UNIVERSITÉ DE STRASBOURG

UNIVERSITÉ DE STRASBOURG
ÉCOLE DOCTORALE SCIENCES DE LA VIE ET DE LA SANTÉ - 414

Thesis presented by:
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To obtain the degree:
Doctor of the University of Strasbourg
Specialty: Virology – Molecular and Medical Aspects

Expression of human protein phosphatases during chronic HCV infection and the development of hepatocellular carcinoma

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Presented in public: *Strasbourg - 29th of November 2019*

Cover art by Nicolle R. Fuller

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Bellevue, WA, USA

Acknowledgements

I would like to acknowledge the support of several people involved during the realization of my PhD:

My parents Armando and Nancy, my brothers Fernando, Jimena, Rodrigo and Adrian. Without their support I would have never been able to start my carrier as a researcher. They have been my motivation all these years and I will never be able to fully express how grateful I am to all of them.

Kristina for always been there in the moments of success and failure. Her understanding and care during these years have been fundamental to accomplish my personal goals, improve as a scientist and become a better man.

My PhD supervisors Dr. Joachim Lupberger and Prof. Thomas F. Baumert. They not only provided me with the opportunity of doing my doctoral studies but also guided me throughout this process in a manner that I now consider them great friends as well.

My colleagues at the UMR-S1110. As for most students doing a PhD, this is not an easy task but having the help and support of these friends definitely made this process easier.

This doctoral thesis was supported by grants from: *L'Agence nationale de recherche sur le sida et les hépatites virales* (ANRS) and *La Fondation pour la Recherche Médicale en France* (FRM).

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Abbreviations

AHC	Acute HCV infection
Akt	Akt serine/threonine kinase
ANG	Angiopoietin
ALT	Alanine aminotransferase
ApoC	Apolipoprotein C
ApoE	Apolipoprotein E
ATP	Adenosine triphosphate
BCLC	Barcelona Clinic Liver Cancer
BRAF	B-raf proto-oncogene
CAT	Catalase
CD81	Cluster of differentiation 81
CDC14s	Cell division cycle 14 phosphatases
CD-HFD	Choline-deficient high-fat diet
CHC	Chronic HCV infection
CLDN1	Claudin 1
cLDs	Cytoplasmic lipid droplets
CPT1A	Carnitine palmitoyltransferase 1A
CypA	Cyclophilin A
DAA	Direct-acting antiviral
DGAT1	Diacylglycerol acyltransferase 1
DMSO	Dimethyl sulfoxide
DSP	Dual-specificity phosphatase
EASL	European Association for the Study of the Liver
ECM	Extracellular matrix
EGFR	Epidermal growth factor receptor
Eph2A	Ephrin receptor type 2A
ES	Enrichment score
ER	Endoplasmic reticulum
FDA	U.S. Food and Drug Administration
FDR	False discovery rate

FN	Fibronectin
GAG	Glycosaminoglycan
GSEA	Gene set enrichment analysis
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HGDN	High-grade dysplastic nodules
HOMA2	Homeostasis model assessment 2
HSC	Hepatic stellate cells
ICTV	International Committee on Taxonomy of Viruses
IFN	Interferon
Ig	Immunoglobulin
IL-6	Interleukin 6
IL-6R	Interleukin 6 receptor
InsR	Insulin receptor
IR	Insulin resistance
IRES	Internal ribosome entry site
IRS-1	Insulin receptor substrate 1
ISG	Interferon-stimulated gene
LDLR	Low-density lipoprotein receptor
LGDN	Low-grade dysplastic nodule
LuLDs	Luminal lipid droplets
LVP	Lipoviroparticle
MDA5	Melanoma differentiation-associated protein 5
miRNA	MicroRNA
MKPs	MAPK phosphatases
MOI	Multiplicity of infection
MTMRs	Myotubularin-related phosphatases
mTORC	Mechanistic target of rapamycin kinase
MTTP	Microsomal triglyceride transfer protein
NC	Nucleocapsid
NES	Normalized enrichment score
NPC1L1	Niemann-Pick C1-like 1

OCLN	Occludin
ORF	Open reading frame
PD-1	Programmed cell death protein 1
PDGFR	Platelet-derived growth factor receptor
PDK1	Phosphoinositide-dependent kinase 1
PIP3	Phosphatidylinositol triphosphate
PKR	Protein kinase R
PP2A	Protein phosphatase 2A
PPARα	Peroxisome proliferator activated receptor alpha
PRLs	Phosphatases of regenerating liver
PTK	Protein tyrosine kinase
PTM	Post-translational modification
PTP	Protein tyrosine phosphatase
PTPNs	Non-receptor protein tyrosine phosphatases
PTPRs	Receptor protein tyrosine phosphatases
PTPRD	Protein tyrosine phosphatase receptor type delta
RIG-I	Retinoic acid-inducible gene I
RNA	Ribonucleic acid
RXR	Retinoid X receptor
SOCS3	Suppressor of cytokine signaling 3
SR-BI	Scavenger receptor, class B type I
SREBP	Sterol regulatory element binding protein
SSHs	Slingshot phosphatases
STAT3	Signal transducer and activator of transcription 3
SVR	Sustained virological response
TACE	Transarterial chemoembolization
TERT	Telomerase reverse transcriptase
TG	Triglycerides
TGF-α	Transforming growth factor alpha
TGF-β	Transforming growth factor beta
TIM-3	T cell immunoglobulin mucin 3
TLR3	Toll-like receptor 3

UTR	Untranslated region
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor

1.- Introduction

1.1.- The global burden of HCV-associated disease:

Since the first observations of non-A non-B posttransfusion hepatitis in the 1970s (Feinstone, Kapikian et al. 1975) and the discovery of the hepatitis C virus (HCV) in 1989 as the etiological agent (Choo, Kuo et al. 1989), the HCV field has undergone a true scientific and therapeutic revolution due to the recent development of direct-acting antivirals (DAA) (Martinello, Hajarizadeh et al. 2018). Indeed, as discussed in later chapters, the treatment of chronic HCV-infected patients with current DAAs allows elimination of the virus in more than 90% of cases (EASL 2018). Despite these important achievements, several challenges remain leaving HCV infection as a public health concern.

Recent estimations suggest that there are approximately 71 million chronically infected individuals worldwide with the highest HCV prevalence in countries with a history of iatrogenic infections (e.g. Egypt, Cameroon and Nigeria) (Polaris 2017). Western countries where injection drug use is an important risk factor (e.g. Australia, Finland and United Kingdom) account only for a small percentage of HCV infections globally (**Fig. 1**). From this population, less than 20% of HCV-infected patients are ever treated due to the low rates of case identification, inadequate health insurance coverage, limited access to regular health care and the high cost of medications (Thrift, El-Serag et al. 2017). Consequently, HCV-infected patients are at an increased risk of developing hepatic complications such as cirrhosis, decompensation and hepatocellular carcinoma (HCC). Additionally, HCV infection induces a series of extrahepatic manifestations including non-Hodgkin lymphoma, cryoglobulinemia and type II diabetes (Cacoub, Gragnani et al. 2014). The total of these HCV-associated complications accounts for more than 500,000 related deaths per year (Mohd Hanafiah, Groeger et al. 2013). Although successful viral elimination significantly reduces the risk of HCC development and liver-related mortality, these remain considerably high in HCV-cured patients (van der Meer, Veldt et al. 2012).

In contrast to the success of DAAs, the clinical management of HCV-associated complications presents several areas that remain to this day without effective intervention. These include for example the early identification of risk-patients using specific and reliable biomarkers, the implementation of chemo-preventive strategies and the development of new systemic therapies. Indeed, to date there are only few systemic agents having received U.S. Food and Drug Administration (FDA) approval for the treatment of

HCC (Villanueva 2019). Therefore, a more detailed understanding of the molecular alterations induced by HCV infection is needed in order to meet these clinical demands and improve the management of its associated complications.

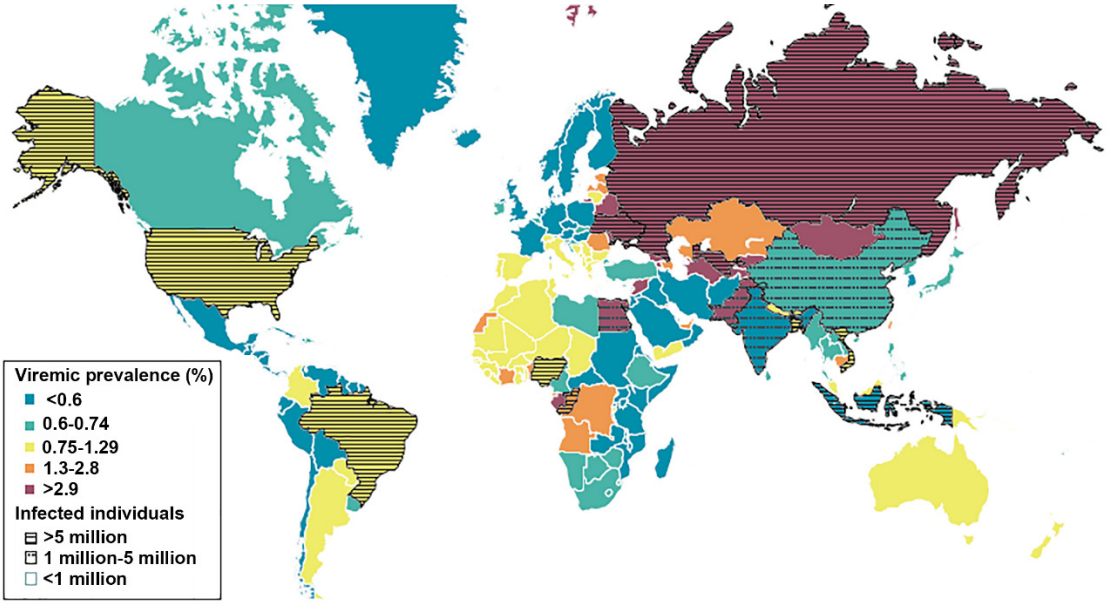


Figure 1: HCV prevalence. Representation of the viremic HCV prevalence and the extrapolated total HCV infections per country (Image modified from Manns, Buti et al. 2017).

1.2.- The hepatitis C virus

1.2.1.- The HCV particle

HCV belongs to the genus *Hepacivirus* within the family *Flaviviridae*. These viruses present a positive-sense non-segmented RNA genome that contains one long open reading frame (ORF) flanked by highly structured untranslated regions (UTRs) (Simmonds, Becher et al. 2017). In the case of HCV, the 5' UTR contains a type III internal ribosome entry site (IRES) that directs translation of the genome in a cap-independent manner. In addition, the 5' UTR presents two binding sites for miR-122, one of the most abundant liver-specific microRNAs (miRNA), which seems to be a positive regulator of HCV replication by stabilizing the viral genome (Jopling 2012). The 3' UTR region contains several *cis*-acting RNA elements that favor translation of the HCV RNA. The ORF encodes a polyprotein of around 3,000 amino acids which is cleaved co- and post-translationally into ten different proteins. The proteins generated from the amino-terminal region of the polyprotein are structural components of the virus particle (i.e. core, E1 and E2) or have a

function during particle assembly while not being incorporated into it (i.e. p7 and NS2). The remaining polyprotein cleavage products (i.e. NS3, NS4A, NS4B, NS5A and NS5B) have distinct roles during viral RNA replication (**Fig. 2a**).

HCV viral particles are 50–80 nanometers in diameter with E1 and E2 glycoprotein heterodimers present in the lipid bilayer which surrounds the nucleocapsid (NC) and the RNA genome within it. The virions exist as lipoviroparticles (LVP) since they are associated with lipids (cholesterol esters and triglycerides) and apolipoproteins, conferring them an unusually low buoyant density (**Fig. 2b-c**).

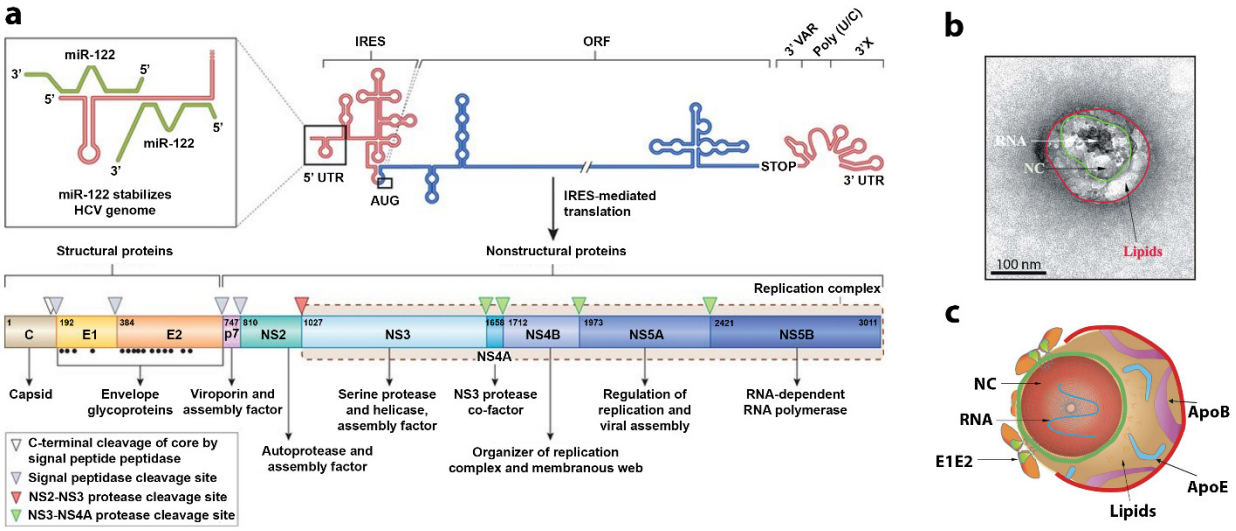


Figure 2: HCV genome and particle organization. a) The HCV genome containing the central ORF (blue) flanked by the 5' and 3' UTRs with their predicted secondary structures (red). Translation of the ORF leads to the polyprotein which is processed into ten viral proteins. Triangles indicate the cellular or viral protease implicated in the cleavage. Glycosylation sites of E1, E2 (black dots) and the function of each protein are indicated (Scheel and Rice 2013). **b**) Electron micrograph of an HCV particle captured from infected patients with red and green lines indicating the outer limits of the NC and the LVP respectively. **c**) Model of the HCV LVP (Piver, Boyer et al. 2017).

1.2.2.- The HCV life cycle

Although HCV RNA, proteins or attached particles have been detected in monocytes (Marukian, Jones et al. 2008), endothelial cells (Fletcher, Wilson et al. 2012), B lymphocytes (Stamataki, Shannon-Lowe et al. 2009), dendritic cells and brain tissue (Meredith, Wilson et al. 2012), the virus presents mainly a tropism

for hepatocytes. As we will see in the following sections, this is due to the specific presence of several host factors in the liver that allow the full HCV cycle to take place (**Fig. 3**).

1.2.2.1.- Viral entry and genome release

Virus attachment to the surface of the hepatocytes involves the glycoproteins E1 and E2, as well as the apolipoproteins present in the LPV which interact with several cellular surface proteins in a complex multi-step process. Glycosaminoglycans (GAG) and the low-density lipoprotein receptor (LDLR) are involved in the initial low-affinity binding, before E2 interaction with the co-receptors scavenger receptor class B type I (SR-BI) and cluster of differentiation 81 (CD81). Claudin-1 (CLDN1), occludin (OCLN), epidermal growth factor receptor (EGFR) and ephrin receptor type 2A (EphA2) are also required for entry (Manns, Buti et al. 2017). Virion-associated cholesterol seems to be involved at a late stage of HCV entry, via its interaction with the Niemann-Pick C1-like 1 (NPC1L1) cholesterol absorption receptor (Sainz, Barretto et al. 2012).

Uptake of the virion occurs via clathrin-mediated endocytosis and requires a low pH compartment encountered in endosomes. In this acidic environment, HCV E2 is primed by CD81 for activation which induces fusion between the viral envelope and the bounding endosomal membrane (Sharma, Mateu et al. 2011). These entry processes lead to the release of the HCV genome into the cytoplasm.

1.2.2.2.- Translation and polyprotein processing

Once the viral genome is present in the cytoplasm, endoplasmic reticulum (ER)-associated translation takes place and is initiated via the IRES located in the 5' UTR of the HCV genome. Although the mechanism has not been fully elucidated, it has been suggested that the HCV genome can circularize by interactions between motifs in the IRES and stem-loop structures residing in the NS5B coding region (Romero-Lopez, Barroso-Deljesus et al. 2014). This circularization of the HCV genome might prevent clashes between translating ribosomes moving in the 5' to 3' direction and the viral replication complex moving in the opposite sense (Paul, Madan et al. 2014). The resulting HCV polyprotein is co- and post-translationally cleaved by cellular proteases (i.e. signal peptidase and signal peptide peptidase) and the viral NS2-NS3 and NS3-NS4A proteases producing the release of each one of the ten HCV proteins.

