcription-3 inhibitor, in patients with advanced hepatocellular carcinoma. *Hepatol Res* **45:** 1283–1291. doi:10.1111/hepr .12504

Omenetti A, Popov Y, Jung Y, Choi SS, Witek RP, Yang L, Brown KD, Schuppan D, Diehl AM. 2008. The Hedgehog pathway regulates remodelling responses to biliary obstruction in rats. *Gut* **57:** 1275–1282. doi:10.1136/gut .2008.148619

Osawa Y, Oboki K, Imamura J, Kojika E, Hayashi Y, Hishima T, Saibara T, Shibasaki F, Kohara M, Kimura K. 2015. Inhibition of cyclic adenosine monophosphate (cAMP)-response element-binding protein (CREB)-binding protein (CBP)/β-catenin reduces liver fibrosis in mice. *EBioMedicine* **2:** 1751–1758.

Pal S, Polyak SJ, Bano N, Qiu WC, Carithers RL, Shuhart M, Gretch DR, Das A. 2010. Hepatitis C virus induces oxidative stress, DNA damage and modulates the DNA repair enzyme NEIL1. *J Gastroenterol Hepatol* **25:** 627–634. doi:10.1111/j.1440-1746.2009.06128.x

Park CY, Choi SH, Kang SM, Kang JI, Ahn BY, Kim H, Jung G, Choi KY, Hwang SB. 2009. Nonstructural 5A protein activates β-catenin signaling cascades: implication of hepatitis C virus-induced liver pathogenesis. *J Hepatol* **51:** 853–864. doi:10.1016/j.jhep.2009.06.026

Paternostro C, David E, Novo E, Parola M. 2010. Hypoxia, angiogenesis and liver fibrogenesis in the progression of chronic liver diseases. *World J Gastroenterol* **16:** 281–288. doi:10.3748/wjg.v16.i3.281

Pavio N, Battaglia S, Boucreux D, Arnulf B, Sobesky R, Hermine O, Brechot C. 2005. Hepatitis C virus core variants isolated from liver tumor but not from adjacent non-tumor tissue interact with Smad3 and inhibit the TGF-β pathway. *Oncogene* **24:** 6119–6132. doi:10.1038/sj.onc .1208749

Perez S, Gal-Tanamy M. 2019. Studying the hepatitis C virus-induced epigenetic signature after cure with direct-acting antivirals. *Methods Mol Biol* **1911:** 191–207. doi:10.1007/978-1-4939-8976-8_13

Petrick JL, Kelly SP, Altekruse SF, McGlynn KA, Rosenberg PS. 2016. Future of hepatocellular carcinoma incidence in the United States forecast through 2030. *J Clin Oncol* **34:** 1787–1794. doi:10.1200/JCO.2015.64.7412

Peyrou M, Clément S, Maier C, Bourgoin L, Branche E, Conzelmann S, Kaddai V, Foti M, Negro F. 2013. PTEN protein phosphatase activity regulates hepatitis C virus

secretion through modulation of cholesterol metabolism. *J Hepatol* **59:** 420–426. doi:10.1016/j.jhep.2013.04.012

Plimack ER, Lorusso PM, McCoon P, Tang W, Krebs AD, Curt G, Eckhardt SG. 2013. AZD1480: a phase I study of a novel JAK2 inhibitor in solid tumors. *Oncologist* **18:** 819–820. doi:10.1634/theoncologist.2013-0198

Plissonnier ML, Lahlali T, Michelet M, Lebossé F, Cottarel J, Beer M, Neveu G, Durantel D, Bartosch B, Accardi R, et al. 2016. Epidermal growth factor receptor-dependent mutual amplification between netrin-1 and the hepatitis C virus. *PLoS Biol* **14:** e1002421. doi:10.1371/journal.pbio.1002421

Polaris Observatory HCV Collaborators. 2017. Global prevalence and genotype distribution of hepatitis C virus infection in 2015: a modelling study. *Lancet Gastroenterol Hepatol* **2:** 161–176. doi:10.1016/S2468-1253(16)30181-9

Rani B, Malfettone A, Dituri F, Soukupova J, Lupo L, Mancarella S, Fabregat I, Giannelli G. 2018. Galunisertib suppresses the staminal phenotype in hepatocellular carcinoma by modulating CD44 expression. *Cell Death Dis* **9:** 373. doi:10.1038/s41419-018-0384-5

Ray S, Broor SL, Vaishnav Y, Sarkar C, Girish R, Dar L, Seth P, Broor S. 2003. Transforming growth factor β in hepatitis C virus infection: in vivo and in vitro findings. *J Gastroenterol Hepatol* **18:** 393–403. doi:10.1046/j.1440-1746.2003.02985.x

Reya T, Clevers H. 2005. Wnt signalling in stem cells and cancer. *Nature* **434:** 843–850. doi:10.1038/nature03319

Roca Suarez AA, Baumert TF, Lupberger J. 2018. Beyond viral dependence: the pathological consequences of HCV-induced EGF signaling. *J Hepatol* **69:** 564–566. doi:10.1016/j.jhep.2018.05.033

Rosen HR. 2013. Emerging concepts in immunity to hepatitis C virus infection. *J Clin Invest* **123:** 4121–4130. doi:10.1172/JCI67714

Rubbia-Brandt L, Giostra E, Mentha G, Quadri R, Negro F. 2001. Expression of liver steatosis in hepatitis C virus infection and pattern of response to α-interferon. *J Hepatol* **35:** 307. doi:10.1016/S0168-8278(01)00087-3

Sanderson N, Factor V, Nagy P, Kopp J, Kondaiah P, Wakefield L, Roberts AB, Sporn MB, Thorgeirsson SS. 1995. Hepatic expression of mature transforming growth factor β1 in transgenic mice results in multiple tissue lesions. *Proc Natl Acad Sci* **92:** 2572–2576. doi:10.1073/pnas.92.7.2572

Schulze K, Imbeaud S, Letouzé E, Alexandrov LB, Calderaro J, Rebouissou S, Couchy G, Meiller C, Shinde J, Soysouvanh F, et al. 2015. Exome sequencing of hepatocellular carcinomas identifies new mutational signatures and potential therapeutic targets. *Nat Genet* **47:** 505–511. doi:10.1038/ng.3252

Shao YY, Hsieh MS, Wang HY, Li YS, Lin H, Hsu HW, Huang CY, Hsu CH, Cheng AL. 2017. Hepatitis C virus core protein potentiates proangiogenic activity of hepatocellular carcinoma cells. *Oncotarget* **8:** 86681–86692. doi:10.18632/oncotarget.21407

Shimizu K, Soroida Y, Sato M, Hikita H, Kobayashi T, Endo M, Sato M, Gotoh H, Iwai T, Tateishi R, et al. 2018. Eradication of hepatitis C virus is associated with the attenuation of steatosis as evaluated using a controlled attenuation parameter. *Sci Rep* **8:** 7845. doi:10.1038/s41598-018-26293-9

Shimoda K, Mori M, Shibuta K, Banner BF, Barnard GF. 1999. Vascular endothelial growth factor/vascular permeability factor mRNA expression in patients with chronic hepatitis C and hepatocellular carcinoma. *Int J Oncol* **14:** 353–359. doi:10.3892/ijo.14.2.353

Shintani Y, Fujie H, Miyoshi H, Tsutsumi T, Tsukamoto K, Kimura S, Moriya K, Koike K. 2004. Hepatitis C virus infection and diabetes: direct involvement of the virus in the development of insulin resistance. *Gastroenterology* **126:** 840–848. doi:10.1053/j.gastro.2003.11.056

Siddiquee K, Zhang S, Guida WC, Blaskovich MA, Greedy B, Lawrence HR, Yip ML, Jove R, McLaughlin MM, Lawrence NJ, et al. 2007. Selective chemical probe inhibitor of Stat3, identified through structure-based virtual screening, induces antitumor activity. *Proc Natl Acad Sci* **104:** 7391–7396. doi:10.1073/pnas.0609757104

Sievert W, Razavi H, Estes C, Thompson AJ, Zekry A, Roberts SK, Dore GJ. 2014. Enhanced antiviral treatment efficacy and uptake in preventing the rising burden of hepatitis C-related liver disease and costs in Australia. *J Gastroenterol Hepatol* **29**(Suppl. 1): 1–9. doi:10.1111/jgh.12677

Stanford SM, Rapini N, Bottini N. 2012. Regulation of TCR signalling by tyrosine phosphatases: from immune homeostasis to autoimmunity. *Immunology* **137:** 1–19. doi:10.1111/j.1365-2567.2012.03591.x

Stepan V, Ramamoorthy S, Nitsche H, Zavros Y, Merchant JL, Todisco A. 2005. Regulation and function of the sonic hedgehog signal transduction pathway in isolated gastric parietal cells. *J Biol Chem* **280:** 15700–15708. doi:10.1074/jbc.M413037200

Street A, Macdonald A, McCormick C, Harris M. 2005. Hepatitis C virus NS5A-mediated activation of phosphoinositide 3-kinase results in stabilization of cellular β-catenin and stimulation of β-catenin-responsive transcription. *J Virol* **79:** 5006–5016. doi:10.1128/JVI.79.8.5006-5016.2005

Syed GH, Amako Y, Siddiqui A. 2010. Hepatitis C virus hijacks host lipid metabolism. *Trends Endocrinol Metab* **21:** 33–40. doi:10.1016/j.tem.2009.07.005

Taniguchi H, Kato N, Otsuka M, Goto T, Yoshida H, Shiratori Y, Omata M. 2004. Hepatitis C virus core protein upregulates transforming growth factor-β1 transcription. *J Med Virol* **72:** 52–59. doi:10.1002/jmv.10545

Tawfik AK, Amin AM, Yousef M, El-Sayd NM, Elashry H, Elkadeem M, Abd-Elsalam S. 2018. IL-1α correlates with severity of hepatitis C virus-related liver diseases. *J Inflamm Res* **11:** 289–295. doi:10.2147/JIR.S166564

Tetsu O, McCormick F. 1999. β-Catenin regulates expression of cyclin D1 in colon carcinoma cells. *Nature* **398:** 422–426. doi:10.1038/18884

Thiery JP, Sleeman JP. 2006. Complex networks orchestrate epithelial-mesenchymal transitions. *Nat Rev Mol Cell Biol* **7:** 131–142. doi:10.1038/nrm1835

Thrift AP, El-Serag HB, Kanwal F. 2017. Global epidemiology and burden of HCV infection and HCV-related disease. *Nat Rev Gastroenterol Hepatol* **14:** 122–132. doi:10.1038/nrgastro.2016.176

Tsushima H, Kawata S, Tamura S, Ito N, Shirai Y, Kiso S, Doi Y, Yamada A, Oshikawa O, Matsuzawa Y. 1999. Reduced

plasma transforming growth factor-β1 levels in patients with chronic hepatitis C after interferon-α therapy: association with regression of hepatic fibrosis. *J Hepatol* **30:** 1–7. doi:10.1016/S0168-8278(99)80001-4

Van Renne N, Roca Suarez AA, Duong FHT, Gondeau C, Calabrese D, Fontaine N, Ababsa A, Bandiera S, Croonenborghs T, Pochet N, et al. 2018. miR-135a-5p-mediated downregulation of protein tyrosine phosphatase receptor delta is a candidate driver of HCV-associated hepatocarcinogenesis. *Gut* **67:** 953–962. doi:10.1136/gutjnl-2016-312270

Vilgrain V, Ronot M, Abdel-Rehim M, Zappa M, d'Assignies G, Bruno O, Vullierme MP. 2013. Hepatic steatosis: a major trap in liver imaging. *Diagn Interv Imaging* **94:** 713–727. doi:10.1016/j.diii.2013.03.010

Villanueva A, Hoshida Y, Battiston C, Tovar V, Sia D, Alsinet C, Cornella H, Liberzon A, Kobayashi M, Kumada H, et al. 2011. Combining clinical, pathology, and gene expression data to predict recurrence of hepatocellular carcinoma. *Gastroenterology* **140:** 1501–1512.e2. doi:10.1053/j.gastro.2011.02.006

Wang JN, Li L, Li LY, Yan Q, Li J, Xu T. 2018a. Emerging role and therapeutic implication of Wnt signaling pathways in liver fibrosis. *Gene* **674:** 57–69. doi:10.1016/j.gene.2018.06.053

Wang Z, Li J, Xiao W, Long J, Zhang H. 2018b. The STAT3 inhibitor S3I-201 suppresses fibrogenesis and angiogenesis in liver fibrosis. *Lab Invest* **98:** 1600–1613. doi:10.1038/s41374-018-0127-3

Wijetunga NA, Pascual M, Tozour J, Delahaye F, Alani M, Adeyeye M, Wolkoff AW, Verma A, Greally JM. 2017. A pre-neoplastic epigenetic field defect in HCV-infected liver at transcription factor binding sites and polycomb targets. *Oncogene* **36:** 2030–2044. doi:10.1038/onc.2016.340

Xu X, Zheng L, Yuan Q, Zhen G, Crane JL, Zhou X, Cao X. 2018. Transforming growth factor-β in stem cells and tissue homeostasis. *Bone Res* **6:** 2. doi:10.1038/s41413-017-0005-4

Ye B, Ge Y, Perens G, Hong L, Xu H, Fishbein MC, Li F. 2013. Canonical Wnt/β-catenin signaling in epicardial fibrosis of failed pediatric heart allografts with diastolic dysfunction. *Cardiovasc Pathol* **22:** 54–57.

Yoo C, Kang J, Lim HY, Kim JH, Lee MA, Lee KH, Kim TY, Ryoo BY. 2019. Phase I dose-finding study of OPB-111077, a novel STAT3 inhibitor, in patients with advanced hepatocellular carcinoma. *Cancer Res Treat* **51:** 510–518. doi:10.4143/crt.2018.226

Yoshida T, Hanada T, Tokuhisa T, Kosai K, Sata M, Kohara M, Yoshimura A. 2002. Activation of STAT3 by the hepatitis C virus core protein leads to cellular transformation. *J Exp Med* **196:** 641–653. doi:10.1084/jem.20012127

Yoshiji H, Kuriyama S, Yoshii J, Ikenaka Y, Noguchi R, Hicklin DJ, Wu Y, Yanase K, Namisaki T, Yamazaki M, et al. 2003. Vascular endothelial growth factor and receptor interaction is a prerequisite for murine hepatic fibrogenesis. *Gut* **52:** 1347–1354. doi:10.1136/gut.52.9.1347

Yu L, Hebert MC, Zhang YE. 2002. TGF-β receptor-activated p38 MAP kinase mediates Smad-independent TGF-β responses. *EMBO J* **21:** 3749–3759. doi:10.1093/emboj/cdf366

Yu H, Lee H, Herrmann A, Buettner R, Jove R. 2014. Revisiting STAT3 signalling in cancer: new and unexpected biological functions. *Nat Rev Cancer* **14:** 736–746. doi:10.1038/nrc3818

Zhang W, Liu HT. 2002. MAPK signal pathways in the regulation of cell proliferation in mammalian cells. *Cell Res* **12:** 9–18. doi:10.1038/sj.cr.7290105

Zhang J, Wang X, Vikash V, Ye Q, Wu D, Liu Y, Dong W. 2016. ROS and ROS-mediated cellular signaling. *Oxid Med Cell Longev* **2016:** 4350965. doi:10.1155/2016/4350965

Zhu C, Liu X, Wang S, Yan X, Tang Z, Wu K, Li Y, Liu F. 2014. Hepatitis C virus core protein induces hypoxia-inducible factor 1α-mediated vascular endothelial growth factor expression in Huh7.5.1 cells. *Mol Med Rep* **9:** 2010–2014. doi:10.3892/mmr.2014.2039

Zhu AX, Kang YK, Yen CJ, Finn RS, Galle PR, Llovet JM, Assenat E, Brandi G, Pracht M, Lim HY, et al. 2019. Ramucirumab after sorafenib in patients with advanced hepatocellular carcinoma and increased α-fetoprotein concentrations (REACH-2): a randomised, double-blind, placebo-controlled, phase 3 trial. *Lancet Oncol* **20:** 282–296. doi:10.1016/S1470-2045(18)30937-9

Zona L, Lupberger J, Sidahmed-Adrar N, Thumann C, Harris HJ, Barnes A, Florentin J, Tawar RG, Xiao F, Turek M, et al. 2013. HRas signal transduction promotes hepatitis C virus cell entry by triggering assembly of the host tetraspanin receptor complex. *Cell Host Microbe* **13:** 302–313. doi:10.1016/j.chom.2013.02.006

Annex IX: Interferon revisited: Peering behind the lines of antiviral defense

My main contribution was to conceptualize and write the editorial.

Interferon revisited: Peering behind the lines of antiviral defense

Alessia Virzì[1,2], Armando Andres Roca Suarez[1,2], Joachim Lupberger[1,2,*]

[1]Université de Strasbourg F-67000, France; [2]Institut National de la Santé et de la Recherche Médicale, U1110, Institut de Recherche sur les Maladies Virales et Hépatiques, Université de Strasbourg (IVH) Strasbourg F-67000, France

See Article, pages 549–558

HCV is a positive-stranded RNA virus, that accounts for approximately 71 million chronically infected individuals worldwide and represents a major risk factor for liver fibrosis, cirrhosis and hepatocellular carcinoma.[1] For a long time, interferon (IFN)-alpha regimens were the cornerstone of HCV therapy, which depending on the HCV genotype, led to a sustained virologic response in 54–75% of patients.[2] However, therapy with IFNs is lengthy and burdensome for many patients due to the broad spectrum of adverse effects.[2] Thus, they have recently been replaced with more efficient and well-tolerated direct-acting antivirals, rendering chronic HCV infection a curable disease.[3] Over the last 30 years, HCV research has provided important insights into the molecular mechanisms of innate IFN responses, as well as the sophisticated viral strategies to evade the host defenses and to persist.[4] Viruses entering the host are detected by cellular sensors of pathogen-associated molecular patterns, leading to the production of type I and III IFNs. Subsequently, this triggers the rapid transcription of hundreds of IFN-stimulated genes (ISGs), which are directly or indirectly antiviral and control the IFN response itself.[5,6] The ISG C19orf66 is induced by several clinically relevant viruses including HCV, and thus may exhibit antiviral activity.[6] Indeed, it was previously described as a potent restriction factor for HIV, Kaposi's sarcoma-associated herpesvirus (KSHV), Zika virus and dengue virus (DENV).[7–10] However, the molecular details related to the function of C19orf66, especially for HCV, remained largely unknown.