1.2.2.3.- Viral genome replication

Following polyprotein cleavage, the formation of the viral replicase complex takes place. This complex is constituted of NS3, NS4A, NS5A, NS5B and the genomic RNA template. Besides its function as protease, NS3 possesses a C-terminal helicase domain which is important for RNA replication and has been shown to be able to unwind RNA *in vitro* (Dumont, Cheng et al. 2006). This RNA helicase function is stimulated by its cofactor NS4A. The actual RNA replication takes place in association with ER-derived membrane compartments. The formation of this so-called membranous web is induced by NS4B and NS5A. NS5A also induces the activation of the NS5B RNA-dependent RNA polymerase, which is suggested to be mediated by the RNA-binding ability of NS5A or by direct interaction with NS5B (Quezada and Kane 2013).
Additional host factors for HCV replication are miR-122 and the peptidyl-prolyl *cis-trans* isomerase cyclophilin A (CypA). CypA, which binds NS5A, is believed to induce conformational changes necessary for HCV RNA replication (Yang, Robotham et al. 2008).

1.2.2.4.- Virion assembly and release

The formation of HCV virions starts with the relocation of core and NS5A from ER membranes to cytoplasmic lipid droplets (cLDs), via their interaction with diacylglycerol acyltransferase-1 (DGAT1) (Herker, Harris et al. 2010). How the HCV genome is directed to the sites of nucleocapsid assembly remains unclear, but it has been suggested that this could be facilitated by the spatial proximity of the sites where replication and assembly take place (Bartenschlager, Penin et al. 2011). Translocation of E1 and E2 to the ER induces a type I membrane protein topology of these glycoproteins. Following heterodimer formation and addition of N-linked sugars, the E1, E2 glycans are then trimmed by glycosidases I and II (Lavie, Goffard et al. 2007).

During later stages of viral assembly, the nucleocapsid is transferred to the precursor of VLDL particles called luminal lipid droplets (luLDs). These intracellular lipid droplets containing the nucleocapsid, fuse with VLDLs containing ApoB to form the LVPs which also acquire ApoE and ApoC (Gastaminza, Cheng et al. 2008) before their final exit through the Golgi (Coller, Heaton et al. 2012).

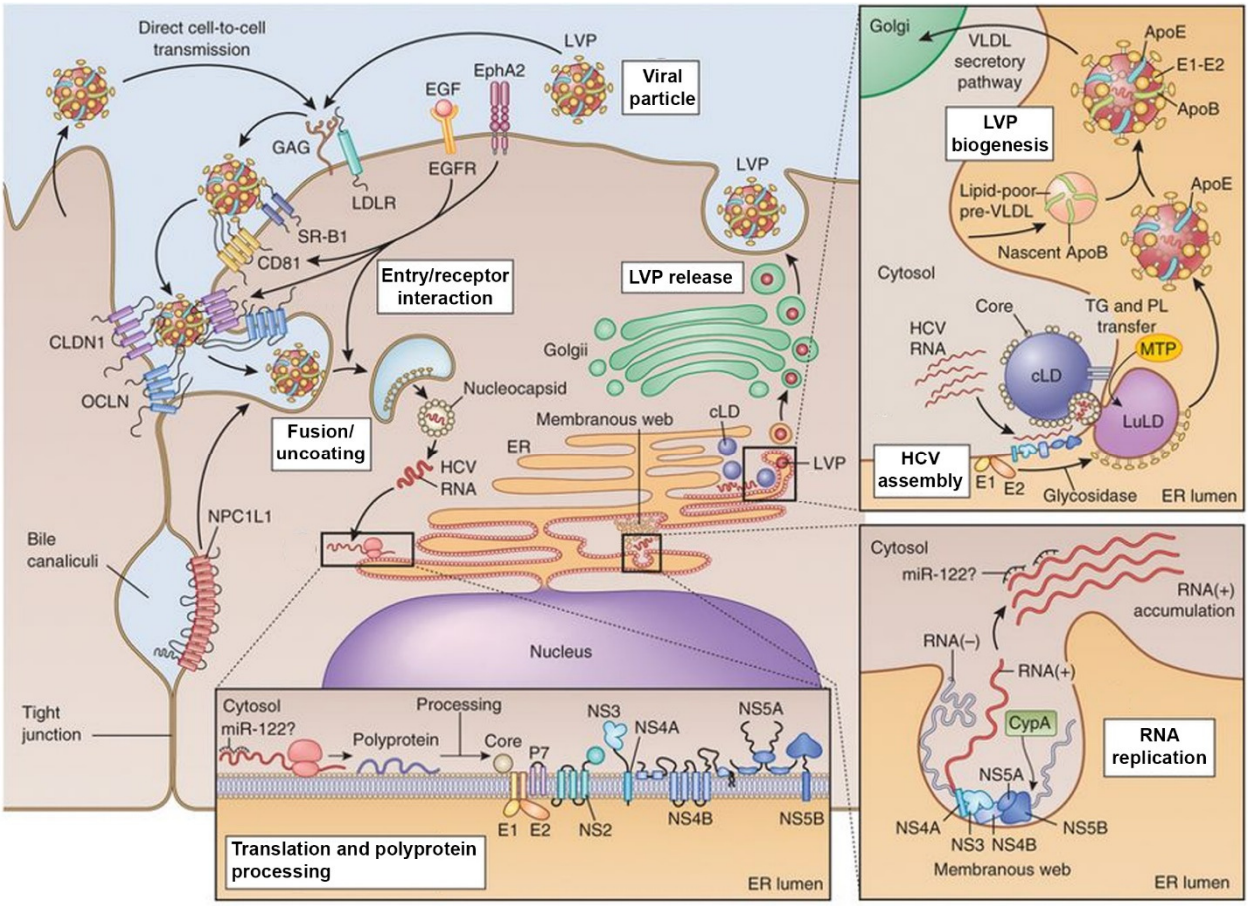


Figure 3: The HCV life cycle. Model of the different stages required for the HCV viral cycle including entry, fusion, translation, genome replication, HCV assembly and LVP release (Image modified from Scheel and Rice 2013).

1.2.3.- HCV genotypes and subtypes

HCV is a highly heterogenous virus and circulates as quasispecies in the blood of infected patients (Fafi-Kremer, Fofana et al. 2010). As most RNA viruses, the main factor of this genetic diversity is the lack of proofreading by its RNA-dependent RNA polymerase NS5B. Although in theory these mutations could be present at random positions in the viral genome, it is well-stablished that certain regions are more prone to variability such as the ones coding for the E1, E2 glycoproteins. The lowest sequence variability is found in the 5' UTR, since these secondary structures are required for an efficient replication and translation (Simmonds 2004).

This variation in nucleotide sequence led to the current classification of HCV into 8 distinct genotypes, which are further subdivided into 90 subtypes according to the International Committee on Taxonomy of

Viruses (ICTV). Each HCV genotype differs from the others by approximately 30% and each subtype by at least 15% (**Fig. 4a**). The distribution of these genotypes varies according to different geographical regions, for example genotype 1 is more prevalent in the Americas, Europe and Australia, genotype 3 in India and Pakistan and genotype 4 is the main one observed in Egypt (Manns, Buti et al. 2017) (**Fig. 4b**). Genotypes 7 and 8 represent only a small minority of cases that have been detected in the Republic of Congo (Murphy, Sablon et al. 2015) and India (Borgia, Hedskog et al. 2018) respectively.

As we will see in a following section, the HCV genotype influences the disease course and the therapeutic approach used to treat chronically infected patients.

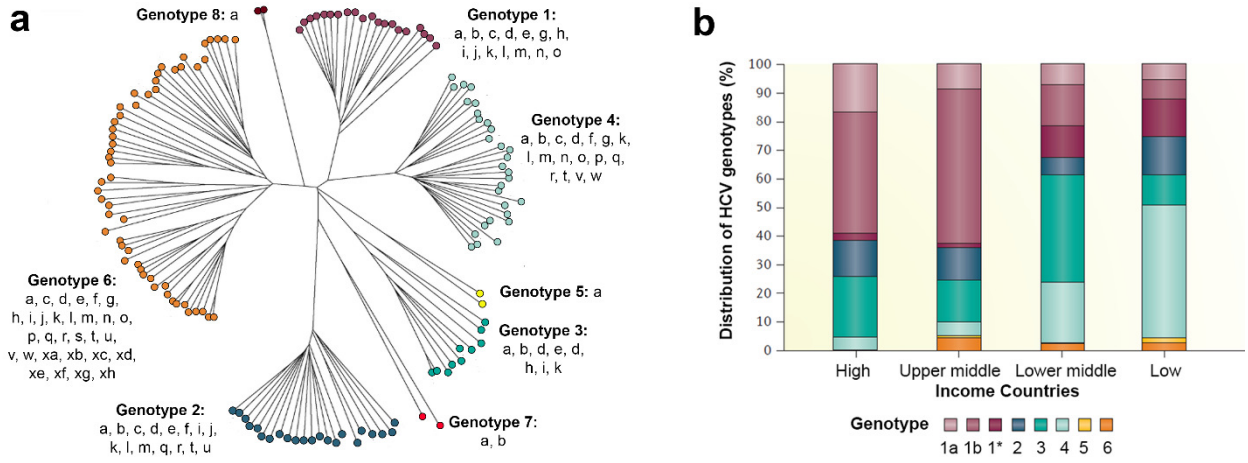


Figure 4: HCV genotypes, subtypes and global distribution. a) Phylogenetic tree of the HCV genotypes and the subtypes of each group (Image modified from the ICTV database). **b**) HCV genotype distribution according to country income category (Image modified from Manns, Buti et al. 2017). * HCV genotype 1 other than 1a or 1b.

1.3.- Hepatitis C virus natural history

1.3.1.- Acute HCV infection

Acute HCV infection (AHC) refers to the 6-month period following infection acquisition. AHC is often unrecognized but can be suspected in patients presenting marked elevation of alanine aminotransferase (ALT) levels and clinical manifestations such as jaundice, fever, headache, malaise, anorexia, nausea, vomiting, diarrhea or abdominal pain. In potentially HCV-infected patients, further tests need to be carried

out employing a quantitative method to detect HCV RNA or core levels in serum or plasma, although these may vary widely and present interludes where they are not detectable. Therefore, individuals presenting a negative HCV test must be retested 12 and 24 weeks after the first results as confirmation (EASL 2018). A self-limited HCV infection occurs in 15-25% of infected patients (Micallef, Kaldor et al. 2006), an outcome that results from the complex interplay between host and viral factors leading to a successful immune response (**Fig. 5**).

After cell entry, HCV RNA is detected by several cellular sensors which initiate an innate immune response against the virus. Some of these sensors include retinoic acid-inducible gene I (RIG-I), melanoma differentiation-associated protein 5 (MDA5), toll-like receptor 3 (TLR3) and protein kinase R (PKR) (Shin, Sung et al. 2016). This induces the production of type I and type III interferons (IFN) suppressing HCV replication via the action of interferon-stimulated genes (ISGs). The initial defense mechanisms mounted by the host are followed by a virus-specific T cell response, which is remarkably delayed despite the early increase in HCV titers and the early induction of the innate response. Consistently, HCV-specific T cells cannot be detected in the blood and liver before 8–12 weeks after infection (Shin, Park et al. 2011) (**Fig. 5a**). A key determinant of infection outcome is the action of virus-specific $CD8^+$ T cells and their robust production of IFN-γ, which is strongly correlated with a spontaneous resolution of acute HCV infection. $CD4^+$ T cells also have an important role in this process since a strong and broad HCV-specific $CD4^+$ T cell response allows the induction and maintenance of effector and memory $CD8^+$ T cells (**Fig. 5b**).

1.3.2.- Chronic HCV infection

The majority of HCV-infected patients (75-85%) progress towards chronicity (Martinello, Hajarizadeh et al. 2018). The diagnosis of chronic HCV infection (CHC) is defined as the presence of both anti-HCV antibodies and HCV RNA or core antigen beyond 4-6 months after infection, since spontaneous viral clearance rarely occurs after this time period (EASL 2018) (**Fig. 5c**). It has been observed that in CHC patients $CD4^+$ T cell proliferation and IL-2 production are diminished, leading to an impaired HCV-specific $CD8^+$ T cell response. Moreover, the function of $CD8^+$ T cells is reduced via *T cell exhaustion* due to the sustained HCV antigen stimulation (Shin, Sung et al. 2016). These exhausted $CD8^+$ T cells are characterized by an increased expression of inhibitory receptors such as programmed cell death protein 1 (PD-1), T cell immunoglobulin mucin 3 (TIM-3) and 2B4 (cd244) (Kroy, Ciuffreda et al. 2014). Finally, the high mutation rate in the HCV genome allows the virus to escape the immune response mediated by HCV-specific $CD8^+$ T cells (**Fig. 5d**).

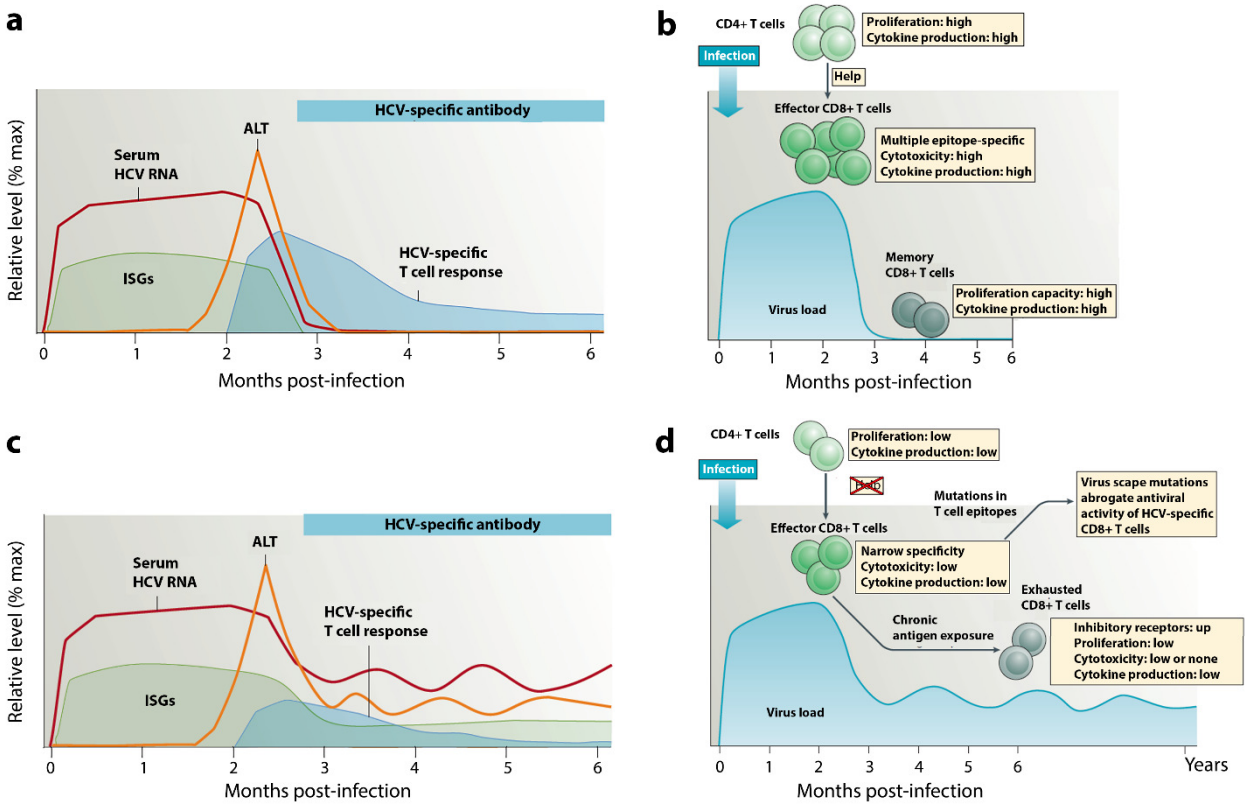


Figure 5: Immune response to HCV infection. a) In the case of self-limited acute HCV infection (AHC), the marked increase of serum HCV RNA is followed by the expression of ISGs. The virus-specific T cell response takes place in a delayed manner and coincides with liver injury (ALT increase) and virus control. **b**) Self-limited HCV infection is associated with a strong $CD8^+$ T cell response. Spontaneous resolution of HCV infection only takes place when $CD4^+$ T cell help is simultaneously maintained. **c**) During chronic HCV infection (CHC), patients present the initial induction of ISGs as in the case of AHC with the difference that this response is maintained as long as the virus persists within the host. **d**) CHC is favored by an impaired $CD4^+$ T cell function, the exhaustion of $CD8^+$ T cells and a diminished antiviral activity due to virus escape mutations (Image modified from Shin, Sung et al. 2016).

1.3.3.- Liver fibrosis and cirrhosis

In patients with CHC, progressive fibrosis and cirrhosis account for the majority of HCV-related morbidity and mortality. Estimates suggest that 15–35% of CHC patients develop this complication after 20–30 years of infection (Freeman, Dore et al. 2001). Hepatic fibrosis is a complex multicellular wound-healing process

that takes place in response to epithelial cell injury and is mediated by the inflammatory response against HCV infection (**Fig. 6a**). In response to this initial injury and hepatocyte death, hepatic stellate cells (HSCs) proliferate and differentiate into myofibroblasts which are the main source of extracellular matrix (ECM) production. This process is further perpetuated by the action of Th2 lymphocytes and macrophages, the later which express profibrogenic molecules such as transforming growth factor beta (TGF-β), TGF-α and interleukin 1β (IL-1β). Additionally, an important component involved during liver fibrosis is angiogenesis which is mediated by the action of sinusoidal endothelial cells and the release of vascular endothelial growth factor (VEGF) and angiopoietin (ANG). Ultimately, the sum of these cellular alterations contributes to the development of liver cirrhosis (Ramachandran and Henderson 2016).