In this regard, a new study published in this issue of *Journal of Hepatology* by Volker Kinast and co-workers, sheds new light on the role of C19orf66 as an IFN-induced restriction factor (Fig. 1).[11] Analyzing primary human hepatocytes infected with cell-culture-derived HCV (HCVcc) and liver biopsies from 25 patients with chronic HCV infection, the authors revealed significantly increased mRNA expression levels of *C19orf66*, that appeared to be largely independent of viral load, METAVIR score and HCV genotype in patients. Consistently, C19orf66 is induced by IFN therapy in

patients with HCV, as revealed by computational analysis of liver transcriptomic data. Combination therapy of pegylated IFN-alpha (pegIFN-α) with ribavirin induced a peak of hepatic *C19orf66* mRNA expression at 4- and 16-hours post-treatment, highlighting it as an early-induced ISG. The authors demonstrated an antiviral effect on HCV using *C19orf66* knockout cell lines generated by CRISPR/Cas9. Disrupted C19orf66 expression restored IFN-α-suppressed replication of HCVcc and a subgenomic HCV replicon, further validating the antiviral effect of C19orf66 on HCV. While the impact of C19orf66 on other steps of the HCV life cycle, such as entry or translation, was not significant, the authors confirmed that C19orf66 is a restriction factor of HCV replication, using over-expression studies in combination with subgenomic replicons. The observed antiviral effect of C19orf66 seems independent from 7 tested HCV genotypes, suggesting an indirect "host targeting" impact of this ISG. This is further supported by the lack of an association of hepatic C19orf66 expression with the underlying HCV genotype in patients. C19orf66 seems to be recruited to lipid droplets in HCV-infected cells, where it partially colocalizes with the viral proteins core, NS3 and NS5A. In contrast, C19orf66 remains homogenously distributed in the cytosol of non-infected cells. These findings indicate that C19orf66 exerts its antiviral action at the HCV replication compartment of the membranous web (MW), which integrates lipid droplet accumulations as the central site of viral processing and particle formation.[12] The MW is formed after a massive remodeling of membranes from the endoplasmic reticulum (ER),[12] which involves a HCV-induced stimulation of phosphatidylinositol 4-kinase (PI(4)K). This leads to an enrichment of phosphatidylinositol 4-phosphate (PI(4)P) at the membranes of the ER,[13] thus provoking a bending and deformation of double-stranded ER membranes in HCV-infected cells. Interestingly, the authors established a functional link between C19orf66 expression and impaired HCV-induced PI(4)P levels in HCV replicating cells. Moreover, expression of C19orf66 with mutated zinc-finger motif (C19orf66-Zinc^mut) impaired its antiviral activity, coinciding with a less perturbed MW morphology and composition compared to cells expressing wild-type C19orf66.

In addition to the identified antiviral role of C19orf66 on MW formation, the authors identified that stress granule-associated nucleoproteins RO60, RBPMS and CELF1 interacted with C19orf66. Since this association required the zinc-finger motif of C19orf66, the authors suggested a role of this ISG in stress granule formation with functional relevance for its antiviral
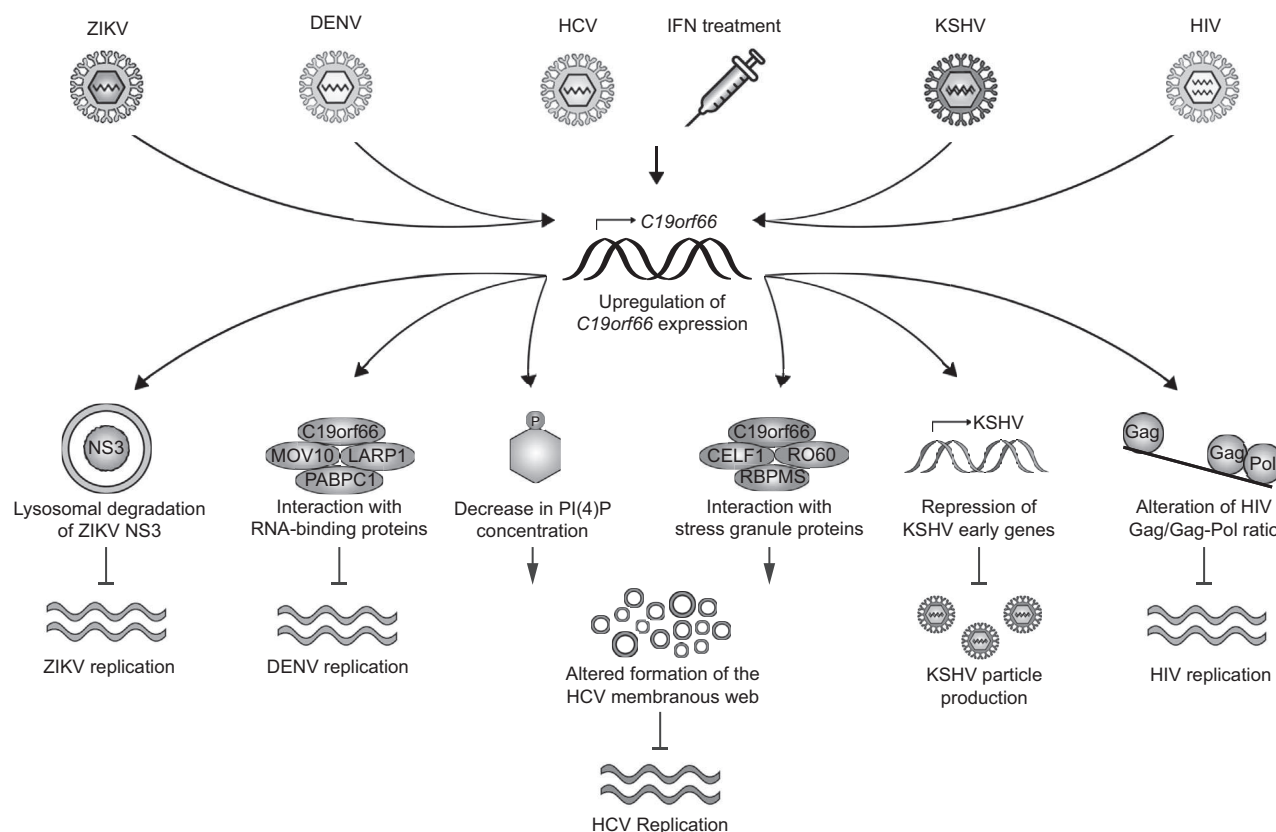
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Fig. 1. C19orf66 is a broadly acting ISG that exhibits a pluripotent and mechanistically diverse antiviral activity on clinically relevant viruses. C19orf66 upregulation has been previously shown to inhibit viral replication via lysosomal degradation of NS3 in the case of ZIKV, by interaction with RNA-binding proteins in the context of DENV infection and through alteration of the Gag/Gag-Pol ratio in the course of HIV life cycle. Additionally, C19orf66 was reported to repress the expression of KSHV early genes, having as a consequence an impaired viral particle production. Volker Kinast and co-workers demonstrate that upregulation of C19orf66 in the context of HCV infection or IFN treatment impairs the HCV cycle specifically at the replication step. The mechanism behind this antiviral effect implicates the altered formation of the HCV MW, originating from the interaction of C19orf66 with stress granule proteins and the downregulation of PI(4)P levels. DENV, dengue virus; IFN, interferon; ISG, IFN-stimulated gene; KSHV, Kaposi's sarcoma-associated herpesvirus; MW, membranous web; PI(4)P, phosphatidylinositol 4-phosphate; ZIKV, Zika virus.

function. This is supported by previous studies on DENV, where infection provoked cytoplasmic ribonucleic C19orf66-containing granule formation, while granule disruption partially rescued viral replication.[7,14] The findings of Volker Kinast and co-workers once more highlight the pluripotent character of the complex IFN response against a pathogen.[5,6] Similar to adaptive immunity, where random pre-existing immunoglobulins react to a novel immunogen and thus lead to the clonal expansion of a pathogen-specific antibody, ISGs are able to target a large variety of host processes that are relevant to previously encountered pathogens, and may be relevant to future pathogens.

C19orf66 is thus another example of how evolution created ISGs as a universal tool set. Like a swiss army knife, ISGs can act with various blades of the same tool against different pathogens. While many putative functions of C19orf66 may not be relevant to HCV infection, this protein certainly inhibits other viruses with different aspects of its pluripotent nature (Fig. 1), *i.e.*, triggering the lysosomal degradation of ZIKA NS3,[8] repressing KSHV gene expression,[9] altering crucial Gag/Pol ratios during HIV replication,[10] stress granule formation during DENV[7] and HCV infection, and most likely additional not yet discovered facets of its action relevant to other pathogens. Interestingly, C19orf66 is also induced in the antiviral response to SARS-CoV,[9] where it escapes the virus-induced mRNA degradation, as has been demonstrated for

KSHV.[9] However, whether C19orf66 has antiviral actions against coronavirus infections remains unclear. Evolution shaped the IFN response as a powerful innate defense mechanism for the eradication of invading pathogens. Understanding the mechanisms of this cellular toolset, as well as the evasion strategies of certain viruses such as HCV, gives important clues on their Achilles' heels and thus may also pave the way to understand and to tackle future emerging viral diseases.

Editorial

Conflict of interest

The authors declare that they do not have anything to disclose regarding conflict of interest with respect to this manuscript.

Please refer to the accompanying ICMJE disclosure forms for further details.

Authors' contributions

All authors conceived, wrote, and reviewed the manuscript.

Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jhep.2020.05.032.

References

Author names in bold designate shared co-first authorship

[1] WHO. Global Hepatitis Report, 2017. 2017.

[2] Heim MH. 25 years of interferon-based treatment of chronic hepatitis C: an epoch coming to an end. Nat Rev Immunol 2013;13(7):535–542.

[3] European Association for the Study of the Liver. EASL recommendations on treatment of hepatitis C 2018. J Hepatol 2018;69(2):461–511.

[4] Schwerk J, Negash A, Savan R, Gale Jr M. Innate immunity in hepatitis C virus infection. Cold Spring Harb Perspect Med 2020.

[5] Hubel P, Urban C, Bergant V, Schneider WM, Knauer B, Stukalov A, et al. A protein-interaction network of interferon-stimulated genes extends the innate immune system landscape. Nat Immunol 2019;20(4):493–502.

[6] Schoggins JW, Wilson SJ, Panis M, Murphy MY, Jones CT, Bieniasz P, et al. A diverse range of gene products are effectors of the type I interferon antiviral response. Nature 2011;472(7344):481–485.

[7] **Suzuki Y**, **Chin WX**, **Han Q**, Ichiyama K, Lee CH, Eyo ZW, et al. Characterization of RyDEN (C19orf66) as an interferon-stimulated cellular inhibitor against dengue virus replication. PLoS Pathog 2016;12(1):e1005357.

[8] **Wu Y**, **Yang X**, **Yao Z**, **Dong X**, Zhang D, Hu Y, et al. C19orf66 interrupts Zika virus replication by inducing lysosomal degradation of viral NS3. PLoS Negl Trop Dis 2020;14(3):e0008083.

[9] Rodriguez W, Srivastav K, Muller M. C19ORF66 broadly escapes virus-induced endonuclease cleavage and restricts Kaposi's sarcoma-associated herpesvirus. J Virol 2019;93(12).

[10] **Wang X**, **Xuan Y**, **Han Y**, Ding X, Ye K, Yang F, et al. Regulation of HIV-1 gag-pol expression by shiftless, an inhibitor of programmed -1 ribosomal frameshifting. Cell 2019;176(3):625–635.e14.

[11] Kinast V, Plociennikowska A, Anggakusuma, Bracht T, Todt D, Brown RJP, et al. C19orf66 is an interferon-induced inhibitor of HCV replication that restricts formation of the viral replication organelle. J Hepatol 2020;73(3):549–558.

[12] Vieyres G, Pietschmann T. HCV pit stop at the lipid droplet: refuel lipids and put on a lipoprotein coat before exit. Cells 2019;8(3).

[13] Berger KL, Kelly SM, Jordan TX, Tartell MA, Randall G. Hepatitis C virus stimulates the phosphatidylinositol 4-kinase III alpha-dependent phosphatidylinositol 4-phosphate production that is essential for its replication. J Virol 2011;85(17):8870–8883.

[14] Balinsky CA, Schmeisser H, Wells AI, Ganesan S, Jin T, Singh K, et al. IRAV (FLJ11286), an interferon-stimulated gene with antiviral activity against dengue virus, interacts with MOV10. J Virol 2017;91(5):e01606–e01616.

Résumé français de la thèse de doctorat

L'analyse phospho-protéomique des cellules infectées par le VHB révèle

un nouveau rôle du collagène VI et de l'histone linker dans la

pathogenèse du foie et le cycle de vie virale

CONTEXTE

Les maladies chroniques du foie constituent un fardeau majeur de santé publique et représentent le principal facteur de risque du carcinome hépatocellulaire (CHC) dans le monde. La fibrose et la cirrhose hépatique sont les principales causes de morbidité et de mortalité chez les patients atteints de maladie chronique du foie. La cirrhose est responsable de 1,16 million de décès au niveau mondial (Asrani, Devarbhavi, Eaton, & Kamath, 2019). Les causes du développement de la cirrhose du foie sont multiples et peuvent varier considérablement d'un pays à l'autre. Les stéatoses hépatiques alcoolique et non alcoolique prédominent dans les pays occidentaux et industrialisés, alors que l'infection chronique par les virus des hépatites B (VHB) et C (VHC) représente la principale cause de progression de la maladie hépatique dans les régions asiatiques (Lozano et al., 2012). Nonobstant la diversité des étiologies, le développement de l'inflammation chronique du foie et son évolution vers la fibrose hépatique et le CHC perturbent des voies de signalisation communes (Virzi, Gonzalez-Motos, Tripon, Baumert, & Lupberger, 2021). Le VHB est l'une des principales causes de CHC, qui est le principal cancer primitif du foie et la quatrième cause de décès associé au cancer dans le monde (Yang et al., 2019). L'infection chronique par le VHB se caractérise par différents stades cliniques qui peuvent durer plusieurs décennies et évoluer vers le CHC et l'insuffisance hépatique. De plus, le VHB peut provoquer directement le développement du CHC via l'effet oncogénique de ses protéines virales (Lupberger & Hildt, 2007). Les thérapies antivirales contre l'infection par le VHB et le VHC ont démontré leur capacité à prévenir ou à

ralentir la progression de la fibrose chez la plupart des patients. Cependant, il s'agit généralement des thérapies antivirales à long terme (Rockey, 2016). De plus, aucune thérapie anti-fibrotique établie n'est disponible pour les patients atteints d'une maladie hépatiques alcoolique et non alcoolique ou de troubles hépatiques auto-immuns (Asrani et al., 2019). Pour toutes ces raisons, des stratégies thérapeutiques capables de bloquer le développement de la fibrose et de prévenir le CHC sont nécessaires. La stratégie mondiale de l'OMS contre l'hépatite vise à réduire les nouvelles infections par les virus des hépatites de 90 % et les décès de 65 % d'ici à 2030 en mettant en œuvre des campagnes de prévention et de vaccination, ainsi qu'en promouvant l'accès au dépistage et aux traitements, qui restent aujourd'hui difficile dans de nombreux pays (Cox et al., 2020) (Thomas, 2019). Dans le contexte de l'infection par le VHB, l'absence d'un traitement résolutif est en grande partie associé à la persistance de l'ADN circulaire fermé par covalence (ADNccc), retenu dans le noyau des cellules infectées sous forme de mini-chromosome, assemblé avec des histones et des protéines non-histones. L'ADNccc est difficile à détecter et à éradiquer car il est insensible à la thérapie antivirale et qu'il agit comme un réservoir pour la réplication virale (Bock et al., 2001) (Kumar, Perez-Del-Pulgar, Testoni, Lebosse, & Zoulim, 2016).

Au niveau moléculaire, une lésion hépatique chronique provoque l'activation de processus profibrogéniques qui sont orchestrés finement par plusieurs voies de signalisation (Roehlen, Crouchet, & Baumert, 2020) et qui aboutissent à un dépôt anormal de collagènes et un remodelage de la matrice extracellulaire (MEC). Cela se traduit par une destruction de l'architecture du foie entrainant une fonction hépatique aberrante. Lorsqu'elles sont activées en réponse à une lésion hépatique, les cellules étoilées hépatiques deviennent la principale source de dépôt de collagène, en particulier le collagène de type I et IV, qui sont les principaux responsables de la fibrose (Berumen, Baglieri, Kisseleva, & Mekeel, 2021). Ainsi, la composition en collagène de la MEC est d'une importance fondamentale pour la fonctionnalité et l'architecture du foie, et la compréhension du rôle de la MEC est essentielle pour mettre au point des thérapies antifibrotiques adéquates.