During liver cirrhosis, the zones of parenchymal loss transform into dense fibrous septa which eventually surround the surviving and regenerating hepatocytes. This constant cycle of hepatocyte death and proliferation leads to the formation of regenerative nodules (**Fig. 6b**) and the architectural alteration of the entire liver (**Fig. 6c**) (Kumar, Abbas et al. 2015).

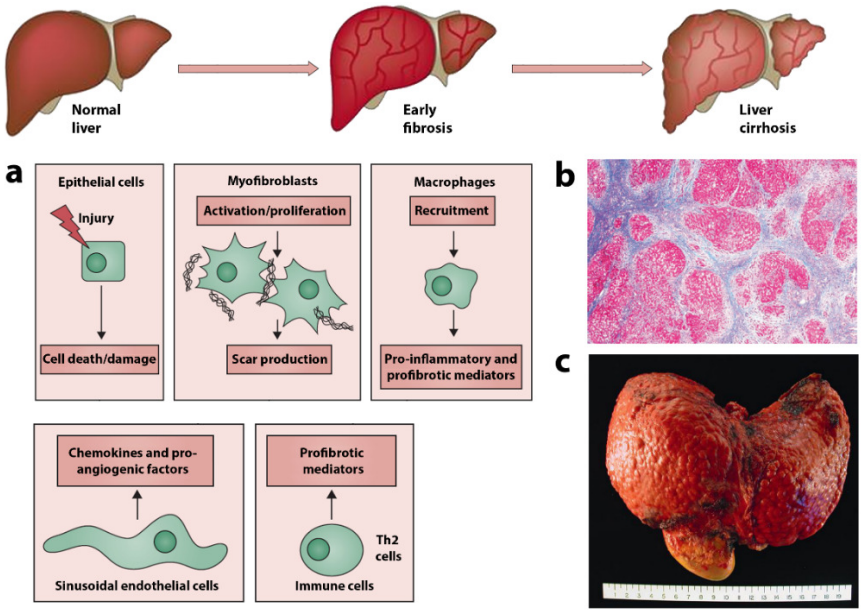


Figure 6: Development of liver fibrosis and cirrhosis. a) Cellular mechanisms implicated during liver fibrosis (Image modified from Ramachandran and Henderson 2016). **b**) Microscopic image of liver cirrhosis showing thick bands of collagen which separate rounded cirrhotic nodules. **c**) Macroscopic image showing depressed areas of dense scar tissue separating bulging regenerative nodules over the liver surface (Kumar, Abbas et al. 2015).

1.3.4.– Hepatocellular carcinoma

Patients affected by HCV-associated cirrhosis present a 4-5% cumulative annual incidence of HCC (El-Serag 2012). The HCV viral cycle in combination with the inflammatory microenvironment observed during liver cirrhosis favors this progression, starting with the development of low-grade dysplastic nodules (LGDNs). One of the early alterations observed in these LGDNs is the presence of mutations that induce the re-expression of the gene telomerase reverse transcriptase (*TERT*), which is not expressed in normal hepatocytes (Kotoula, Hytiroglou et al. 2002). This situation is encountered even more often when LGDNs develop into high-grade dysplastic nodules (HGDNs) and progress to early-stage HCC. Additional signaling alterations in these early steps are related to the activation of the Wnt-β-catenin pathway, the response to oxidative phosphorylation and the deregulation of components associated to protein folding pathways (Llovet, Zucman-Rossi et al. 2016).

In later stages of HCC progression, a further deregulation of cell signaling pathways is observed (Schulze, Imbeaud et al. 2015). Cell cycle control is altered by mutations of tumor suppressor genes such as p53 (although this is more common in hepatitis B patients), retinoblastoma 1 (*RB1*) and deletions of cyclin-dependent kinase inhibitor 2A (*CDKN2*). Epigenetic regulators are similarly often altered including mutations of AT-rich interaction domain 1A (*ARID1A*) and in the lysine methyltransferase 2 (*KMT2*) family of genes. Interestingly, HCV-induced epigenetic alterations are not exclusively associated to mutations at later stages of HCC development and can be observed already during chronic HCV infection (Hamdane, Juhling et al. 2019). Finally, inactivating mutations in genes such as tuberous sclerosis 1 (*TSC1*) and phosphatase and tensin homologue (*PTEN*) mediate the activation of the Akt-mTOR pathway (**Fig. 7**).

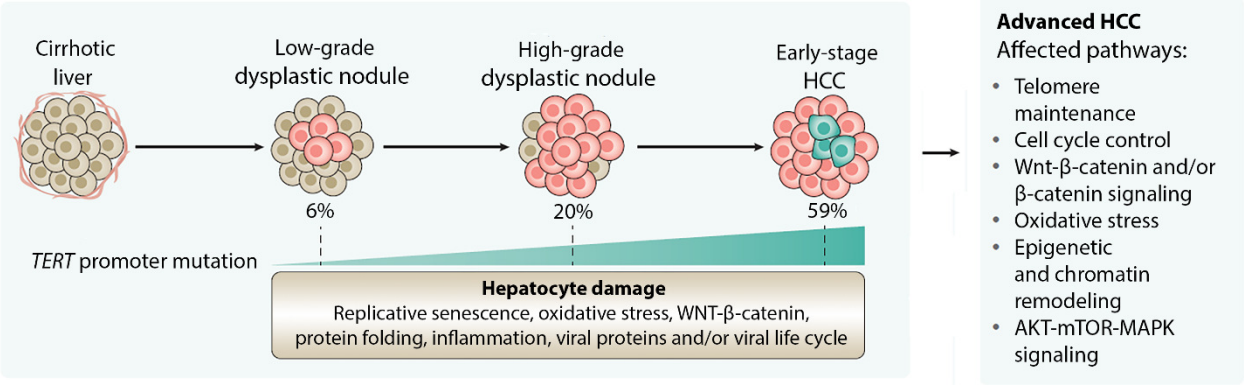


Figure 7: Molecular alterations associated with hepatocellular carcinoma progression. (Image modified from Llovet, Zucman-Rossi et al. 2016).

1.3.5.- HCV-associated metabolic alterations

Besides the previously mentioned HCV-associated complications, chronically infected patients present a wide range of comorbidities including extrahepatic cancers (See Pol, Vallet-Pichard et al. 2018 for review) and the development of metabolic alterations. Two noteworthy metabolic complications are hepatic steatosis and type II diabetes, since HCV has been described to play a direct role in their pathogenesis.

Estimates suggest that around 55% of HCV-infected patients develop hepatic steatosis (Lonardo, Loria et al. 2006), which is defined as an excessive accumulation of triglycerides (TGs) within the hepatocyte cytoplasm. Although this has been observed for several HCV genotypes, steatosis is most frequent and severe in patients infected with genotype 3 (Leandro, Mangia et al. 2006). One of the main mechanisms implicated in HCV-induced steatosis is the downregulation of peroxisome proliferator activated receptor alpha (PPARα) (Lupberger, Croonenborghs et al. 2019). It has been suggested that this downregulation decreases PPARα dimerization with retinoid X receptor (RXR) and account for the impaired expression of enzymes involved in fatty-acid β-oxidation such as carnitine palmitoyl transferase I (CPT1A) (Dharancy, Malapel et al. 2005).

Given the tight association of HCV particles with VLDLs, it does not surprise that alteration of this signaling pathway is also associated with steatosis development. HCV-mediated downregulation of microsomal triglyceride transfer protein (MTTP), a key enzyme implicated in the assembly of VLDLs, is inversely correlated with the levels of circulating cholesterol and the severity of steatosis in genotype 3 infected-patients (Mirandola, Realdon et al. 2006). Finally, it has been reported that HCV activates *in vitro* sterol regulatory element binding proteins (SREBP) which leads to an increased synthesis of fatty acids and cholesterol (Waris, Felmlee et al. 2007) (**Fig. 8a**).

HCV is a major risk factor for the development of type II diabetes and its mechanism involves hepatic and extrahepatic insulin resistance (IR) (Mehta, Brancati et al. 2000). In a physiological context, insulin stimulation induces the phosphorylation of insulin receptor substrate 1 (IRS-1) allowing the recruitment of the lipid kinase PI3K at the plasma membrane. PI3K generates the necessary phosphatidylinositol triphosphate (PIP3) to recruit the phosphoinositide-dependent kinase 1 (PDK1) which directly phosphorylates Akt serine/threonine kinase (Akt) inducing its activation. Finally, activated Akt mediates the effects of insulin which include decreased glucose production and increased glycogen synthesis (Haeusler, McGraw et al. 2018).

The balance between the components of the insulin signaling pathway is altered by HCV via several mechanism that converge on an impaired Akt activity. HCV core expression is associated with the upregulation of suppressor of cytokine signaling 3 (SOCS3) which favors the ubiquitination and proteasomal degradation of IRS-1 (Kawaguchi, Yoshida et al. 2004). Similarly, HCV core impairs IRS-1 expression via the upregulation of mTORC1 (Bose, Shrivastava et al. 2012). Finally, the HCV-mediated upregulation of protein phosphatase 2A (PP2A) induces the direct dephosphorylation and inactivation of Akt (Bernsmeier, Duong et al. 2008) (**Fig. 8b**).

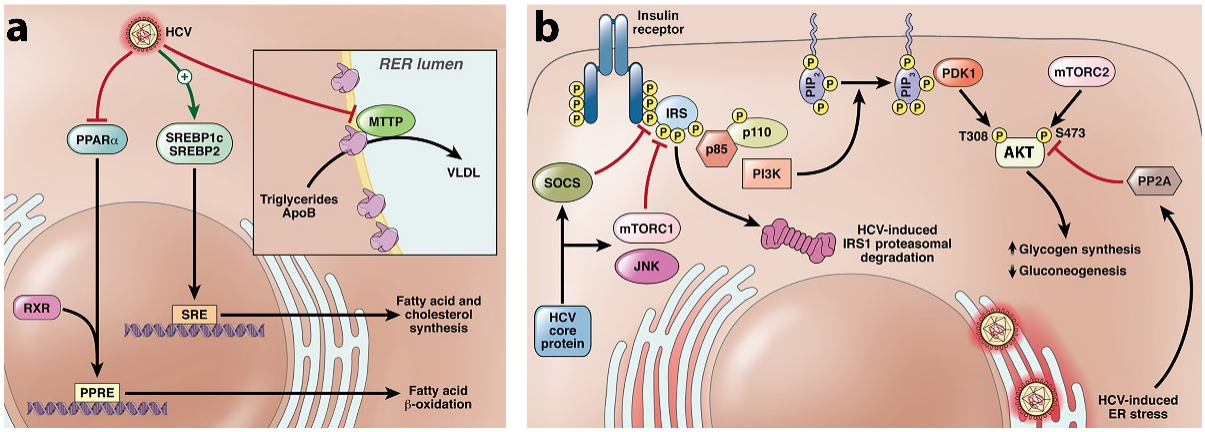


Figure 8: Mechanisms associated with metabolic alterations during HCV infection. a) Signaling interactions between HCV and lipid metabolism pathways leading to hepatic steatosis. **b**) Means by which HCV affects insulin signaling and contributes to hepatic IR (Image modified from Negro 2012).

1.4.- Management of HCV and HCV-associated complications

The current goal of therapy for HCV-infected patients is the elimination of the virus in order to decrease the risk of hepatic and extrahepatic complications, improve the quality of life and prevent onward transmission of the virus (EASL 2018). The endpoint of therapy is the achievement of a sustained virological response (SVR), which is defined as undetectable HCV RNA in serum or plasma 12 weeks (SVR12) or 24 weeks (SVR24) after the end of therapy. In most patients, SVR corresponds to a definitive cure of HCV infection (Swain, Lai et al. 2010).

1.4.1.- HCV antiviral therapy

Even before the formal identification of HCV as the etiological agent of non-A non-B hepatitis, it was clear that this was a transmissible disease of likely viral origin. Therefore, at that time IFN-α was a natural choice as therapeutic agent. Indeed, IFN-α monotherapy induced the normalization of ALT levels in these patients although this improvement usually ceased following treatment discontinuation (Hoofnagle, Mullen et al. 1986). The subsequent introduction of a combination therapy including IFN-α with ribavirin, a nucleoside analogue, substantially improved the SVR rates (28-31%) of HCV genotype 1-infected patients as compared to IFN- α monotherapy (7-11%). Even higher SVR rates were achieved by attaching polyethylene glycol to IFN (Peg-IFN), which allowed to have a steady level of active drug with weekly injections (Pawlotsky, Feld et al. 2015).

Despite these improvements, IFN-based regimes came with the cost of a wide variety of adverse effects such as flu-like symptoms (20-30%), fatigue due to anemia (60-90%) and depression (20-60%) (Sulkowski, Cooper et al. 2011). This picture slowly changed thanks to the advances in the molecular characterization of the HCV cycle, leading to the approval of first-generation DAAs in 2011 (Ghany, Nelson et al. 2011). This event marked the advent of IFN-free regimens and the further development of drug combinations that nowadays achieve SVR rates of more than 90% (EASL 2018). The current DAAs recommended for the treatment of HCV fall into three main categories depending on the viral component they target: NS3/4A protease inhibitors which end in 'previr' (e.g. paritaprevir, grazoprevir, glecaprevir, voxaliprevir), NS5A replication complex inhibitors which end in 'asvir' (e.g. veltapasvir, priventasvir, ledipasvir, ombitasvir) and NS5B polymerase inhibitors which end in 'buvir' (e.g. sofosbuvir, dasabuvir) (**Table 1**).

Although there are pan-genotypic drug combinations available, HCV genotyping is still useful to adapt the treatment regimen and its duration. This should be performed using the sequence of the 5'UTR plus the sequence of other regions such as the ones coding for core or NS5B (EASL 2018).

Achievement of SVR is associated with a reversal of metabolic alterations and hepatic fibrosis in patients without cirrhosis. DAA treatment is also beneficial in patients with advanced fibrosis or cirrhosis, however the risk of HCC development and liver-related mortality is reduced but not completely eliminated (van der Meer and Berenguer 2016).

Regimen	Genotype						
	1a	1b	2	3	4	5	6
Sofosbuvir/velpatasvir	X	X	X	X	X	X	X
Sofosbuvir/velpatasvir/voxilaprevir				X			
Glecaprevir/priventasvir	X	X	X	X	X	X	X
Sofosbuvir/ledipasvir	X	X			X	X	X
Paritaprevir/ombitasvir/ritonavir*		X					
Dasabuvir		X					
Grazoprevir/elbasvir	X	X			X		

Table 1: HCV DAA combinations approved in Europe in 2018 and recommended by the EASL. DAA regimens for treatment-naïve or treatment-experienced patients without cirrhosis or with compensated cirrhosis for each HCV genotype (EASL 2018). * The CYP3A inhibitor ritonavir is used as a pharmacokinetic booster of paritaprevir by preventing its hepatic metabolism.

1.4.2.- Management of hepatocellular carcinoma

The management of HCC is a complex process that requires a multidisciplinary approach in order to estimate the benefits and potential harms of therapy for each individual patient. The initial assessment consists on the evaluation of tumor burden and hepatic function in order to classify patients into five different categories that range from very early to terminal HCC stage as suggested by the Barcelona Clinic Liver Cancer (BCLC) algorithm (Llovet, Bru et al. 1999) (**Fig. 9**).

Based on the disease extent, this algorithm provides treatment recommendations for each stage. Early stage HCC patients (stage 0-A) with a preserved liver function are ideal candidates for surgical resection in the cases presenting a solitary nodule. If resection is not an option, liver transplantation and tumor ablation can be performed. Liver transplantation has the benefit of curing the underlying disease but is contraindicated in the presence of vascular invasion due to the risk of tumor recurrence. Tumor ablation consists on the induction of a high intratumoral temperature in order to achieve tumor necrosis. Ablation has fewer complications than tumor resection but is not suitable for the treatment of large tumors (> 3 cm). Patients with intermediate stage tumors (stage B) are candidates for transarterial chemoembolization (TACE). This approach consists on the infusion of a cytotoxic agent followed by the occlusion (embolization) of the vessels feeding the tumor.

Once the disease has progressed to an advanced stage (stage C), the recommended approach consists on the use of systemic therapies. The multikinase-inhibitor sorafenib was the first systemic drug to receive FDA approval for the treatment of inoperable liver cancer patients (Lang 2008) after having shown to

increase median survival by three months as compared to placebo (Llovet, Ricci et al. 2008). Its mechanism of action consists on the inhibition of VEGFR, PDGFR and BRAF (Llovet, Zucman-Rossi et al. 2016). Lenvatinib, a similar multikinase-inhibitor, was approved in 2018 based on an observed median survival of 13.6 months as compared to 12.3 months with sorafenib (Kudo, Finn et al. 2018). In patients with tumor progression during sorafenib treatment, the second-line therapeutic approach consists on the use of regorafenib which has been shown to increase survival by three months as compared to placebo (Bruix, Qin et al. 2017).