De nombreuses études ont prouvé que les protéines virales interagissent avec les protéines de l'hôte et exploitent une variété de voies cellulaires pour leur propre bénéfice (He et al., 2010; Pant, Dsouza, & Yang, 2021). Notamment, les virus peuvent manipuler le cycle cellulaire ou même l'arrêter dans une phase particulière, afin d'augmenter l'efficacité de la réplication du génome viral ou promouvoir l'assemblage du virus (Fan, Sanyal, & Bruzzone, 2018). Jusqu'à récemment, l'étude des voies de signalisation perturbé par le VHB était limitée par l'absence de modèle cellulaire robuste permissif à l'infection. Depuis la découverte du « sodium taurocholate cotransporter polypeptide » (NTCP) comme récepteur d'entrée du VHB, il a été possible de développer des modèles cellulaires permissif à l'infection virale, comme la lignée cellulaire humane d'hépatoblastome HepG2-NTCP, qui surexprime ce transporteur (Ni et al., 2014; Yan et al., 2012). Précédemment, il a été rapporté que le VHB exploite une voie de signalisation de l'hôte, via l'«Epidermal Growth Factor Receptor » (EGFR) et le « Signal Transducer and Activator of Transcription » (STAT3), pour l'entrée du virus et l'infection de la cellule hôte (Hosel et al., 2017; Iwamoto et al., 2019; Roca Suarez, Van Renne, Baumert, & Lupberger, 2018). Cela indique que le VHB perturbe notamment des voies impliquées dans la progression de la maladie hépatique. Cependant, les voies de signalisation intracellulaire perturbées par le VHB, et notamment la phosphorylation des protéines associée (qui est la modification post-traductionnelle la plus retrouvée dans la transduction du signal (Ubersax & Ferrell, 2007)), restent encore inconnues lorsque j'ai débuté mes travaux de thèse.

OBJECTIFS

L'objectif principal de ma thèse était l'identification de l'ensemble protéomique et phospho-protéomique des voies de signalisations perturbées par le VHB, en utilisant le modèle cellulaire d'hépatoblastome HepG2-NTCP permissif à l'infection virale. Notamment, notre objectif était la délinéation des voies de phosphorylation les plus significatives impliquées dans la pathogenèse du foie. Notre analyse protéomique et phospho-protéomique veut aussi fournir un atlas global des cibles thérapeutique prometteuse contre les maladies chronique du foie associés au VHB.

RESULTATS ET DISCUSSION

L'analyse protéomique et phospho-protéomique révèle de nouveaux aspects associés à la progression de la fibrose et à l'impact du VHB sur le cycle cellulaire

Pour visualiser l'ensemble des voies de signalisation dérégulées par le VHB, des analyses protéomiques et phospho-protéomiques ont été effectuées 2 et 10 jours après l'infection de cellules HepG2-NTCP par la technique de spectrométrie de masse tandem en collaboration avec le Max-Delbrück Center (MDC), à Berlin (Mertins et al., 2018). Afin d'évaluer les effets du VHB sur les voies de signalisation, j'ai identifié l'ensemble des catégories fonctionnelles les plus dérégulées par le VHB (à partir de données protéomiques), en effectuant des analyses bio-informatiques, tel que le « Gene Set Enrichment Analysis » (GSEA). J'ai ainsi pu démontrer que les hépatocytes contribuent à la perturbation de la MEC, notamment à travers la production de collagène de type VI, type XVIII et FN1. L'augmentation de certaines de ces protéines a déjà été observée dans un modèle de culture d'hépatocytes de rat (Farkas, Bhat, Mandapati, Wishnok, & Tannenbaum, 2005). L'analyse protéomique, et la validation par expérience in vitro, a révélé également le rôle de l'apolipoprotéine E (APOE) en tant que promotrice de l'infection par le VHB, ce qui était déjà connu (Qiao & Luo, 2019).

Grâce à l'analyse phospho-protéomique, j'ai contribué à fournir un atlas détaillé des protéines impliquées dans la progression du cycle cellulaire et dérégulé par le VHB, confirmant des études précédentes (Eller et al., 2020; Wang et al., 2011; Xia et al., 2018). Notre atlas a révélé une hypophosphorylation de la protéine RB1 (protéine suppresseur de tumeur) aux sites T356 et S788 lors de l'infection. Les deux positions hypophosphorylés lors de l'infection, sont des sites fréquemment phosphorylés par Cdk2/Cdk4/Cdk5 (Connell-Crowley, Harper, & Goodrich, 1997; Futatsugi et al., 2012; Lees, Buchkovich, Marshak, Anderson, & Harlow, 1991; Zarkowska & Mittnacht, 1997). La phosphorylation de RB1 par la cycline D/Cdk4/6 et la cycline E/Cdk2 permet la transcription des gènes de phase S par la dissociation de E2F (Macdonald & Dick, 2012). L'analyse phospho-protéomique révèle aussi que l'infection VHB provoque la

phosphorylation des plusieurs acides aminés de la protéine Polo-like-kinase (PLK1), connu depuis quelques années comme important facteur provirale pour le VHB (Diab et al., 2017).

Par ailleurs, notre atlas phospho-protéomique a révélé que l'infection par le VHB provoque l'hyperphosphorylation des linker histones, protéines impliquées dans le remodelage de la chromatine. En particulier, le variant H1.4 (HIST1H1E) s'est avérée hyperphosphorylée aux premiers stades de l'infection et sur des acides aminés distincts. De plus, notre phosphoprotéome a permis de fournir des données supplémentaires et de confirmer des découvertes récentes liées aux mécanismes de l'endocytose médiée par la clathrine au cours de l'entrée du VHB (Herrscher et al., 2020). L'ensemble de ces données indique que cette analyse protéomique et phospho-protéomique représente une source valide pour la communauté scientifique, pour le développement d'hypothèses et la validation des expériences.

Le collagène VI est surexprimé dans les hépatocytes infectés par le VHB agissant comme inducteur de la réplication du VHB et favorisant la fibrose hépatique

La combinaison de la protéomique sur les cellules HepG2-NTCP et de la transcriptomique sur les PHHs (GEO accession number : GSE69590), infectés et non-infectés par le VHB, suggère que les hépatocytes remodèlent activement l'environnement de la MEC, surtout par l'expression du collagène VI, collagène XVIII et FN1. J'ai étudié le rôle du collagène VI dans le contexte des maladies hépatiques. Le collagène VI est une molécule d'importance fondamentale pour maintenir l'intégrité des tissus tels que les vaisseaux sanguins, les poumons et la peau. Les mutations dans le gène du collagène VI sont associées à la myopathie de Bethlem et au syndrome d'Ullrich, différentes formes d'atrophie musculaires (Camacho Vanegas et al., 2001; Scacheri et al., 2002). Néanmoins, l'expression de la protéine du collagène VI et ses fragments est élevée dans de nombreuses conditions fibrotiques, telles que la maladie pulmonaire idiopathique et les maladies chroniques du foie et des reins (Freise et al., 2021; Guadagnin et al., 2021; Sivakumar et al., 2021; Sparding et al., 2021). Avant de caractériser le rôle du collagène VI, j'ai d'abord validé sa surexpression dans les cellules HepG2-NTCP infectées par le VHB à l'aide de la cytométrie en flux. Les cellules infectées ont

été sélectionnées sur la présence de HBsAg. Ensuite, j'ai pu observer une augmentation de l'expression de la protéine de collagène VI dans les cellules positives pour le VHB en comparaison aux cellules non infectées. De plus, j'ai détecté une augmentation de l'expression de COL6A1 dans des cultures primaires d'hépatocytes (PHHs) infectées par le VHB. L'expression de *COL6A1* est aussi augmentée chez les patients VHB fibrotiques avec un score S et G Scheuer (Scheuer, 1991) élevé (S 3-4 ; G 3-4) comparé à des patients ayant un score inférieur, indiquant une corrélation directe entre l'expression de COL6A1 et la progression de la maladie hépatique. Ensuite, j'ai démontré que les cellules où le gène COL6A1 était éteint répliquaient moins le génome viral.

Afin de comprendre les interactions entre les hépatocytes et les cellules étoilées hépatiques, principales responsables du développement de la fibrose, nous avons émis l'hypothèse que le collagène VI, sécrété par les hépatocytes infectés, agit comme activateur des cellules étoilées hépatiques du foie. Ainsi, j'ai exposé des cellules étoilées hépatiques fraîchement isolées à partir de différents donneurs à du collagène VI, du collagène I ou du Transforming Growth Factor beta (TGFB) en absence de sérum. Le TGFB est un contrôle positif pour l'activation des cellules étoilées. Les cellules traitées par le collagène VI ont un niveau d'expression des gènes ACTA2, COL1A1 et TGFBR1 plus élevé par rapport aux cellules non traitées. De plus, Freise et al., ont montré des résultats similaires sur des cellules étoilées hépatiques de souris (Freise et al., 2021). Dans leur ensemble, ces données suggèrent une contribution du collagène VI en faveur de la réplication virale et au cours de la fibrogénèse.

La phosphorylation du linker histone H1.4 augmente la transcription du VHB

Notre analyse phosphoprotéomique a montré une hyperphosphorylation du linker histone H1.4 principalement au stade précoce de l'infection par le VHB. L'hyperphosphorylation de H1 se traduit par le détachement de la protéine de l'ADN (Alexandrow & Hamlin, 2005; Bannister & Kouzarides, 2011). Nous avons émis l'hypothèse que l'élimination partielle des linker histones imite la phosphorylation des histones ayant un impact sur le compactage de la chromatine et l'expression des gènes (Dou, Mizzen, Abrams, Allis, & Gorovsky, 1999). Ainsi, nous avons

réduit au silence l'expression du gène HIST1H1E dans des cellules HepG2-NTCP et nous avons analysé l'effet sur la réplication du VHB. L'expression du génome du VHB était augmentée, démontrant que la phosphorylation de H1.4 imitée par silencing s'est avérée avantageuse pour la replication du VHB. Ce résultat peut avoir un impact significatif sur la transcription de l'ADNccc.

CONCLUSION

Grâce à ces travaux de thèse, nous avons obtenu un faisceau d'évidence concernant des aspects spécifiques au cycle de vie viral ou associé à la progression de la maladie chronique du foie et au développement de la fibrose hépatique. Notre atlas phospho-protéomique peut servir de base pour l'identification de nouveaux mécanismes régulant le cycle viral et la progression de la maladie hépatique. La solidité et l'utilité de cet atlas est confirmée par la validation de plusieurs protéines et facteurs déjà connu pour leur rôle dans l'infection par le VHB, comme la protéine APOE et le PLK1. De plus, notre étude fournit un large éventail de candidats qui ont un impact significatif sur la progression du cycle cellulaire, avec un potentiel rôle dans le développement du cancer du foie. Nos résultats suggèrent un rôle encore peu connu du collagène VI et du linker histone H1.4 dans la réplication du VHB avec un impact sur la progression de la fibrose hépatique et la transcription de l'ADNccc, respectivement. Pour finir, l'utilisation des cellules primaires confère plus de valeur à ces résultats, qui peuvent représenter donc une référence pour la validation de cibles thérapeutiques et par conséquent le développement de médicaments antiviraux.

Références :

Alexandrow, M. G., & Hamlin, J. L. (2005). Chromatin decondensation in S-phase involves recruitment of Cdk2 by Cdc45 and histone H1 phosphorylation. J Cell Biol, 168(6), 875-886. doi:10.1083/jcb.200409055

Asrani, S. K., Devarbhavi, H., Eaton, J., & Kamath, P. S. (2019). Burden of liver diseases in the world. J Hepatol, 70(1), 151-171. doi:10.1016/j.jhep.2018.09.014

Bannister, A. J., & Kouzarides, T. (2011). Regulation of chromatin by histone modifications. Cell Res, 21(3), 381-395. doi:10.1038/cr.2011.22

Berumen, J., Baglieri, J., Kisseleva, T., & Mekeel, K. (2021). Liver fibrosis: Pathophysiology and clinical implications. WIREs Mech Dis, 13(1), e1499. doi:10.1002/wsbm.1499

Bock, C. T., Schwinn, S., Locarnini, S., Fyfe, J., Manns, M. P., Trautwein, C., & Zentgraf, H. (2001). Structural organization of the hepatitis B virus minichromosome. J Mol Biol, 307(1), 183-196. doi:10.1006/jmbi.2000.4481

Camacho Vanegas, O., Bertini, E., Zhang, R. Z., Petrini, S., Minosse, C., Sabatelli, P., . . . Pepe, G. (2001). Ullrich scleroatonic muscular dystrophy is caused by recessive mutations in collagen type VI. Proc Natl Acad Sci U S A, 98(13), 7516-7521. doi:10.1073/pnas.121027598

Connell-Crowley, L., Harper, J. W., & Goodrich, D. W. (1997). Cyclin D1/Cdk4 regulates retinoblastoma protein-mediated cell cycle arrest by site-specific phosphorylation. Mol Biol Cell, 8(2), 287-301. doi:10.1091/mbc.8.2.287

Cox, A. L., El-Sayed, M. H., Kao, J. H., Lazarus, J. V., Lemoine, M., Lok, A. S., & Zoulim, F. (2020). Progress towards elimination goals for viral hepatitis. Nat Rev Gastroenterol Hepatol, 17(9), 533-542. doi:10.1038/s41575-020-0332-6

Diab, A., Foca, A., Fusil, F., Lahlali, T., Jalaguier, P., Amirache, F., . . . Durantel, D. (2017). Polo-like-kinase 1 is a proviral host factor for hepatitis B virus replication. Hepatology, 66(6), 1750-1765. doi:10.1002/hep.29236

Dou, Y., Mizzen, C. A., Abrams, M., Allis, C. D., & Gorovsky, M. A. (1999). Phosphorylation of linker histone H1 regulates gene expression in vivo by mimicking H1 removal. Mol Cell, 4(4), 641-647. doi:10.1016/s1097-2765(00)80215-4

Eller, C., Heydmann, L., Colpitts, C. C., El Saghire, H., Piccioni, F., Juhling, F., . . . Baumert, T. F. (2020). A genome-wide gain-of-function screen identifies CDKN2C as a HBV host factor. Nat Commun, 11(1), 2707. doi:10.1038/s41467-020-16517-w

Fan, Y., Sanyal, S., & Bruzzone, R. (2018). Breaking Bad: How Viruses Subvert the Cell Cycle. Front Cell Infect Microbiol, 8, 396. doi:10.3389/fcimb.2018.00396

Farkas, D., Bhat, V. B., Mandapati, S., Wishnok, J. S., & Tannenbaum, S. R. (2005). Characterization of the secreted proteome of rat hepatocytes cultured in collagen sandwiches. Chem Res Toxicol, 18(7), 1132-1139. doi:10.1021/tx0500225

Freise, C., Lee, H., Chronowski, C., Chan, D., Cziomer, J., Ruhl, M., . . . Sahin, E. (2021). Alpha-single chains of collagen type VI inhibit the fibrogenic effects of triple helical collagen VI in hepatic stellate cells. PLoS One, 16(9), e0254557. doi:10.1371/journal.pone.0254557

Futatsugi, A., Utreras, E., Rudrabhatla, P., Jaffe, H., Pant, H. C., & Kulkarni, A. B. (2012). Cyclin-dependent kinase 5 regulates E2F transcription factor through phosphorylation of Rb protein in neurons. Cell Cycle, 11(8), 1603-1610. doi:10.4161/cc.20009

Guadagnin, E., Mohassel, P., Johnson, K. R., Yang, L., Santi, M., Uapinyoying, P., . . . Bonnemann, C. G. (2021). Transcriptome analysis of collagen VI-related muscular dystrophy muscle biopsies. Ann Clin Transl Neurol, 8(11), 2184-2198. doi:10.1002/acn3.51450

He, Y., Xu, K., Keiner, B., Zhou, J., Czudai, V., Li, T., . . . Sun, B. (2010). Influenza A virus replication induces cell cycle arrest in G0/G1 phase. J Virol, 84(24), 12832-12840. doi:10.1128/JVI.01216-10

Herrscher, C., Pastor, F., Burlaud-Gaillard, J., Dumans, A., Seigneuret, F., Moreau, A., . . . Blanchard, E. (2020). Hepatitis B virus entry into HepG2-NTCP cells requires clathrin-mediated endocytosis. Cell Microbiol, 22(8), e13205. doi:10.1111/cmi.13205

Hosel, M., Quasdorff, M., Ringelhan, M., Kashkar, H., Debey-Pascher, S., Sprinzl, M. F., . . . Protzer, U. (2017). Hepatitis B Virus Activates Signal Transducer and Activator of Transcription 3 Supporting Hepatocyte Survival and Virus Replication. Cell Mol Gastroenterol Hepatol, 4(3), 339-363. doi:10.1016/j.jcmgh.2017.07.003

Iwamoto, M., Saso, W., Sugiyama, R., Ishii, K., Ohki, M., Nagamori, S., . . . Watashi, K. (2019). Epidermal growth factor receptor is a host-entry cofactor triggering hepatitis B virus internalization. Proc Natl Acad Sci U S A, 116(17), 8487-8492. doi:10.1073/pnas.1811064116

Kumar, R., Perez-Del-Pulgar, S., Testoni, B., Lebosse, F., & Zoulim, F. (2016). Clinical relevance of the study of hepatitis B virus covalently closed circular DNA. Liver Int, 36 Suppl 1, 72-77. doi:10.1111/liv.13001

Lees, J. A., Buchkovich, K. J., Marshak, D. R., Anderson, C. W., & Harlow, E. (1991). The retinoblastoma protein is phosphorylated on multiple sites by human cdc2. EMBO J, 10(13), 4279-4290.