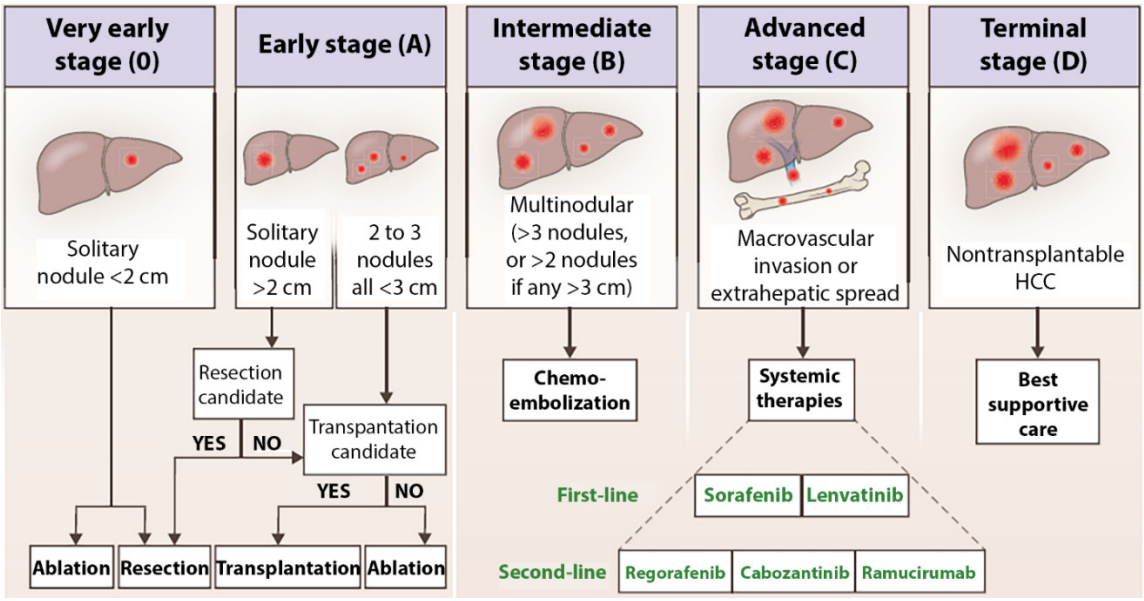
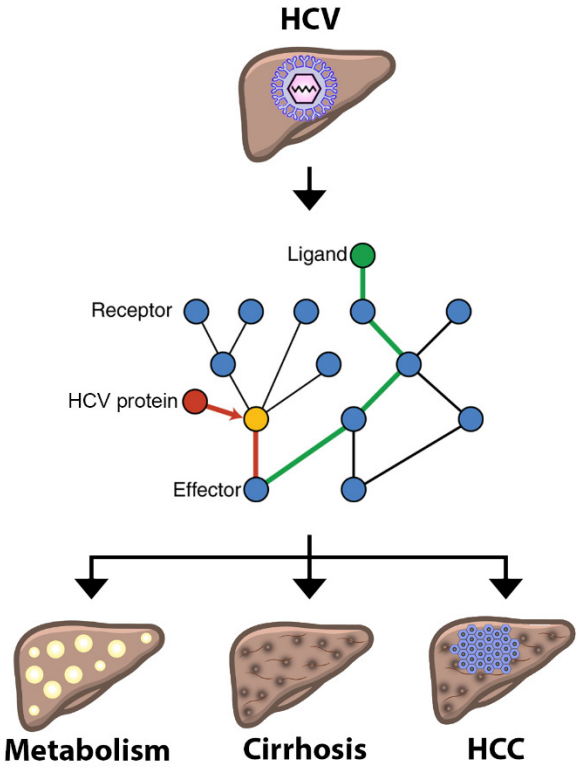


Figure 9: Management of hepatocellular carcinoma. Classification of HCC patients into five different stages and the clinical guidelines to follow accordingly (Image modified from Villanueva 2019).

1.5.- Signal transduction alterations implicated in HCV-associated disease

When studying HCV-host interactions, it soon became clear that HCV not only requires signaling molecules to maintain and promote its viral lifecycle, but it also actively induces signaling processes (Virzi, Roca Suarez et al. 2019). This has important consequences for host pathogenesis. The understanding of virus-induced signaling pathway alterations are a prerequisite to pave the way for potential preventive and therapeutic strategies targeting liver disease. This was explored to a certain extent in the previous sections and a more detailed overview of the current knowledge of HCV/host interactions is provided in the article below, followed by a discussion on the importance of protein phosphorylation in signal transduction with a focus on the signal transducer and activator of transcription 3 (STAT3) signaling pathway.

1.5.1.- Introductory article I – HCV in liver pathogenesis



Virzi, A.; **Roca Suarez, A. A.**; Baumert, T. F.; Lupberger, J. Rewiring Host Signaling: Hepatitis C Virus in Liver Pathogenesis. Hepatitis C viruses: The Story of a Scientific and Therapeutic Revolution. *Cold Spring Harbor Perspectives* **2019**, 10.1101/cshperspect.a037366.

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Rewiring Host Signaling: Hepatitis C Virus in Liver Pathogenesis

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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

Editors: Arash Grakoui, Jean-Michel Pawlotsky, and Glenn Randall
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Advanced Online Article. Cite this article as *Cold Spring Harb Perspect Med* doi: 10.1101/cshperspect.a037366

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).

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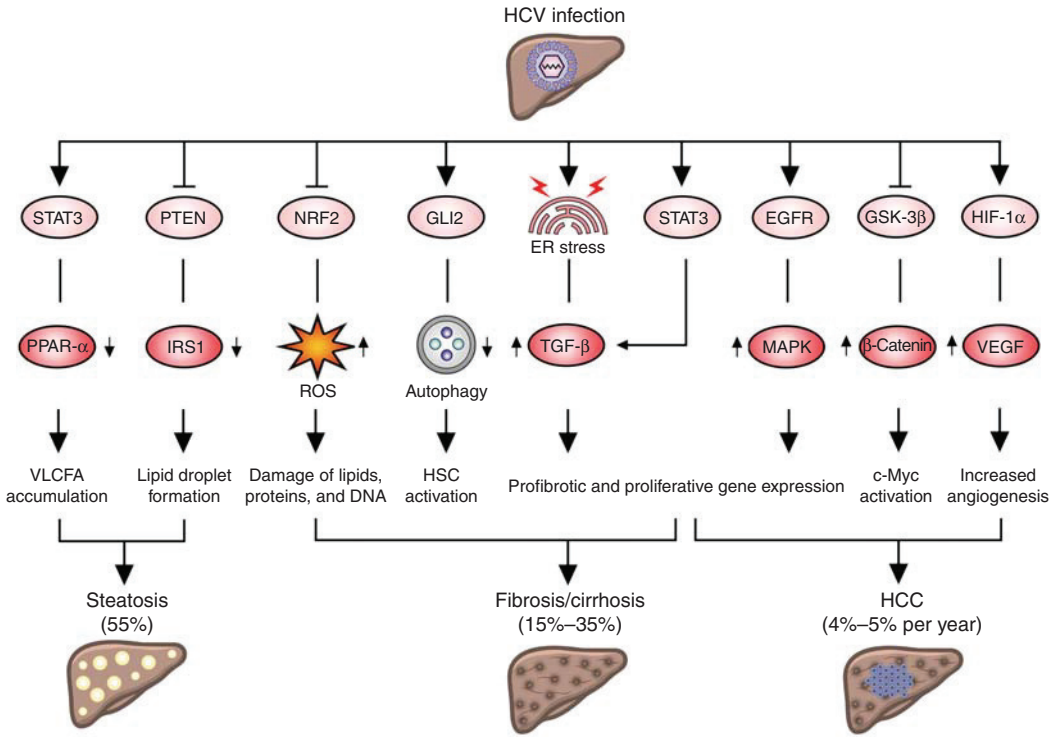

Figure 1. Hepatitis C virus (HCV) infection alters signaling pathways relevant for liver disease. HCV-mediated activation of signal transducer and activator of transcription 3 (STAT3) causes very long-chain fatty acid (VLCFA) accumulation in the infected hepatocytes via down-regulation of peroxisome 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.

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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 procancerogenic 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

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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

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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 (NCT012 46986). 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

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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.

ACKNOWLEDGMENTS

This work was supported by the European Union (ERC-AdG-2014 HEPCIR to T.F.B., EU H2020 HEPCAR 667273 to T.F.B. and J.L.), the French Cancer Agency (ARC IHU201301187 to T.F.B.), the Agence Nationale de Recherche sur le Sida et les Hépatites Virales (ECTZ4236 to J.L. and ECTZ4446 to A.A.R.S.), Fondation pour la Recherche Médicale en France (FDT20180500 5763 to A.A.R.S.), the United States Department of Defense (W81XWH-16-1-0363 to T.F.B.), the National Institutes of Health (NIAID R03AI1 31066, NCI 1R21CA209940, NIAID 5U19AI12 3862-02 to T.F.B), the Fondation de l'Université de Strasbourg (HEPKIN) (TBA-DON-0002) and the Inserm Plan Cancer 2019-2023 to T.F.B. This work has benefitted from support by the Initiative of Excellence IDEX-Unistra (2018-383i to A.V.) and has been published under the framework of the LABEX ANR-10-LAB-28 (HEPSYS). Inserm Plan Cancer, IDEX, and LABEX are initiatives from the French program "Investments for the Future."

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1.5.2.- Phosphorylation-mediated signal transduction – the role of tyrosine phosphatases

As we have seen, cell signaling comprises an incredibly complex and tightly regulated system involved in the sensing, transmission and interpretation of biological information. These responses to external cues are mediated by a series of post-translational modifications (PTMs) such as phosphorylation, ubiquitination and acetylation. One of the best studied PTMs is protein phosphorylation as it is involved in almost every signaling pathway by the regulation of protein activity, interaction, turnover and localization (Humphrey, James et al. 2015). Protein phosphorylation is a reversible process that involves the addition of phosphate groups to amino acids originating from adenosine triphosphate (ATP). Protein phosphorylation is catalyzed by protein kinases and dephosphorylation by protein phosphatases (**Fig. 10a**). In eukaryotic cells this occurs mainly at amino acids serine (86%), threonine (12%) and tyrosine (2%) (Olsen, Blagoev et al. 2006).

Although rare compared to serine and threonine phosphorylation, tyrosine phosphorylation represents a fundamental mechanism of signal transduction (Hunter 2014). EGFR for example was one of the first receptor tyrosine kinases discovered (Cohen, Ushiro et al. 1982). This is relevant in the context of HCV and liver disease since EGFR is a key entry factor regulating viral entry (Roca Suarez, Baumert et al. 2018) and a main driver of liver fibrosis and HCC as observed in animal models (Fuchs, Hoshida et al. 2014).

Counteracting the role of protein tyrosine kinases (PTK), there is a large family of protein tyrosine phosphatases (PTP) which comprises 107 members. PTPs are classified into four categories based on the amino acid sequence of their catalytic domain (H/V)C(X)$_5$R(S/T) (**Fig. 10b**). The cysteine and arginine residues in this motif are invariant and essential for the activity of class I, II and III PTPs. By contrast, class IV PTPs are different in the sense that an aspartic acid is a key element for the reaction. Class I PTPs are divided into classic PTPs which are tyrosine specific and dual-specificity phosphatases (DSPs) which in addition present serine/threonine activity. Classic PTPs are further divided into receptor PTPs (PTPRs) and non-receptor PTPs (PTPNs). This subdivision is wider for DSPs since they are classified into seven groups: PTEN phosphatases, slingshot phosphatases (SSHs), myotubularin-related phosphatases (MTMRs), cell division cycle 14 phosphatases (CDC14s), phosphatases of regenerating liver (PRLs), MAPK phosphatases (MKPs) and atypical DSPs (Julien, Dube et al. 2011).

Similar to PTKs, several PTPs have been implicated in the HCV cycle or liver disease development including PTPN2 (Brenndorfer, Brass et al. 2014), PTEN (Peyrou, Clement et al. 2013) and PTP1B (Carbone, Zheng et al. 2012).

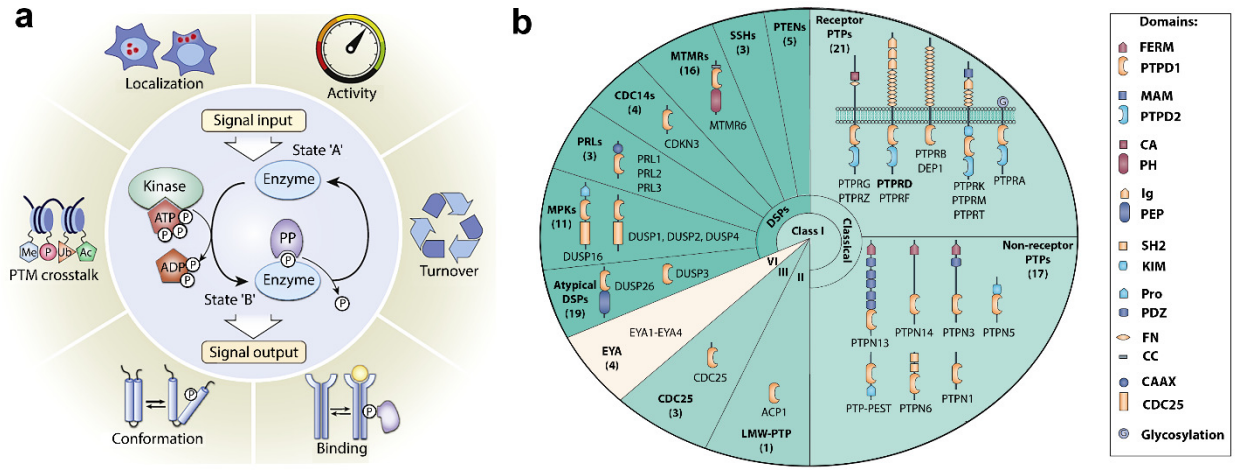


Figure 10: Protein phosphorylation as a key signal transduction component. a) Cellular functions relaying on reversible protein phosphorylation by kinases and phosphatases (Humphrey, James et al. 2015). **b**) The human protein tyrosine phosphatase superfamily and the domains composing each member (Image modified from Julien, Dube et al. 2011).

1.5.3.- Protein tyrosine phosphatase receptor type delta

As described in the following sections, my doctoral work has been focused on the study of a particular phosphatase which is protein tyrosine phosphatase receptor type delta (PTPRD). The *PTPRD* gene is found in the short arm of the human chromosome 9 spanning 2.3 Mb and containing 46 exons, making it one of the largest genes in the human genome (Humphray, Oliver et al. 2004). The canonical *PTPRD* encodes a type I transmembrane protein of 1912 amino acids, containing extracellular immunoglobulin (Ig) and fibronectin (FN) III domains, a transmembrane part and two intracellular phosphatase domains. The extracellular region of PTPRD has been shown to mediate homophilic interactions and bind to substrates such as SLITRK proteins, IL1-RAcP and IL1RAPL1 (Takahashi and Craig 2013). The first of the intracellular domains is active while the second does not present a functional phosphatase activity.

Numerous genetic alterations have been reported for *PTPRD* in association with human malignancies such as glioblastoma, lung, head and neck and colorectal cancer (Julien, Dube et al. 2011) (**Fig. 11a**). Although the significance of these alterations has not been studied for each of these pathologies, the analysis of glioblastoma patients suggests that PTPRD has a role as tumor suppressor (Veeriah, Brennan et al. 2009). This is mediated by the negative regulatory action of PTPRD over the activation of the oncogene STAT3,

preventing its nuclear translocation and the consequent STAT3-induced expression of genes related to cellular proliferation (**Fig. 11b**). As discussed in the following article, this observation is of particular interest since the activity of STAT3 is induced by a wide variety of viruses including HCV (Lupberger, Duong et al. 2013). Moreover, STAT3 activation is associated to a poor prognosis in HCC patients (Calvisi, Ladu et al. 2006).