Lozano, R., Naghavi, M., Foreman, K., Lim, S., Shibuya, K., Aboyans, V., . . . Memish, Z. A. (2012). Global and regional mortality from 235 causes of death for 20 age groups in 1990 and 2010: a systematic analysis for the Global Burden of Disease Study 2010. Lancet, 380(9859), 2095-2128. doi:10.1016/S0140-6736(12)61728-0

Lupberger, J., & Hildt, E. (2007). Hepatitis B virus-induced oncogenesis. World J Gastroenterol, 13(1), 74-81. doi:10.3748/wjg.v13.i1.74

Macdonald, J. I., & Dick, F. A. (2012). Posttranslational modifications of the retinoblastoma tumor suppressor protein as determinants of function. Genes Cancer, 3(11-12), 619-633. doi:10.1177/1947601912473305

Mertins, P., Tang, L. C., Krug, K., Clark, D. J., Gritsenko, M. A., Chen, L., . . . Carr, S. A. (2018). Reproducible workflow for multiplexed deep-scale proteome and phosphoproteome analysis of tumor tissues by liquid chromatography-mass spectrometry. Nat Protoc, 13(7), 1632-1661. doi:10.1038/s41596-018-0006-9

Ni, Y., Lempp, F. A., Mehrle, S., Nkongolo, S., Kaufman, C., Falth, M., . . . Urban, S. (2014). Hepatitis B and D viruses exploit sodium taurocholate co-transporting polypeptide for species-specific entry into hepatocytes. Gastroenterology, 146(4), 1070-1083. doi:10.1053/j.gastro.2013.12.024

Pant, A., Dsouza, L., & Yang, Z. (2021). Alteration in Cellular Signaling and Metabolic Reprogramming during Viral Infection. mBio, 12(5), e0063521. doi:10.1128/mBio.00635-21

Qiao, L., & Luo, G. G. (2019). Human apolipoprotein E promotes hepatitis B virus infection and production. PLoS Pathog, 15(8), e1007874. doi:10.1371/journal.ppat.1007874

Roca Suarez, A. A., Van Renne, N., Baumert, T. F., & Lupberger, J. (2018). Viral manipulation of STAT3: Evade, exploit, and injure. PLoS Pathog, 14(3), e1006839. doi:10.1371/journal.ppat.1006839

Rockey, D. C. (2016). Liver Fibrosis Reversion After Suppression of Hepatitis B Virus. Clin Liver Dis, 20(4), 667-679. doi:10.1016/j.cld.2016.06.003

Roehlen, N., Crouchet, E., & Baumert, T. F. (2020). Liver Fibrosis: Mechanistic Concepts and Therapeutic Perspectives. Cells, 9(4). doi:10.3390/cells9040875

Scacheri, P. C., Gillanders, E. M., Subramony, S. H., Vedanarayanan, V., Crowe, C. A., Thakore, N., . . . Hoffman, E. P. (2002). Novel mutations in collagen VI genes: expansion of the Bethlem myopathy phenotype. Neurology, 58(4), 593-602. doi:10.1212/wnl.58.4.593

Scheuer, P. J. (1991). Classification of chronic viral hepatitis: a need for reassessment. J Hepatol, 13(3), 372-374. doi:10.1016/0168-8278(91)90084-o

Sivakumar, P., Ammar, R., Thompson, J. R., Luo, Y., Streltsov, D., Porteous, M., . . . Christie, J. D. (2021). Integrated plasma proteomics and lung transcriptomics reveal novel biomarkers in idiopathic pulmonary fibrosis. Respir Res, 22(1), 273. doi:10.1186/s12931-021-01860-3

Sparding, N., Genovese, F., Rasmussen, D. G. K., Karsdal, M. A., Neprasova, M., Maixnerova, D., . . . Tesar, V. (2021). Endotrophin, a collagen type VI-derived matrikine, reflects the degree of renal fibrosis in patients with IgA nephropathy and in patients with ANCA-associated vasculitis. Nephrol Dial Transplant. doi:10.1093/ndt/gfab163

Thomas, D. L. (2019). Global Elimination of Chronic Hepatitis. N Engl J Med, 380(21), 2041-2050. doi:10.1056/NEJMra1810477

Ubersax, J. A., & Ferrell, J. E., Jr. (2007). Mechanisms of specificity in protein phosphorylation. Nat Rev Mol Cell Biol, 8(7), 530-541. doi:10.1038/nrm2203

Virzi, A., Gonzalez-Motos, V., Tripon, S., Baumert, T. F., & Lupberger, J. (2021). Profibrotic Signaling and HCC Risk during Chronic Viral Hepatitis: Biomarker Development. J Clin Med, 10(5). doi:10.3390/jcm10050977

Wang, T., Zhao, R., Wu, Y., Kong, D., Zhang, L., Wu, D., . . . Jin, X. (2011). Hepatitis B virus induces G1 phase arrest by regulating cell cycle genes in HepG2.2.15 cells. Virol J, 8, 231. doi:10.1186/1743-422X-8-231

Xia, Y., Cheng, X., Li, Y., Valdez, K., Chen, W., & Liang, T. J. (2018). Hepatitis B Virus Deregulates the Cell Cycle To Promote Viral Replication and a Premalignant Phenotype. J Virol, 92(19). doi:10.1128/JVI.00722-18

Yan, H., Zhong, G., Xu, G., He, W., Jing, Z., Gao, Z., . . . Li, W. (2012). Sodium taurocholate cotransporting polypeptide is a functional receptor for human hepatitis B and D virus. Elife, 1, e00049. doi:10.7554/eLife.00049

Yang, J. D., Hainaut, P., Gores, G. J., Amadou, A., Plymoth, A., & Roberts, L. R. (2019). A global view of hepatocellular carcinoma: trends, risk, prevention and management. Nat Rev Gastroenterol Hepatol, 16(10), 589-604. doi:10.1038/s41575-019-0186-y

Zarkowska, T., & Mittnacht, S. (1997). Differential phosphorylation of the retinoblastoma protein by G1/S cyclin-dependent kinases. J Biol Chem, 272(19), 12738-12746. doi:10.1074/jbc.272.19.12738

Curriculum vitae

Alessia Virzì

Contact: +33 767709251 - virzi@unistra.fr

WORK EXPERIENCE

2018-2022 (October - May)	**PhD student at the Institute of Viral and Liver Disease, Inserm UMRS_1110 (Laboratory of Excellence HepSYS) Strasbourg, France** Characterization of the proteomic and phosphoproteomic landscape of signaling pathways deregulated by hepatitis B viral infection. **Funding award: Initiative d'Excellence (IdEx)**
2018 (March - September)	**Ingénieur d'étude en techniques biologiques at Institute of Viral and Liver Disease, INSERM UMRS_1110 (laboratory of Excellence HepSYS), Strasbourg, France** Host-virus interactions implicated in hepatitis C and B chronic infection.
2017 (March - June)	**Internship at Inte:Ligand (Computer Science, Software and Algorithms Group), Vienna, Austria** Creation of structure and ligand -based pharmacophore models for the identification of selective drugs for Adenosine A2 receptor using computational tools (Inte:ligand; KNIME). **Funding award: Erasmus+**
2016-2017	**Master thesis project in computational chemistry at Faculty of Pharmaceutical Chemistry, Palermo, Italy** Identification of potential brain human targets for Indicaxanthin, natural compound extracted from Opuntia ficus-indica, by the establishment of new reverse pharmacophore mapping and reverse docking approaches.

EDUCATION

2017	Qualified pharmacist, University of Palermo, Italy
2017	Five-year Master's degree in Pharmaceutical Chemistry and Technology, University of Palermo, Italy Grade: 110/110 *cum Laude*
2016	Volunteering Exchange at the community of Reconciliation, Worcestershire, United Kingdom
2015 - 2016	University training at Dr. Fontana's pharmacy, Trapani, Italy

CONFERENCE AND CURRICULAR ACTIVITIES

22-26 June 2022	Selected for oral presentation at the International Liver congress EASL, 2022
2021	DATAETHICS pilot school
2021, June	International Liver congress, EASL, 2021 (poster session)
2021, June	ANRS meeting, 2021 (oral presentation)
2020, February	ANRS meeting, 2020, Paris, France
2019 – 2020	Vice-secretary at Association of International Researchers in Strasbourg (StrasAIR)
2019, September	EUROPIN Summer School on Drug Design, Vienna, Austria (poster session)
2019, July	ERC HEPICIR SYMPOSIUM, IRCAD Research institute, Strasbourg, France
2019, June	International summer school in transplantation: Transplantex NG: Immunology and Genomic Medicine, Strasbourg, France
2018, November	Winter school LabEx Hepsys "Addressing the next challenges in liver disease", Strasbourg, France
2018, July	RICT – Rencontres Internationales de Chimie Thérapeutique, Strasbourg, France (poster session)

PROFESSIONAL SKILLS

Scientific techniques: Cell culture, RT-qPCR, western blotting, immunofluorescence, infection models (Biosafety level 3), molecular biology and oncology, Gene Set Enrichment Analysis (GSEA).

Software: GenePattern, Graphpad Prism, Adobe Illustrator, Rstudio (EdX DataScience: R Basics certificate), LigandScout, Schrodinger Suite MS office.