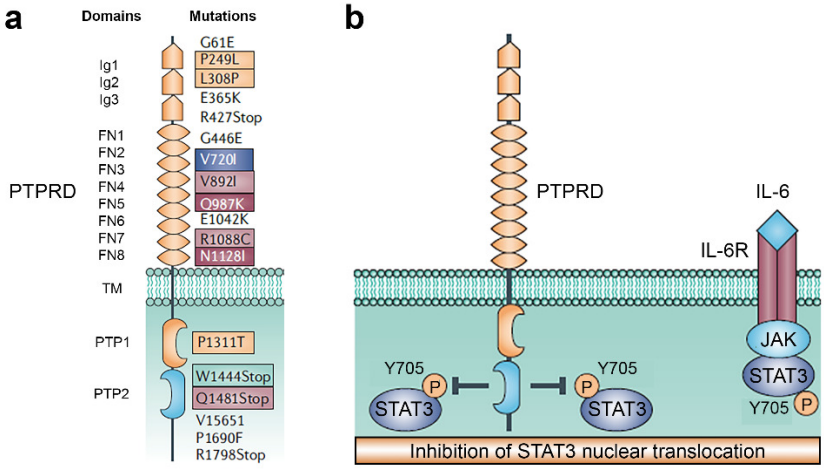
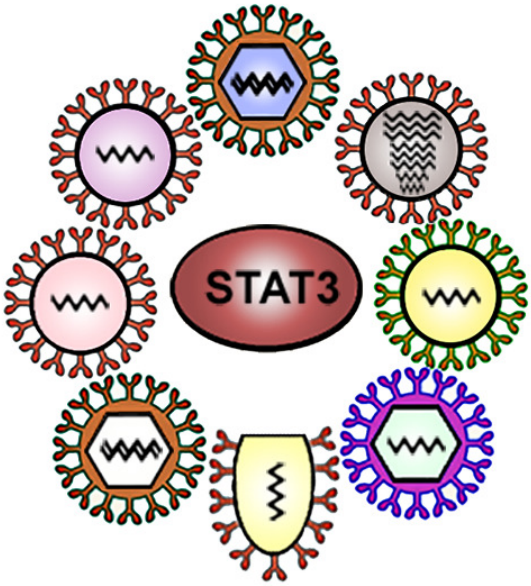


Figure 11: Protein tyrosine phosphatase receptor type delta (PTPRD). a) Schematic representation of the protein domains present in PTPRD and its sequence alterations associated with human cancers. Melanoma = black text, glioblastoma = pink, lung cancer = green, head and neck cancer = blue, colorectal cancer = red. **b**) PTPRD induces the tyrosine dephosphorylation (Y705) of STAT3 following interleukin 6 (IL-6) stimulation, thus preventing its nuclear translocation (Image modified from Julien, Dube et al. 2011).

1.5.4.- Introductory article II – Viral manipulation of STAT3



Roca Suarez, A. A.; Van Renne, N.; Baumert, T. F.; Lupberger, J., Viral manipulation of STAT3: Evade, exploit, and injure. *PLoS Pathog* **2018**, *14* (3), e1006839.

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REVIEW

Viral manipulation of STAT3: Evade, exploit, and injure

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OPEN ACCESS

Citation: Roca Suarez AA, Van Renne N, Baumert TF, Lupberger J (2018) Viral manipulation of STAT3: Evade, exploit, and injure. PLoS Pathog 14 (3): e1006839. https://doi.org/10.1371/journal.ppat.1006839

Editor: Tom C. Hobman, University of Alberta, CANADA

Published: March 15, 2018

Funding: This work was supported by grants from the Wilhelm Sander-Stiftung (Förderantrag 2010.023.1 to TFB - www.wilhelm-sander-stiftung.de), the European Union (Interreg IV-Rhin Supérieur-FEDER-Hepato-Regio-Net to TFB - www.interreg-rhin-sup.eu; ERC-AdG-2014 HEPCIR to TFB - www.cordis.europa.eu/project/rcn/198487_en.html; EU H2020 HEPCAR to TFB - www.hep-car.eu), the French Cancer Agency (ARC IHU201301187 to TFB - www.fondation-arc.org), the University of Strasbourg (IdEx, Projet Attractivité 2014, ANR, to JL - www.en.unistra.fr), the French ANRS (ECTZ4236, ECTZ4446 to JL and AARS - www.anrs.fr) and Cancéropôle Est (AAP Emergence du CGE / 2017 to JL, - www.canceropole-ge.org). The funders had no role in

Abstract

Signal transducer and activator of transcription 3 (STAT3) is a key regulator of numerous physiological functions, including the immune response. As pathogens elicit an acute phase response with concerted activation of STAT3, they are confronted with two evolutionary options: either curtail it or employ it. This has important consequences for the host, since abnormal STAT3 function is associated with cancer development and other diseases. This review provides a comprehensive outline of how human viruses cope with STAT3-mediated inflammation and how this affects the host. Finally, we discuss STAT3 as a potential target for antiviral therapy.

Signal transduction through the STAT3 pathway

STAT3 is a transcription factor activated by tyrosine phosphorylation

Signal transducer and activator of transcription 3 (STAT3) was first described in 1994 as a central transcription factor in acute inflammation [1]. Since then, STAT3 has been shown to regulate a wide spectrum of biological programs, including inflammation, tissue regeneration, cell proliferation, cell survival, cellular differentiation, angiogenesis, chemotaxis, and cell adhesion. This functional pleiotropy can be partially explained by the broad number of ligands that lead to STAT3 activation after binding to their respective cytokine receptors [2]. Upon cytokine binding, there is typically recruitment and reciprocal trans-phosphorylation of tyrosine kinases of the Janus kinase (JAK) family comprising JAK1, JAK2, JAK3, and tyrosine kinase 2 (TYK2) [3,4,5]. They, in turn, recruit and phosphorylate STAT3 (p-STAT3) at the highly conserved tyrosine residue 705 (pY705) [6], resulting in the formation of STAT3 homo- or heterodimers with signal transducer and activator of transcription 1 (STAT1) or signal transducer and activator of transcription 5 (STAT5) [7]. Subsequently, the activated signal transducer and activator of transcription (STAT) dimers translocate to the nucleus and facilitate gene transcription after binding to genomic DNA. Many pathways thus converge in STAT3-mediated gene-expression (Fig 1).

study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing interests: The authors have declared that no competing interests exist.

Regulation of STAT3 activation

STAT3 activity is additionally regulated by several post-translational modifications. First, phosphorylation at serine 727 (pS727) by a variety of serine/threonine kinases, such as the mitogen-activated protein (MAP) kinases, mechanistic target of rapamycin (mTOR), and protein kinase C delta type (PKCδ), increases transcriptional activity even further [8]. In mitochondria, pS727 promotes cellular respiration independently from pY705 [9]. Second, STAT3 can be reversible acetylated on K685 by histone acetyltransferase CBP/p300, prolonging transcriptional activity [10]. Contrarily, K140 methylation by histone methyltransferase SET9 impairs transcription [11].

Additional negative feedback regulators include the protein phosphatases receptor-type tyrosine-protein phosphatase C (PTPRC), receptor-type tyrosine-protein phosphatase D (PTPRD), receptor-type tyrosine-protein phosphatase T (PTPRT), and dual specificity protein phosphatase 2 (DUSP2) that hydrolyze p-STAT3 or upstream pathway members [12, 13, 14, 15]. Suppressor of cytokine signaling 3 (SOCS3) prevents STAT3 activation by shielding phospho-tyrosine residues of upstream kinases [16,17], while protein inhibitor of activated STAT protein 3 (PIAS3) prevents binding of STAT3 dimers to DNA [18]. In the nucleus, the phosphorylation and transcriptional activity of STAT3 pS727 is negatively regulated by tripartite motif-containing protein 28 (TRIM28), which binds directly to the central coiled-coil and DNA-binding domains of STAT3 [19]. Furthermore, several microRNAs (miRNAs) directly target *STAT3* mRNA, including Let-7a [20], miR-17-5p [21], miR-29b [22], miR-124 [23], and miR-519a [24]. Let-7a also exerts an indirect effect on STAT3 by promoting SOCS3 expression [25]. STAT3-activating miRNAs include miR-24 and miR-629 that impair miR-124 expression via *HNF4A* mRNA silencing [26]. Similarly, miR-135a-5p and miR-19a enhance pY705 phosphorylation by respectively targeting the mRNA of *PTPRD* and *SOCS3* [27,28].

Although STAT3 phosphorylation is often considered a prerequisite for its transcriptional activity, unphosphorylated STAT3 (u-STAT3) can promote the expression of genes related to cell cycle progression [29,30]. Finally, cytoplasmic STAT3 promotes cell migration by interacting with stathmin, a microtubule destabilizer [31].

Physiological role of STAT3 in inflammation

In mammalian organisms, tissue injuries inflicted by pathogens are met by the release of inflammatory mediators and local infiltration of white blood cells. This eliminates foreign material, removes damaged tissue components, and clears the way for repair. STAT3 plays an essential role in these processes by enabling the expression of a variety of genes in response to specific external signals sensed by cell-surface receptors [32]. Not all cell types and tissues have the same expression patterns of these receptors and their signaling cascade mediators. Therefore, the functional consequence of STAT3 activation is highly context-dependent, which can often lead to conflicting information. As illustrated in the following examples, this is particularly true for the role of STAT3 in inflammation, since it is either able to promote or suppress this process.

IL-6/STAT3 pathway promotes inflammation

Interleukin 6 (IL-6) is a classic proinflammatory cytokine that signals through STAT3 as part of the acute phase response (APR), a nonspecific reaction of the innate immune system to pathogen infection. During acute inflammation, IL-6 is produced in the lesion site to attract neutrophils and increase granulopoiesis [33]. Upon extravasation at the site of injury, neutrophils produce soluble interleukin 6 receptor alpha (sIL-6Rα), which in complex with IL-6 binds to glycoprotein 130 (gp130) at the membrane of resident tissue cells. This process is

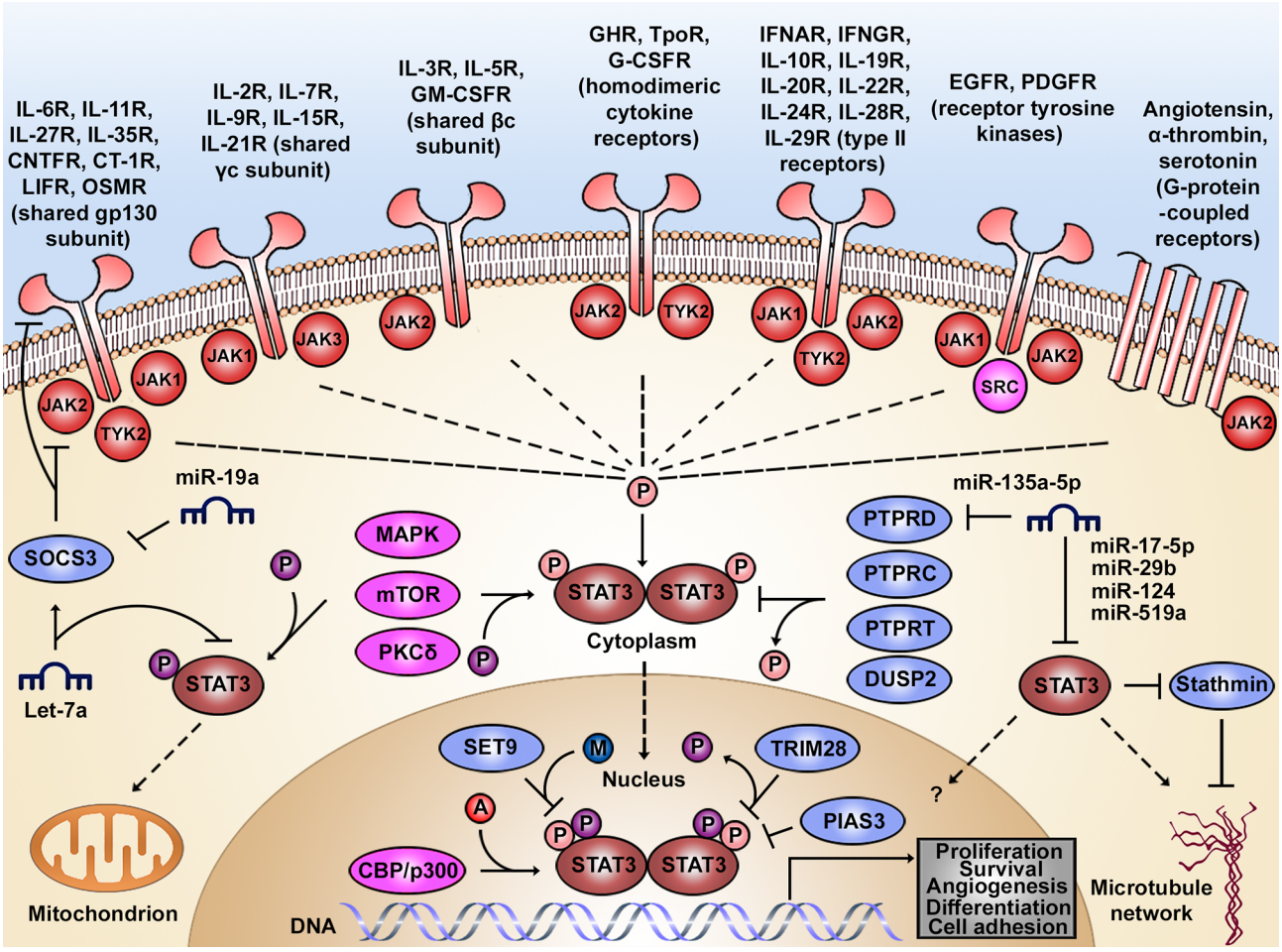

Fig 1. Regulatory circuits of the STAT3 signaling pathway. STAT3 can be activated by a wide range of ligands binding to cytokine, growth factor, or G-protein-coupled receptors. With the exception of receptor tyrosine kinases, these receptors lack intrinsic kinase activity and thus act by recruiting adaptor kinases (e.g., JAKs, SRC) to propagate downstream signals. As a result, STAT3 is phosphorylated at tyrosine 705 (pY705, pink), forms homodimers or heterodimers, and translocates to the nucleus, where it transcribes regulators of various cellular processes. Additionally, STAT3 can be phosphorylated at serine 727 (pS727, purple) by serine/threonine kinases (e.g., MAPK, mTOR, PKCδ), which enhance STAT3 transcriptional activity in the nucleus or direct STAT3 to mitochondria. Acetylation at lysine 685 (K685, red) by histone acetyltransferases (e.g., CREB binding protein CBP/histone acetyltransferase p300) or methylation at lysine 140 (K140, blue) by histone methyltransferases (e.g., SET9) favor or impair STAT3 transcriptional activity, respectively. Unphosphorylated STAT3 exhibits regulatory functions in the nucleus or can be retained in the cytoplasm, where it associates with microtubules and focal adhesions. The activity of STAT3 is tightly regulated by phosphatases (e.g., PTPRD), SOCS3, PIAS3, and miRNAs that fine-tune the temporal pattern of STAT3 activity and its other pathway components. All miRNAs are degrading the mRNAs of the indicated proteins. A, acetylation; CBP, CREB-binding protein; CT-1R, cardiotrophin 1 receptor; CNTFR, ciliary neurotrophic factor receptor; DUSP2, dual specificity protein phosphatase 2; EGFR, epidermal growth factor receptor; GHR, growth hormone receptor; G-CSFR, granulocyte colony-stimulating factor receptor; GM-CSFR, granulocyte-macrophage colony-stimulating factor receptor; gp130, glycoprotein 130; IFNAR, interferon alpha receptor; IFNGR, interferon gamma receptor; IL, interleukin; JAK, Janus kinase; K140, lysine 140; K685, lysine 685; LIFR, leukemia inhibitory factor receptor; MAPK, mitogen-activated protein kinase; M, methylation; miRNA, microRNA; mTOR, mechanistic target of rapamycin; OSMR, oncostatin-M-specific receptor; P, phosphorylation; p300, histone acetyltransferase p300; PDGFR, platelet-derived growth factor receptor; PIAS3, protein inhibitor of activated STAT protein 3; PKCδ, protein kinase C delta type; pS727, phospho-serine 727; PTPRC, receptor-type tyrosine-protein phosphatase C; PTPRD, receptor-type tyrosine-protein phosphatase D; PTPRT, receptor-type tyrosine-protein phosphatase T; pY705, phospho-tyrosine 705; SET9, histone-lysine N-methyltransferase SET9; SOCS3, suppressor of cytokine signaling 3; SRC, proto-oncogene tyrosine-protein kinase; STAT3, signal transducer and activator of transcription 3; TpoR, thrombopoietin receptor; TRIM28, tripartite motif-containing protein 28.

https://doi.org/10.1371/journal.ppat.1006839.g001

known as the *trans*-signaling pathway [34], which subsequently leads to a switch in chemokine expression attracting monocytic and T cells [35,36]. Upon the arrival of monocytic cells in the inflammation site, IL-6 signals govern their transformation into macrophages [37]. Pathogens

are thus initially confronted in their initial microenvironment with a potent IL-6 stimulus, which is mounted by the host to combat their very presence.

Apart from the lesion site, the IL-6/STAT3 proinflammatory signaling axis functions in many other cellular and tissue compartments. In secondary lymphoid tissues, where the adaptive immune response takes place, IL-6-mediated STAT3 activation promotes the proliferation and survival of T and B cell populations [38,39]. In addition, together with transforming growth factor beta (TGF-β), the IL-6/STAT3 axis is crucial for differentiating naive $CD4^+$ T cells into Th17 cells [40,41], limiting the generation of regulatory $CD4^+$ T cells (T_{reg} cells) [42]. Moreover, IL-6 promotes the differentiation of follicular helper T cells (T_{FH} cells) via STAT3 [43,44], effectively linking together T and B cell responses [45].

IL-10/STAT3 pathway suppresses inflammation

Interleukin 10 (IL-10) also activates STAT3, but unlike IL-6 the IL-10/STAT3 axis has powerful anti-inflammatory properties. Its function is essential to restrain unwanted immune responses and prevent autoimmune pathologies [46]. IL-10 only exerts an effect on immune cells, as they are the only cells to have the interleukin 10 receptor alpha (IL-10RA). This IL-10 receptor is highly expressed in monocytic cells and macrophages but also to a lesser extent in NK cells, $CD4^+$ and $CD8^+$ T cells, B cells, dendritic cells (DCs), and mast cells [47]. Until recently it was unclear how, in cells responsive to both IL-6 and IL-10, STAT3 orchestrates such opposing functions. In fact, SOCS3 is critical for selecting the transcriptional response. While IL-6 signaling is selectively inhibited by SOCS3 binding to gp130, SOCS3 does not interfere with IL-10R-mediated STAT3 activation [48]. As an effect, STAT3 activation is transient and proinflammatory in response to IL-6, while long lasting and anti-inflammatory in IL-10 [49].