Languages: English (fluent), French (intermediate), Spanish (intermediate), Italian (mother tongue).

SCIENTIFIC OUTPUT

ORIGINAL ARTICLES:

Virzì A, Boulahtouf Z, Heydmann L, Durand C. S, Ramberger E, Popp O, Moehlin J, Juhling F, Felli E, Mertins P, Pessaux P, Verrier E. R, Schuster C, Baumert T, Lupberger J. Phosphoproteomic atlas of HBV infection reveals a novel role of collagen VI and linker histone for liver pathogenesis and viral life cycle. Prepared for submission in *Cell Host and Microbe* as short article.

Roca Suarez A.A, Mailly L, **Virzì A**, Brignon N, Jühling F, Thumann C, Durand S, Oudot M, Schaeffer E, Martin R, Heydmann L, Bach C, Parent R., Jamey C, Brumaru D, Dali-Youcef N, Felli E, Pessaux P, Mukherji A, Schuster C, Baumert T, Lupberger J. Impaired hepatic protein tyrosine phosphatase delta is a driver of metabolic syndrome. *In preparation.*

Butterworth J, Gregoire D, Peter M, Roca Suarez AA, Desandré G, Simonin Y, **Virzì A**, Zine El Aabidine A, Guivarch M, Andrau JC, Bertrand E, Assenat E, Lupberger J, Hibner U. GOLT1B activation in hepatitis C virus-infected hepatocytes links er trafficking and viral replication. Pathogens. 2021 Dec 31;11(1):46.

Lupberger J, Croonenborghs T, Roca Suarez AA, Van Renne N, Jühling F, Oudot MA, **Virzì A,** Bandiera S, Jamey C, Meszaros G, Brumaru D, Mukherji A, Durand SC, Heydmann L, Verrier ER, El Saghire H, Hamdane N, Bartenschlager R, Fereshetian S, Ramberger E, Sinha R, Nabian M, Everaert C, Jovanovic M, Mertins P, Carr SA, Chayama K, Dali-Youcef N, Ricci R, Bardeesy NM, Fujiwara N, Gevaert O, Zeisel MB, Hoshida Y, Pochet N, Baumert TF. Combined analysis of metabolomes, proteomes, and transcriptomes of hepatitis C virus-infected cells and liver to identify pathways associated with disease development. Gastroenterology. 2019 Aug;157(2):537-551.e9.

Tutone M, **Virzì A**, Almerico AM. Reverse screening on indicaxanthin from opuntia ficus-indica as natural chemoactive and chemopreventive agent. J Theor Biol. 2018 Oct 14;455:147-160.

REVIEWS, BOOK CHAPTERS AND EDITORIALS:

Boulahtouf Z, **Virzì A,** Baumert TF, Verrier ER, Lupberger J. Signaling Induced by Chronic Viral Hepatitis: Dependence and Consequences. International Journal of Molecular Sciences. 2022 Mar 3;23(5):2787. (Review).

Virzì A, Gonzalez-Motos V, Tripon S, Baumert TF, Lupberger J. Profibrotic signaling and HCC risk during chronic viral hepatitis: biomarker development. J Clin Med. 2021 Mar 2;10(5):977. (Review).

Virzì A, Roca Suarez AA, Lupberger J. Interferon revisited: peering behind the lines of antiviral defense. J Hepatol. 2020 Sep;73(3):496-498. Epub 2020 Jul 11. (Editorial).

Virzì A, Roca Suarez AA, Baumert TF, Lupberger J. Rewiring host signaling: hepatitis C virus in liver pathogenesis. Cold Spring Harb Perspect Med. 2020 Jan 2;10(1):a037366. (Book Chapter).

Saviano A, Roehlen N, **Virzì A**, Roca Suarez AA, Hoshida Y, Lupberger J, Baumert TF. Stromal and immune drivers of hepatocarcinogenesis. 2019 Aug 6. In: Hoshida Y, editor. Hepatocellular Carcinoma: Translational Precision Medicine Approaches. Cham (CH): Humana Press; 2019. Chapter 15. (Book Chapter).

Virzì A, Roca Suarez AA, Baumert TF, Lupberger J. Oncogenic signaling induced by HCV infection. Viruses. 2018 Oct 2;10(10):538. (Review).

ORAL PRESENTATION AND POSTER SESSION:

Virzì A, Boulahtouf Z, Heydmann L, Durand C. S, Ramberger E, Popp O, Moehlin J, Juhling F, Felli E, Mertins P, Pessaux P, Verrier E. R, Schuster C, Baumert T, Lupberger J. Phospho-proteomic analysis of HBV infection revealed novel mechanisms for the regulation of viral transcription and pro-fibrotic stellate cell activation. EASL, 2022 (upcoming oral presentation).

Virzì A, Heydmann L, Durand S, Felli E, Pessaux P, Verrier E. R, Schuster C, Baumert F. T, Lupberger J. HBV-infection induces collagen VI expression by hepatocytes promoting stellate cell-activation and liver fibrosis. at International Liver congress, EASL, 2021 (Poster session).

Virzì A. HBV proteomic analysis on hepatocytes identifies collagen VI as driver for liver fibrosis. ANRS meeting, 2021 (Oral presentation).

Virzì A. Proteogenomic and phospho-proteomic atlas of HBV infection - new insights into virus entry and liver pathogenesis. ANRS meeting, Paris, France 2020 (Oral presentation).

Virzì A, Bryant S., Selective A2A Pharmacophore Models Discriminating Agonist from Antagonist Compounds. EUROPIN Summer School on Drug Design, Vienna, Austria, 2019 (Poster session).

Tutone M, **Virzì A,** Almerico AM. Reverse screening on indicaxanthin from opuntia ficus-indica as natural chemoactive and chemopreventive agent. National Meeting on Medicinal Chemistry (NMMC2018), Palermo, Italy, 2018 (Poster session).

Virzì A, Bryant S., Selective A2A Pharmacophore Models Discriminating Agonist from Antagonist Compounds. at RICT – Rencontres Internationales de Chimie Thérapeutique, Strasbourg, France, 2018 (Poster session).

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"Progress can be slow and gradual. Continue putting in effort with patience, enthusiasm and faith". Mata Amritanandamayi'

Alessia VIRZI

L'ANALYSE PHOSPHO-PROTEOMIQUE DES CELLULES INFECTEES PAR LE VHB REVELE

UN NOUVEAU ROLE DU COLLAGENE VI ET DE L'HISTONE LINKER DANS LA

PATHOGENESE DU FOIE ET LE CYCLE DE VIE VIRALE

Résumé

L'hépatite B chronique est une des causes majeures des maladies du foie. L'éradication totale du virus de l'hépatite B (VHB) est difficilement réalisable en raison de la persistance de l'ADNccc. De plus, les antiviraux utilisés actuellement nécessitent un traitement à long terme et ne peuvent pas garantir une inversion de la fibrose hépatique. En conséquence, il est urgent de comprendre les voies de signalisation impliquées dans le cycle de vie viral et la pathogenèse hépatique. Ici, nous avons fourni un atlas protéomique et phospho-protéomique complet des cellules infectées par le VHB qui a fortement mis en évidence une dérégulation des voies de signalisation impliquées dans le remodelage de la matrice extracellulaire et le contrôle du cycle cellulaire. Nous avons découvert un rôle proviral du collagène VI dans les hépatocytes qui, en outre, renforce l'activation des cellules étoilées hépatiques. De plus, nous avons montré une phosphorylation des histones de liaison induite par le VHB dans la phase G1 du cycle cellulaire, qui a un impact spécifique sur la transcription du VHB, possiblement en rapport avec l'ADNccc. Ces résultats permettent de mieux comprendre les maladies hépatiques liées au VHB et peuvent contribuer à l'identification de nouvelles stratégies thérapeutiques.

Mots-clés : virus de l'hépatite B ; voies de signalisations ; ADNccc ; cellules étoilées hépatiques.

Résumé en anglais

Chronic hepatitis B virus (CHB) is the major cause of liver disease progression. Total eradication of the virus is hardly achieved due to cccDNA persistence. Moreover, the current antivirals require long-term treatment and cannot guarantee a reversal of liver fibrosis. Considering the lack of curative antiviral treatment, the understanding of the signaling pathways involved in the HBV life cycle and CHB liver pathogenesis is urgently needed. Here, we provided a comprehensive proteomic and phosphoproteomic atlas of the complete HBV viral cycle. Our atlas strongly highlighted a dysregulation of signaling pathways involved in extra-cellular matrix remodeling and cell cycle checkpoint. We uncovered a pro-viral role of collagen VI in hepatocytes which in addition reinforces the hepatic stellate cells activation. Moreover, we revealed an HBV-induced linker histone phosphorylation in the G1 phase of the cell cycle, which specifically impacts HBV transcription with link to cccDNA. These findings provide insights on HBV-related liver disease and may help in the identification of druggable targets for novel therapeutic strategies.

Key words: Hepatitis B virus, signal transduction, liver fibrosis, cccDNA, hepatic stellate cells.