IL-10 exerts its anti-inflammatory effect by suppressing T helper 1 (T_H1) cell responses [50] and regulating apoptosis in B cells [51]. In addition, IL-10/STAT3 is necessary for generation of tolerogenic DCs and of induced T_{reg}s out of naïve $CD4^+$ T cells [52].

Interferon activation of STAT3

Upon viral infection, type I and type II interferons (IFNs) initiate a canonical antiviral transcriptional program through STAT1 and STAT2, which results in an inflammatory, proapoptotic, and antiproliferative state [53]. At the same time, IFNs induce STAT3 activation [54,55], which provides a negative feedback by favoring cell proliferation and survival and thus resulting in gene expression with anti-inflammatory properties [56]. In support of this model, silencing of STAT1 or STAT3 expression by RNA interference confirmed the role of STATs as important determinants of IFN-α receptor (IFNAR) function [57] and emphasizes the role of STAT3 to restrain STAT1-mediated proinflammatory signaling [58].

In this context, an initial proinflammatory response to IFNs is mediated by STAT1, which expression is far more abundant, while STAT3-mediated gene induction is prevented by the SIN3 transcription regulator family member A complex (SIN3A). This multimolecular complex, containing histone deacetylases 1 (HDAC1) and 2 (HDAC2), inactivates STAT3 by deacetylation [59]. It has been suggested that only in a second phase is STAT3 activity increased, leading to a sequential counterbalance to the initial flare of apoptosis and decrease in proliferation mediated by IFNs [60].

A potential regulatory layer that remains poorly understood is the role of STAT1 and STAT3 heterodimers induced by IFNs. On one hand, STAT1 and STAT3 heterodimers have been described to bind regulatory elements present in promoters of interferon-stimulated genes (ISGs) such as γ-activated sequence (GAS), supporting a potential antiviral role of STAT1 and STAT3 heterodimers [61]. On the other hand, it has been proposed that STAT1 and STAT3

heterodimers can effectively quench STAT1 and thus provide negative feedback in a later phase of the IFN response [57]. Whatever the effect of STAT1 and STAT3 heterodimers on viral infection, either proviral or antiviral, it provides another layer of potential manipulation for viral gene products that warrants further research.

The suggested temporal dynamics of STAT biology may explain the serious consequences of persistent viral infections, as in the case of hepatitis C virus (HCV) [60]. Here, sustained type I and II IFN signaling may drastically alter the initial STAT dimerization balance, enabling a more pronounced proliferative role of STAT3 and hence increasing oncogenic pressure on hepatocytes.

Role of STAT3 in regeneration and disease

Upon infection, inflammatory cytokines trigger cell signaling in local stem cells or differentiated cells. Among other transcription factors, this eventually leads to the activation of STAT3 that mediates regenerative gene-expression programs. These genes include growth factors, cell-cycle stimulators, cell death inhibitors, and genes promoting dedifferentiation and cell motility and migration [62]. The task of STAT3 in regenerative inflammation is well studied in the liver, a model for organ regeneration as it can easily restore functional capacity after partial resection through compensatory hyperplasia [63,64]. In the liver, the inflammatory response following injury instigates the regenerative process [65]. As part of the APR, liver-residing macrophages (Kupffer cells) release proinflammatory cytokines such as IL-6 and tumor necrosis factor alpha (TNF-α) [66]. These inflammatory cytokines are important components of priming pathways that help sensitize hepatocytes to proliferative signals, such as hepatocyte growth factor (HGF) and epidermal growth factor (EGF) [67]. However, when liver injury persists, as in the case of chronic viral hepatitis, liver inflammation paired with constant STAT3 activity fosters the development of hepatocellular carcinoma (HCC) [27]. A similar oncogenic role of STAT3 has been observed in a wide variety of other malignancies such as colorectal, lung, prostate, gastric, and breast cancers [68].

Given the extensive role of STAT3 in many physiological processes, it is only logical that its perturbation entails a wide variety of pathological consequences. This is exemplified by loss-of-function mutations in the STAT3 gene that lead to the autosomal dominant hyper-immunoglobulin E (IgE) syndrome (AD-HIES) [69]. These patients exhibit an immunodeficiency complex that presents with recurrent episodes of pneumonia and other lung abnormalities, abnormally high levels of IgE, eosinophilia, eczema, and skeletal and connective tissue abnormalities. Inadequate inflammatory capacity due to a broken IL-6/STAT3 axis curtails the APR and leads to "cold" skin abscesses (i.e., without inflammatory signs). As STAT3 is necessary for generating Th17 cells, a defective Th17 response and increased susceptibility for microbial infections are hallmarks of AD-HIES. On the other hand, the defects in the anti-inflammatory IL-10/STAT3 pathway lead to reduced peripheral tolerance, which is clinically translated in atopic dermatitis. Finally, AD-HIES patients exhibit a marked reduction in memory T cells and increased latency of herpesviruses such as varicella-zoster virus (VZV) and Epstein–Barr virus (EBV) [70].

Molecular mechanisms of viral STAT3 manipulation

Viral stimulation of STAT3 function

As STAT3 activation is a pivotal event in the APR elicited by pathogen invasion, many viruses have evolved to thrive in a STAT3-driven microenvironment and have developed strategies to stimulate STAT3 signaling (Fig 2A, Table 1). For example, hepatitis B virus (HBV) promotes the formation of p-STAT3 dimers that bind specifically to an androgen-responsive element

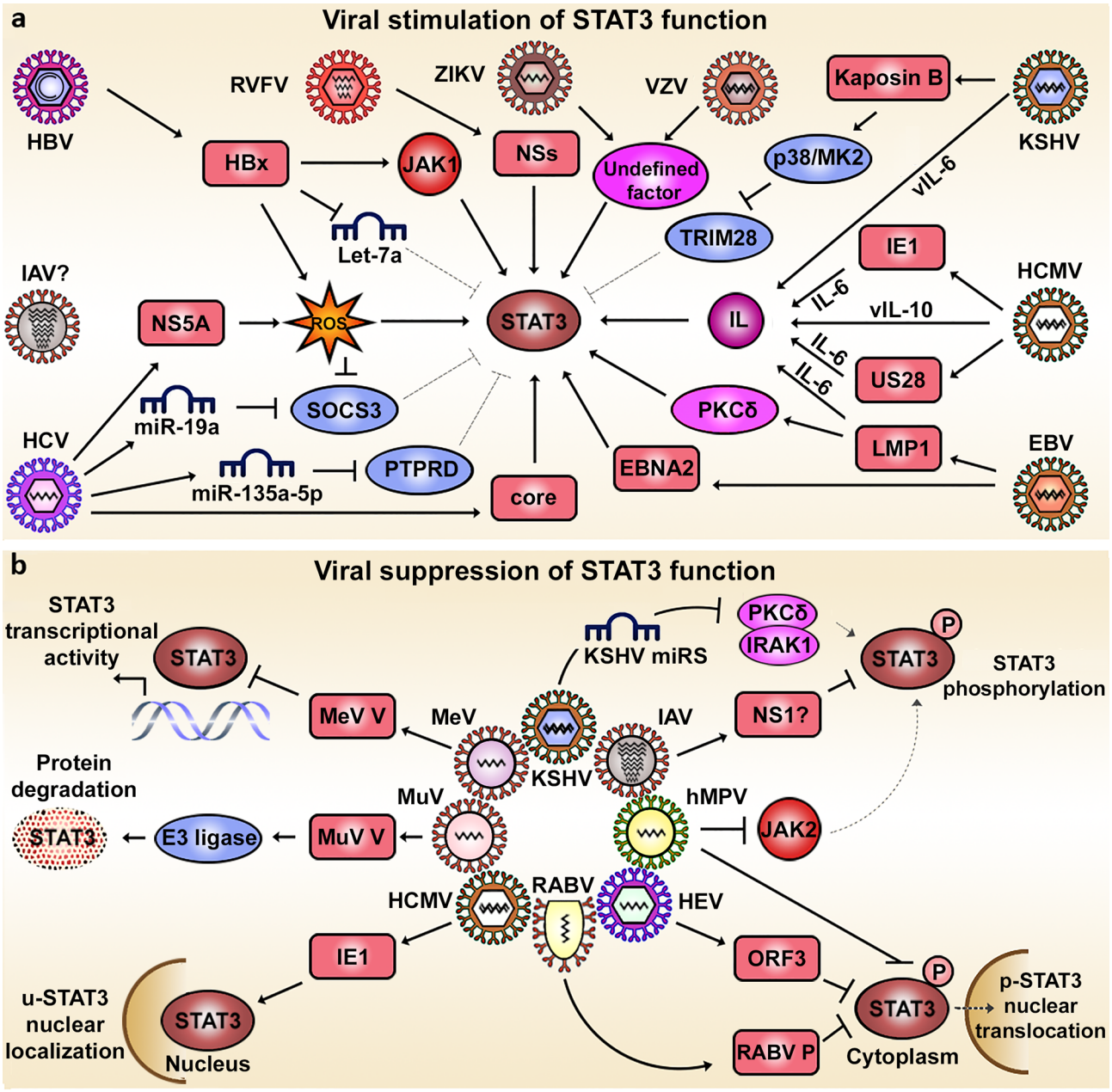

Fig 2. Viral manipulation of the STAT3 signaling pathway. (A) Viruses activating STAT3 function and the mechanisms involved. Viral proteins such as HBx, NS5A, core, NSs, EBNA2, LMP1, US28, and IE1 induce STAT3 activation either directly or by favoring the action of upstream positive regulators. Viruses like HCMV and KSHV code for homologues of human interleukins such as IL-10 and IL-6. Alternatively, virus-induced activation of STAT3 can be achieved by the inhibition of negative regulators such as SOCS3, PTPRD, TRIM28, and Let-7a. In the case of some viruses, STAT3 activation (VZV and ZIKV) or STAT3-mediated effects (IAV) have been described, but the mechanisms involved have not been fully elucidated. All miRNAs are degrading the mRNAs of the indicated proteins. **(B)** Viruses suppressing STAT3 function and the mechanisms involved. Virus-mediated inactivation of STAT3 can be attained by decreasing its phosphorylation (KSHV, IAV, and hMPV), inducing STAT3 protein degradation (MuV), hampering its transcriptional activity (MeV), or altering its subcellular localization (HCMV, RABV, HEV, and hMPV). EBNA2, Epstein–Barr virus nuclear antigen 2; EBV, Epstein–Barr virus; HBV, hepatitis B virus; HBx, hepatitis B virus X protein; HCMV, human cytomegalovirus; HCV, hepatitis C virus; HEV, hepatitis E virus; hMPV, human metapneumovirus; IAV, influenza A virus; IE1, intermediate-early protein 1; IL-6, interleukin 6; IL-10, interleukin 10; IRAK1, interleukin 1 receptor-associated kinase 1; JAK1, Janus kinase 1; KSHV, Kaposi's sarcoma-associated herpesvirus; LMP1, latent membrane protein 1; miRNA, microRNA; MeV, measles virus; MK2, mitogen-activated protein kinase 2; MuV, mumps virus; NS5A, non-structural protein 5A; NSs, non-structural proteins; P, phosphorylation; PKCδ, protein kinase C delta type; PTPRD, receptor-type tyrosine-protein phosphatase D; RABV, rabies virus; ROS, reactive oxygen species; RVFV, Rift Valley fever virus; SOCS3, suppressor of cytokine signaling 3; STAT3, signal transducer and activator of transcription 3; TRIM28, tripartite motif-containing protein 28; u-STAT3, unphosphorylated STAT3; vIL-10, viral IL-10; vIL-6, viral IL-6; VZV, varicella-zoster virus; ZIKV, Zika virus.

https://doi.org/10.1371/journal.ppat.1006839.g002

Table 1. Virus/STAT3 interactions: Summary of observations and employed methods.

Virus	Observation	Method	Virus strain	Experimental system	Reference
HBV	Increased STAT3 (pY705) phosphorylation	In vitro viral protein expression (HBx)	adr4-derived sequence (genotype C)	Mouse hepatoma cell line (Hepa 1–6)	[72]
	Increased STAT3 protein and mRNA expression	In vitro viral protein expression (HBx)	adw-derived sequence (genotype A)	Human hepatoma cell lines (HepG2, SNU-182)	[20]
	Increased STAT3 (?) phosphorylation	HBV-expressing cells and patient-derived samples	ayw (genotype D)	Human hepatoma cell line (HepG2.2.15), HBV-positive HCC samples	[73]
HCV	Increased STAT3 (pY705) phosphorylation	In vitro HCV genomic replicon and virus infection	JFH-1 (genotype 2a)	Human hepatoma cell lines (Huh-7, NNeoC-5B)	[74]
	Increased STAT3 (pY705) phosphorylation	In vitro and in vivo viral protein expression (core)	Patient-derived sequence (genotype 1b)	Human hepatoma cell line (HepG2), Tg mice (C57BL/6)	[75]
	Increased STAT3 (pY705) phosphorylation	In vitro viral protein expression (NS5A)	Patient-derived sequence (genotype 1b)	Human hepatoma cell line (Huh-7)	[76]
	Up-regulation STAT3 responsive genes	In vitro HCV infection and patient-derived samples	Jc1 (genotype 2a chimera)	Human hepatoma cell line (Huh7.5.1) and HCV-positive HCC samples	[27]
	Increased STAT3 (?) phosphorylation	In vitro exposition to HCV-derived exosomes	JFH-1 (genotype 2a)	Primary HSCs	[28]
RVFV	Increased STAT3 (pY705) phosphorylation	In vitro viral protein expression (NSs) and RVFV infection	Recombinant MP12	Vero cells, HSAECs, and MEFs	[77]
HCMV	Increased STAT3 (pY705) phosphorylation	In vitro viral protein expression (US28) and HCMV infection	Titan	HEK293 and astrocytoma cell line (U373 MG)	[78]
	Increased STAT3 (?) phosphorylation	In vitro HCMV infection	HCMV-AD169, HCMV-DB	Human hepatoma cell line (HepG2) and PHHs	[79]
	Increased STAT3 (?) phosphorylation	In vitro vIL-10 stimulation		DCs	[81]
	Increased STAT3 (pY705/pS727) phosphorylation	In vitro vIL-10 stimulation		Primary human monocytic cells	[82]
	Increased u-STAT3 nuclear localization	In vitro viral protein expression (IE2) and HCMV infection	HCMV-AD169	Human embryonic lung fibroblasts (MRC-5) and astrocytoma cell line (U373)	[101]
EBV	Increased STAT3 (pY705) phosphorylation	In vitro viral protein expression (LMP1) and EBV infection	Recombinant EBV (Bx1)	HeLa cells, NPC cell line (CNE2)	[83]
	Increased STAT3 (pY705/pS727) phosphorylation	In vitro viral protein expression (LMP1)		Cervical carcinoma cell line (C33A)	[84]
	Increased STAT3 DNA-binding and transcriptional activity	In vitro viral protein expression (EBNA2)		HeLa, HEK293, and human Burkitt's lymphoma B cell line (DG75)	[85]
KSHV	Increased STAT3 (pY705) phosphorylation	In vitro viral protein expression or stimulation (vIL-6)		Human hepatoma cell line (Hep3B)	[88]
	Increased STAT3 (pY705/pS727) phosphorylation	In vitro KSHV infection	BCBL-1-cell line-derived	HUVECs	[19]
	Increased STAT3 (pY705) phosphorylation	In vitro KSHV infection	BC3-cell line-derived	DCs	[89]
	Decreased STAT3 (pY705) phosphorylation	In vitro viral miRNAs expression	BCBL-1-cell line-derived	HUVECs	[102]
VZV	Increased STAT3 (pY705) phosphorylation	In vitro and in vivo VZV infection	Recombinant VZV (ORF10-GFP)	HELFs, primary tonsil T cells and human skin xenografts (mouse)	[90]
ZIKV	Increased STAT3 (pY705) phosphorylation	In vitro ZIKV infection	FSS13025	Primary Müller cells (mouse)	[92]
	Increased STAT3 pathway activity	In vivo ZIKV infection	Brazil-ZKV2015, PRVABC59	PBMCs (rhesus monkeys)	[93]

(*Continued*)

Table 1. (Continued)

Virus	Observation	Method	Virus strain	Experimental system	Reference
MuV	STAT3 protein degradation	In vitro viral protein expression (MuV V) and MuV infection	Enders strain	Human fibrosarcoma-derived cell line (2fTGH)	[94]
MeV	Reduced STAT3 transcriptional activity	In vitro viral protein expression (MeV V)	Edmonston strain-derived sequence	Human fibrosarcoma-derived cell line (2fTGH)	[95]
IAV	Decreased STAT3 (pY705) phosphorylation	In vitro IAV infection	H1N1/54, H5N1/483	Alveolar epithelial cells	[96]
	Increased STAT3-dependent transcription (ANGPTL4)	In vivo IAV infection	H1N1 A/PR/8/34	BALB/c mice	[103]
HEV	p-STAT3 impaired nuclear translocation	In vitro viral protein expression (ORF3)	Hyderabad strain-derived sequence (genotype 1)	Human hepatoma cell line (Huh7)	[98]
RABV	p-STAT3 impaired nuclear translocation	In vitro viral protein expression (RABV P)	CVS strain-derived sequence	Fibroblast-derived cell line (COS-7)	[99]
hMPV	Decreased STAT3 (pY705) phosphorylation and nuclear translocation	In vitro hMPV infection	CAN97-83	Lung adenocarcinoma cell line (A549)	[100]

Abbreviations: ANGPTL4, angiopoietin-like protein 4; CVS, challenge virus standard; DCs, dendritic cells; EBNA2, Epstein–Barr virus nuclear antigen 2; EBV, Epstein–Barr virus; HBV, hepatitis B virus; HBx, hepatitis B virus X protein; HCC, hepatocellular carcinoma; HCMV, human cytomegalovirus; HCV, hepatitis C virus; HELFs, human embryonic lung fibroblasts; HEV, hepatitis E virus; hMPV, human metapneumovirus; HSAECs, human small airway epithelial cells; HSCs, hepatic stellate cells; HUVECs, human umbilical vein endothelial cells; IAV, influenza A virus; IE1, intermediate-early protein 1; IE2, intermediate-early protein 2; JFH-1, Japanese fulminant hepatitis; KSHV, Kaposi's sarcoma-associated herpesvirus; LMP1, latent membrane protein 1; MEFs, mouse embryonic fibroblasts; MeV, measles virus; MeV V, measles virus viral protein V; miRNA, microRNA; MuV, mumps virus; MuV V, mumps virus viral protein V; NPC, nasopharyngeal carcinoma; NS5A, non-structural protein 5A; NSs, non-structural proteins; PBMCs, peripheral blood mononuclear cells; PHHs, primary human hepatocytes; RABV, rabies virus; RVFV, Rift Valley fever virus; STAT3, signal transducer and activator of transcription 3; Tg, transgenic; u-STAT3, unphosphorylated STAT3; vIL-10, viral IL-10; vIL-6, viral IL-6; VZV, varicella-zoster virus; ZIKV, Zika virus.

https://doi.org/10.1371/journal.ppat.1006839.t001

site present in the HBV enhancer 1 region and hence stimulates viral gene expression [71]. This is in part mediated by hepatitis B virus X protein (HBx), which induces pY705 phosphorylation via JAK1 [72] and down-regulates miRNA let-7a, a negative regulator of *STAT3* mRNA [20]. Additionally, HBV favors STAT3 activation by inducing reactive oxygen species (ROS), which results in epigenetic silencing of *SOCS3* mRNA via up-regulation of snail family transcriptional repressor 1 (SNAIL1) [73]. HCV requires STAT3 and therefore promotes STAT3 signaling to maintain infection [74]. HCV stimulates STAT3 directly by interaction with the HCV core protein [75] and indirectly through non-structural protein 5A (NS5A), which activates STAT3 via ROS induction [76]. Furthermore, miR-135a-5p is a negative regulator of STAT3 phosphatase PTPRD and is up-regulated in HCV-infected hepatocytes, leading to an enhanced STAT3 transcriptional activity [27]. Furthermore, HCV-infected hepatocytes secrete miR-19a within exosomes, down-regulating the expression of SOCS3 in hepatic stellate cells (HSCs) and promoting STAT3 phosphorylation [28]. Similarly, Rift Valley fever virus (RVFV) infection induces STAT3 (pY705) phosphorylation by the viral non-structural protein s (NSs) [77]. STAT3 activation is also a frequent feature of the Herpesviridae family. Human cytomegalovirus (HCMV) activates STAT3 through various mechanisms, depending on virus strain and cell type. In U373 MG astrocytes, viral protein US28 of the Titan strain induces IL-6 production, which in turn activates STAT3 in an auto- and paracrine fashion [78]. In hepatoma cells and primary human hepatocytes (PHHs), strains AD169 and HCMV-DB also activate STAT3 via IL-6 in an autocrine and/or paracrine manner, which is independent of US28 [79]. Additionally, HCMV codes for a homologue of the human IL-10, viral interleukin 10 (vIL-10), [80] that induces STAT3 (pY705/pS727) phosphorylation [81,82]. EBV infection in HeLa cells creates a positive feedback loop where the viral protein

latent membrane protein 1 (LMP1) induces IL-6 expression and STAT3 phosphorylation, which in turn reinforces LMP1 expression [83]. In addition, LMP1 promotes pS727 phosphorylation through the kinase PKCδ [84], while the viral protein Epstein–Barr virus nuclear antigen 2 (EBNA2) fosters STAT3 DNA binding, enhancing its transcription [85]. EBV also codes for a viral IL-10 homologue [86], but unlike its cellular counterpart it is not able to mount a strong STAT3 response [81] due to a point mutation at I87A [87]. Kaposi's sarcoma-associated herpesvirus (KSHV) encodes a viral homologue of IL-6 (vIL-6) that signals through the same receptors as cellular IL-6 (IL-6Rα/gp130) but can also activate STAT3 in an IL-6Rα-independent manner in Hep3B liver cells [88]. In human endothelial cells, KSHV increases both pY705 and pS727 phosphorylation [19]. Though pY705 phosphorylation is transient, pS727 persists because the viral protein kaposin B activates the p38/MK2 pathway to suppress TRIM28, which is a negative regulator of pS727 phosphorylation [19]. STAT3 activation in DCs is believed to stem from virions interacting with dendritic cell-specific ICAM-3-grabbing nonintegrin 1 (DC-SIGN) at the cell's surface, as antibody blockage of DC-SIGN reduces pY705 levels [89]. VZV induces pY705 phosphorylation in epidermal cells and T cells in vivo as well as in fibroblasts in vitro through unknown mechanisms [90]. Resveratrol, an inhibitor of kinases phosphorylating STAT3, hampers VZV infection, suggesting the involvement of host kinases [91]. Similarly, ZIKA virus (ZIKV) infection induces pY705 in primary retinal glial cells [92] and favors the activity of the IL-6/STAT3 pathway in blood mononuclear cells from infected rhesus monkeys, albeit without any known molecular mechanism [93].

Viral suppression of STAT3 function

In the acute phase, viral suppression of STAT3 reduces the host cell's ability to respond to inflammatory cytokines. On the other hand, inhibiting STAT3 also removes negative feedback on the antiviral response. To understand the beneficial effect of blocking STAT3 for viruses, it thus requires a temporal dissection of each individual virus/STAT3 interaction. Most viruses that suppress STAT3, however, do this to avoid the antiviral pressure exerted by STAT3 responsive genes in the acute phase of infection (Fig 2B, Table 1). Mumps virus (MuV) viral protein V (MuV V) induces STAT3 degradation by promoting STAT3-directed ubiquitin E3 ligase complexes [94]. Similarly, measles virus (MeV) viral protein V (MeV V) reduces STAT3-mediated transcription but through an unknown mechanism that is, however, independent of ubiquitin ligase subunits [95]. Influenza A virus (IAV) infection induces STAT3 activation in the early phase of the inflammatory response. As the infection progresses, STAT3 activity is suppressed to a degree that inversely correlates with the pathogenicity of each IAV strain. For instance, the highly pathogenic avian influenza strain H5N1 impairs pY705 phosphorylation, but in the case of the low pathogenic seasonal H1N1 strain this decrease is even more pronounced [96]. This inhibition could be partly mediated by viral protein NS1, which increases *SOCS3* expression [97]. Other viruses have developed alternative strategies to impair STAT3 function, such as manipulating its subcellular localization during infection. Hepatitis E virus (HEV) ORF3 protein blocks the nuclear translocation of p-STAT3 [98]. Likewise, in rabies virus (RABV) infections, viral protein P associates with p-STAT3 in the cytoplasm, impeding its nuclear translocation. In addition, P protein interferes with gp130 receptor signaling [99]. Human metapneumovirus (hMPV) infection prevents the nuclear translocation of STAT3 in a cytokine-specific manner, as this was only observed following stimulation with IL-6 and not in case of interleukin 22 (IL-22) [100]. Contrary to the occasions where HCMV induces STAT3 phosphorylation [78,79], HCMV can also rapidly disrupt IL-6/STAT3 signaling in U-373 cells by sequestering u-STAT3 to the nucleus via viral protein IE1 [101]. Apart from inducing STAT3 activation, KSHV can also target and inhibit STAT3 or its activators in vitro through a

panel of virally encoded miRNAs. KSHV miR-K6-5, miR-K8, and miR-K9* reduce STAT3 levels, while upon IL-6 treatment, miR-K6-5 and miR-K9 decrease PKCδ and interleukin 1 receptor-associated kinase 1 (IRAK1) expression, respectively, which is accompanied by reduced p-STAT3 levels [102]. Whether in the end KSHV-induced STAT3 activation or the negative regulation of STAT3 by viral miRNAs act predominantly in endothelial cells remains unclear. But it is conceivable that both opposing mechanisms are required in a time-dependent manner to regulate the transition from the latent to the lytic stage of the viral life cycle.

Consequences of viral perturbations in STAT3 activity

Recalibration of apoptosis dynamics

Apoptosis is perhaps the most primordial response of a host cell to infection, designed to thwart the virus spread. Generally, viruses need to prevent host cell apoptosis to maintain a compartment of infected cells [104]. However, there are also examples where viruses induce apoptosis to spark the release of virions and galvanize viral spread [105]. STAT3 is mainly considered a negative regulator of apoptosis by up-regulating the expression of several antiapoptotic factors [106] (Fig 3A). IAV H5N1 causes higher pY705 levels than seasonal H1N1. Therefore, apoptosis is delayed during H5N1 infection, allocating additional time to infected cells for progeny virus production. Ultimately, this leads to an accumulation of apoptotic cells at later stages [96]. Similarly, VZV prevents apoptosis by increasing STAT3 phosphorylation, which up-regulates baculoviral IAP repeat-containing protein 5 (*BIRC5*) expression, a VZV host factor belonging to the family of inhibitors of apoptosis (IAP) [90]. During EBV infection, virus-induced STAT3 activation up-regulates poly(rC)-binding protein 2 (*PCBP2*) expression, limiting susceptibility of latently infected cells to lytic signals and fostering persistence [107]. This goes as well for KSHV, in which STAT3 restrains the exit from latency into the lytic cycle by repressing the expression of the viral protein R transactivator (RTA) [108]. MuV is yet another example in which the cytopathic effects of infection are associated with the induction of apoptosis, partly via V protein-mediated STAT3 degradation [94]. Finally, RVFV reins in apoptosis by enhancing the nuclear translocation of phosphorylated STAT3 and impairs the expression of proapoptotic genes such as proto-oncogene c-Fos (*FOS*), proto-oncogene c-Jun (*JUN*), and nuclear receptor subfamily 4 group A member 2 (*NR4A2*) [77].

Perturbing the immune response

The benefit for a virus to dampen STAT3 signaling lies in controlling antiviral innate immunity responses such as the APR (Fig 3B). Many of the APR genes are modulators of inflammation. C-reactive protein (CRP) for example is a target gene of STAT3 and has several biological functions related to nonspecific host defense [109]. Increased plasma levels of metal-binding APRs (e.g., haptoglobin and hemopexin) help protect host cells from iron loss during infection and the associated injury. Moreover, they act as scavengers for potentially damaging free oxygen radicals. Protease inhibitors among APR genes (e.g., alpha-1-antitrypsin) neutralize lysosomal proteases. These inhibiting factors are released in response to tissue infiltration of activated neutrophils and macrophages, modulating the activity of proinflammatory enzyme cascades. HEV impairs the expression of these APR genes by inhibiting STAT3, attenuating inflammatory responses and creating a favorable environment for viral replication and survival [98].

In contrast to HEV, the KSHV-mediated activation of STAT3 is associated with increased expression of C-C motif chemokine ligand 5 (CCL5) [19], a potent chemoattractant for monocytic cells, eosinophils, NKs, and DCs [110]. Many of these cell types have been shown to be present in Kaposi's sarcoma lesions, suggesting that STAT3 contributes to the chronic

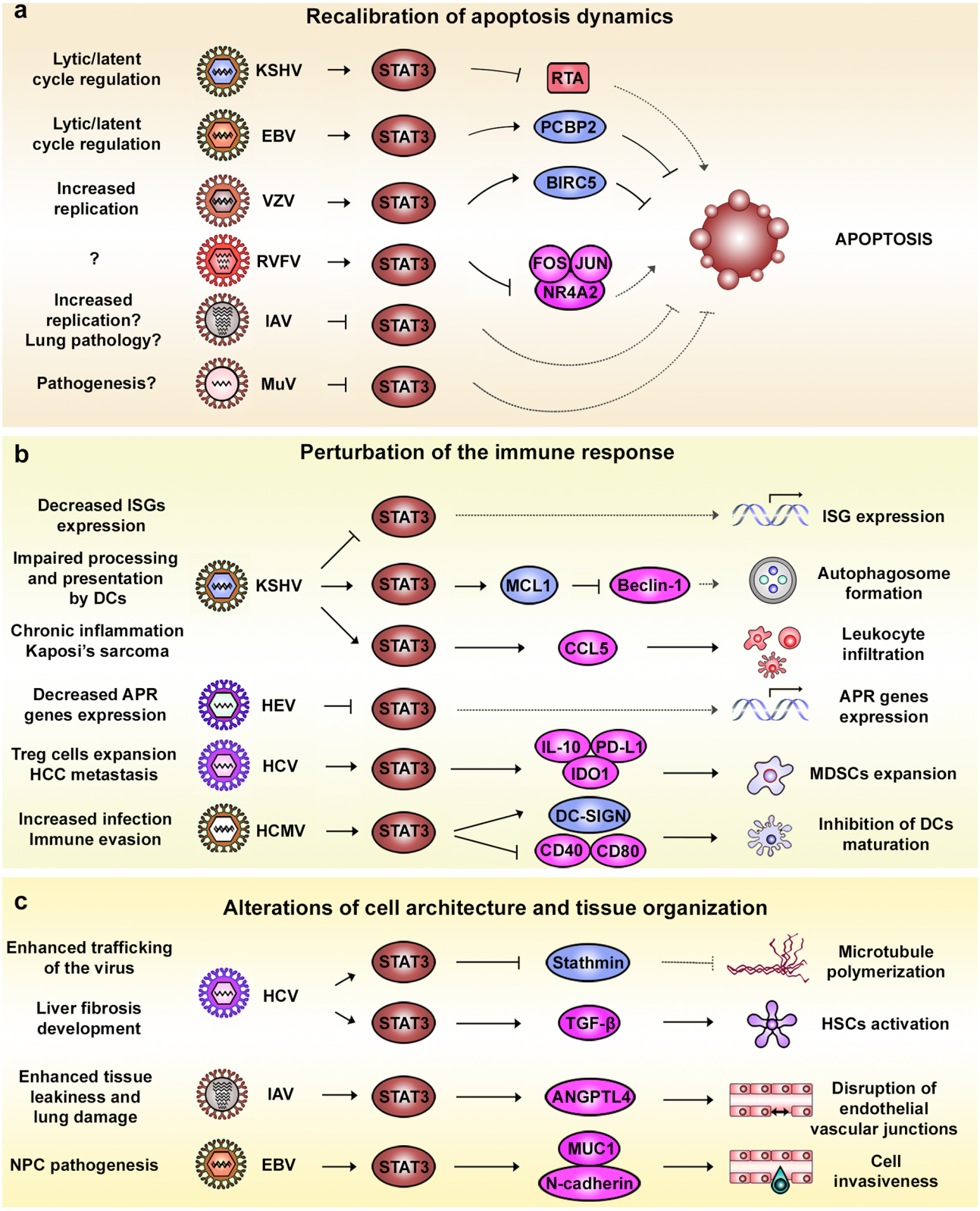

Fig 3. Viral replicative advantages and pathological consequences related to STAT3-altered function. (A) Virus-induced perturbation of STAT3 as regulator of apoptosis. In the context of viral infections, apoptosis can be restrained via STAT3, since it favors the expression of antiapoptotic factors (e.g., *PCBP2* and *BIRC5*) or prevents proapoptotic ones (e.g., RTA, *FOS*, *JUN*, and *NR4A2*). In contrast, inhibition of STAT3 by viruses such as IAV and

MuV has been associated with the induction of the apoptotic process. **(B)** Viral manipulation of STAT3 and its effect on immune responses. Viral inhibition of STAT3 can induce a decrease of ISG and APR gene expression and favor immune evasion, as in the case of KSHV and HEV. Virus-mediated STAT3 activation can also have immunosuppressive actions such as impairing DC function (KSHV and HCMV) and favoring the expansion of MDSCs (HCV). In other cases, the proinflammatory actions of STAT3 have been associated with the development of host pathologies such as cancer (KSHV). **(C)** Virus-induced alteration of STAT3 and its impact on cell and tissue organization. STAT3 activation during HCV infection has been associated with alterations of the MT network. This represents a potential advantage for HCV by favoring virus trafficking along MTs. At the tissue and organ level, STAT3 activation has been associated with the development of fibrosis (HCV), the disruption of endothelial vascular junctions (IAV), and enhanced cell invasion, which favors cancer development (EBV). ANGPTL4, angiopoietin-like protein 4; APR, acute phase response; BIRC5, baculoviral IAP repeat-containing protein 5; CCL5, C-C motif chemokine ligand 5; DCs, dendritic cells; DC-SIGN, dendritic cell-specific ICAM-3-grabbing non-integrin 1; EBV, Epstein–Barr virus; FOS, proto-oncogene c-Fos; HCC, hepatocellular carcinoma; HCMV, human cytomegalovirus; HCV, hepatitis C virus; HEV, hepatitis E virus; HSCs, hepatic stellate cells; IAV, influenza A virus; IDO1, indoleamine 2,3-dioxygenase 1; IL-10, interleukin 10; ISG, interferon-stimulated gene; JUN, proto-oncogene c-Jun; KSHV, Kaposi's sarcoma-associated herpesvirus; MCL1, induced myeloid leukemia cell differentiation protein Mcl-1; MDSCs, myeloid-derived suppressor cells; MT, microtubule; MUC1, mucin 1 cell surface associated; MuV, mumps virus; NPC, nasopharyngeal carcinoma; NR4A2, nuclear receptor subfamily 4 group A member 2; PCBP2, poly(rC)-binding protein 2; PD-L1, programmed cell death 1 ligand 1; RTA, R transactivator; RVFV, Rift Valley fever virus; STAT3, signal transducer and activator of transcription 3; TGF-β, transforming growth factor beta; T_{reg}, regulatory $CD4^+$ T cell; VZV, varicella-zoster virus.

https://doi.org/10.1371/journal.ppat.1006839.g003

inflammatory state observed in this pathology [19]. Moreover, KSHV-induced STAT3 activation correlates with up-regulated induced myeloid leukemia cell differentiation protein Mcl-1 (MCL1) expression levels, which can be reverted by inhibiting STAT3 [89]. MCL1 inhibits Beclin-1, a positive regulator of autophagosome formation, to interfere with antigen processing and presentation by DCs to avoid recognition and clearance [89]. KSHV also inhibits STAT3 via the action of viral miRNAs, and by doing so it hinders the expression of ISGs such as *CXCL10*, *ISG15*, *IFITM1*, *IRF1*, *OAS2*, and *MX1* [102]. The vIL-10 coded by HCMV up-regulates expression of its receptor DC-SIGN in DCs, their target cells [81,111]. vIL-10 stimulation of DCs also prevents the expression of costimulatory molecules (i.e., CD40, CD80, and CD86), inhibiting maturation of DCs, enhancing their susceptibility to infection, and hampering the immune response [81]. Chronic HCV infection has been associated with the presence of myeloid-derived suppressor cells (MDSCs), a heterogeneous population of myeloid cells that suppress the function of NK, $CD4^+$, and $CD8^+$ T cells [112]. Analysis of myeloid and lymphoid cells from chronically HCV-infected patients has shown that activation of STAT3 up-regulates the expression of suppressive genes (i.e., IL-10, programmed cell death 1 ligand 1 [PD-L1], indoleamine 2,3-dioxygenase 1 [IDO1]) in monocytic cells. They acquire MDSC-like characteristics and favor the expansion of T_{reg} cells [113,114]. MDSCs have been linked to an increased tumor burden and a higher metastasis rate in patients with HCC and in liver cancer mouse models [115]. Thus, by the STAT3-mediated induction of MDSCs, HCV can establish a microenvironment that supports viral immune evasion and accelerates HCC development.

Altering cell architecture and tissue organization

STAT3 also plays a role in cell morphology, which viruses exploit to promote viral persistence, with grave consequences for host cell physiology (Fig 3C). HCV-induced p-STAT3 directly controls microtubule (MT) dynamics through contact inhibition with stathmin [74]. Both HCV core and NS5A are transported along MTs [116]. Moreover, HCV core integrates into the MT lattice by a direct binding to tubulin [117]. Viral attenuation of stathmin enhances intracellular trafficking of the virus and increases replication [74]. In addition, regenerative STAT3 activation in HSCs precipitates fibrotic gene expression (i.e., *TGF-β1*, *TIMP-1*) [28], eventually leading to cirrhosis, which constitutes the procarcinogenic field on which most HCCs grow [118]. IAV triggers a STAT3-mediated up-regulation of angiopoietin-like protein 4 (ANGPTL4), a protein that compromises the integrity of endothelial vascular junctions. This leads to enhanced tissue leakiness and exacerbation of inflammatory lung damage in infected mice [103]. EBV is the most distinct etiological agent for the development of nasopharyngeal

carcinoma (NPC), a type of cancer in which STAT3 activation or overexpression is associated with more than 75% of tumors in regions where EBV is endemic [119]. EBV-mediated activation of STAT3 spurs cell invasiveness in vitro, and constitutive expression of STAT3 in NPC cell lines results in an increase of mesenchymal markers such as fibronectin and N-cadherin [120]. In accordance, STAT3 activation via LMP1 induces the expression of mucin 1 cell surface-associated (MUC1), a glycoprotein involved in the early steps of cancer cell detachment [121].

Disruption of STAT3 function as antiviral therapy

In the cases where STAT3 activity has a proviral or pathogenic effect, blocking STAT3 represents an interesting therapeutic strategy. Unfortunately, no molecule directly targeting STAT3 has received Food and Drug Administration (FDA) approval for any pathology so far [122], and candidate compounds targeting viral disease have not advanced beyond preclinical evaluation (Table 2). Small-molecule inhibitors targeting STAT3 phosphorylation (e.g., Cpd188, IB-32, Stattic) or dimerization (e.g., STA-21, S3I-201) have been evaluated as antivirals in vitro or in animal models. For instance, HCV replication but not entry is inhibited by STA-21, S3I-201, Cpd188, and IB-32 in Huh7 hepatoma cells or derivatives thereof [58,74,123]. Similarly, S3I-201 and Stattic reduce HCMV replication in cell culture [101], while S3I-201 limits VZV infection both in vitro and in animal models [90]. Oligodeoxynucleotide decoys (ODNs) are DNA-binding domain inhibitors that compete for binding of transcription factors with endogenous promoter sequences in their target genes. STAT3-targeting ODNs significantly decrease HBV RNA expression and DNA replication in hepatoma cell lines [124].

In addition, several natural products such as resveratrol or curcumin have been described to exhibit STAT3 inhibitory properties [125]. Resveratrol impairs EBV and VZV infection. For

Table 2. STAT3 signaling inhibitors, their mechanisms and in vitro antiviral applications.

Molecule	Targets	Molecule class	Mechanism of action	Antiviral effect	Refs
Cpd188	STAT3	Non-peptide small molecule	Inhibition of STAT3 phosphorylation	HCV	[58]
IB-32	STAT3	Non-peptide small molecule	Inhibition of STAT3 phosphorylation	HCV	[123]
STA-21	STAT3	Non-peptide small molecule	Inhibition of STAT3 dimerization	HCV	[74]
S3I-201	STAT3	Non-peptide small molecule	Inhibition of STAT3 dimerization	HCV VZV HCMV	[74] [90] [101]
Stattic	STAT3	Non-peptide small molecule	Inhibition of STAT3 phosphorylation	HCMV	[101]
Sorafenib	VEGFR PDFGR BRAF JAK2 STAT3	Tyrosine kinase inhibitor	Inhibition of STAT3 phosphorylation	HCMV*	[130]
Resveratrol	JAK1 STAT3	Natural product	Inhibition of STAT3 phosphorylation	VZV* EBV	[91] [126,127]
Curcumin	JAK1 JAK2 JAK3 STAT3	Natural product	Inhibition of STAT3 nuclear localization	HCMV	[101]
Oligodeoxynucleotide decoy	STAT3	DNA-binding modifier	Inhibition of STAT3 transcriptional activity	HBV	[124]

*Antiviral effect via STAT3 not determined.

Abbreviations: BRAF, serine/threonine-protein kinase B-raf; EBV, Epstein–Barr virus; HBV, hepatitis B virus; HCMV, human cytomegalovirus; HCV, hepatitis C virus; JAK1, Janus kinase 1; JAK2, Janus kinase 2; JAK3, Janus kinase 3; PDFGR, platelet-derived growth factor receptor; STAT3, signal transducer and activator of transcription 3; VEGFR, vascular endothelial growth factor receptor; VZV, varicella-zoster virus.

https://doi.org/10.1371/journal.ppat.1006839.t002

EBV, at least, it has been demonstrated that resveratrol suppresses STAT3 phosphorylation [126,127], while the antiviral mechanism by which resveratrol inhibits VZV is not yet understood [91]. Curcumin hinders HCMV replication in U373 cells by reducing nuclear accumulation of STAT3 [101], and while it exerts antiviral properties for IAV [128] and HCV [129], a mechanistic link to STAT3 has not been demonstrated yet.

The multikinase inhibitor sorafenib exhibits an antiviral effect against various HCMV strains by inhibiting the expression of immediate early genes of HCMV at clinically relevant concentrations [130]. However, sorafenib is not selective for STAT3; therefore, it is likely that a combination of unspecific effects may account for the observed antiviral effect of sorafenib on HCMV.

Outlook

STAT3 is a key regulator in inflammation and tissue regeneration triggered by almost every pathogenic infection. Therefore, viruses must deal with STAT3 activity by either curtailing it or employing it. STAT3 dependencies of viruses put a spotlight on the diverse role of signal transduction during viral infections and represent a target for potential antiviral strategies. Deregulated STAT3 signaling is an oncogenic driver and is associated with virus-induced complications, including cancers. However, targeting STAT3 during viral infection and cancer is currently an untapped reservoir, and the question still remains as to why it has not yet resulted in a broad range of clinical applications.

Currently, unspecific tyrosine kinase inhibitors (e.g., sorafenib) and monoclonal antibodies (e.g., tocilizumab) that block upstream components in the STAT3 pathway are readily administered to patients as cancer chemotherapeutics [131,132]. Similarly, other indirect STAT3-targeting strategies, including the modulation of STAT3 regulators, are promising. These include the use of histone deacetylase or proteasome inhibitors that promote expression of the endogenous STAT3 inhibitors SOCS3 and PIAS3, respectively [133]. While the use of approved indirect STAT3 modulators in clinical practice allows an indirect safety evaluation for STAT3-targeting strategies, their use does not allow conclusions on the specific clinical tolerance and efficacy of a STAT3-based antiviral approach.

Several natural products targeting STAT3 are currently being explored and seem promising; however, many (including curcumin and resveratrol) have been described as pan-assay interference compounds (PAINs). In other words, it currently cannot be ruled out that the observed effects of these natural compounds are due to an interference with the experimental readout rather than an interaction with their specific targets [134].

Due to multiple and redundant pathways that converge in STAT3 activation, direct STAT3-targeting agents would be a gold standard to assess the potential benefit of this approach. One reason why we have not observed a breakthrough in STAT3-targeting drugs so far may be that transcription factors are notoriously difficult to target and that many of the STAT3 inhibitors evaluated to date have shown to be problematic regarding their potency, bioavailability, and specificity [122]. Nevertheless, as we have explored in this review, there is strong scientific rationale to continue the development of novel STAT3-targeting therapies. Recently emerged agents that appear encouraging include AZD9150, an antisense oligonucleotide targeting *STAT3* mRNA that is in early phase I and II studies for advanced solid and hematological cancers [135–137], and napabucasin, a small-molecule inhibitor that has advanced to phase III clinical trials [138]. The evaluation of these and similar compounds for the treatment of cancers is expected to result in a broad range of clinical applications and holds great promise for future antiviral strategies as well.

Acknowledgments

We would like to thank the investigators whose work is cited here and apologize to those whose work could not be cited due to space restrictions. The authors thank Philippe Georgel, Université de Strasbourg, for helpful discussions.

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1.6.- Understanding cell signaling alterations in the era of multi-omics

During the last few decades, the study of biological phenomena has been slowly moving from a classical approach based on the acquisition of data via single-molecule experiments towards a more compressive systems biology approach. The technological advances driving this revolution have provide us with the tools to generate data at multiple levels, such as genomic, epigenomic, transcriptomic, proteomic and metabolomic. However, understanding the biological complexity in a given organism through omic studies is not just about the large-scale quantification of single molecules, rather it is a hypothesis-driven process the requires this type of data to validate the predictions of a model (Chuang, Hofree et al. 2010).

Moreover, it is not enough to map the components of a system using a single-data-type analysis. In this context, data integration and network analysis from multiple layers can achieve a more comprehensive evaluation of the link between a molecular alteration and a phenotype. Additionally, it can compensate for missing values and decrease the likelihood of false positives by using different sources of information (Ritchie, Holzinger et al. 2015). This integrative systems approach can be used to reveal phenotypic differences and to outline the molecular mechanisms involved, subsequently leading to the discovery of novel biomarkers or drug targets in translational medicine (**Fig. 12**).

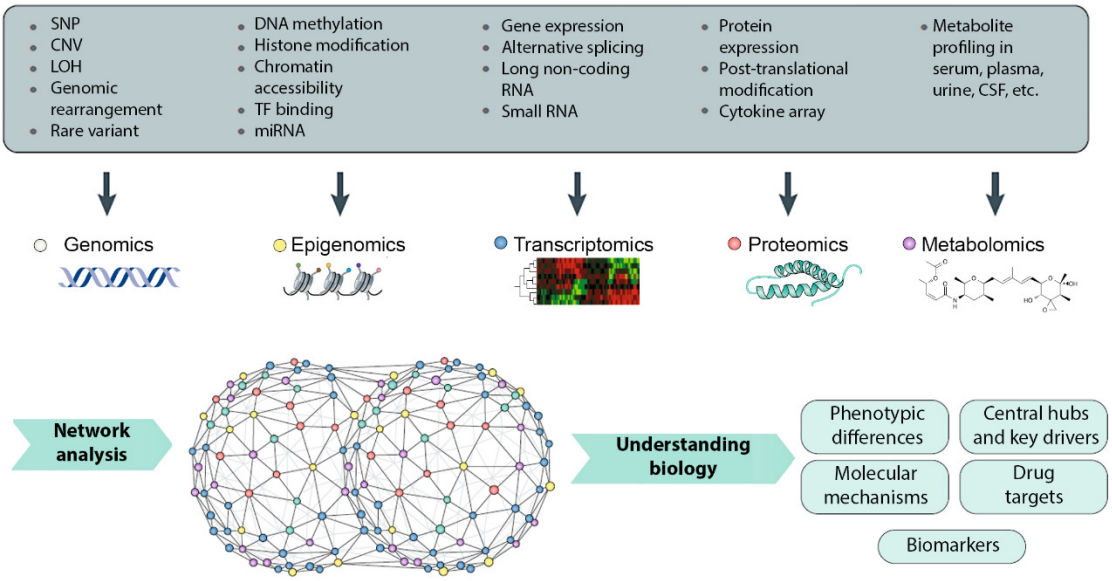


Figure 12: Biological systems multi-omics from the genome, epigenome, transcriptome, proteome and metabolome to understand complex traits and phenotypes. (Image modified from Ritchie, Holzinger et al. 2015 and Mardinoglu, Boren et al. 2018).

1.6.1.- Gene set enrichment analysis

Following omics data acquisition, the challenge relies in making sense of this large amount of information in order to gain insights into a biological process. If a classical approach is employed focusing on single genes presenting the biggest difference between two conditions, there is a risk of missing important information about the pathway as a whole. In other words, a modest increase of all genes belonging to a certain pathway may be biologically more relevant than a 20-fold increase in a single gene (Subramanian, Tamayo et al. 2005).

One of the most widely used tools for the identification of signaling pathway alterations from omics data is gene set enrichment analysis (GSEA) (**Fig. 13a**).

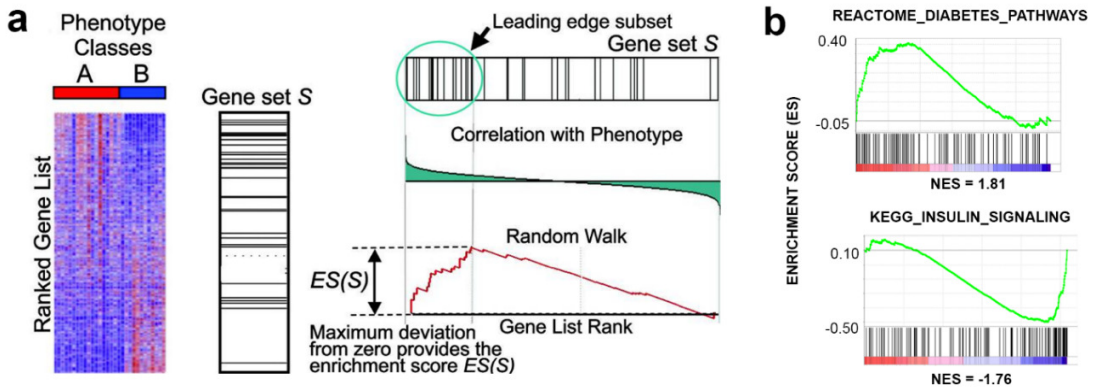


Figure 13: Gene Set Enrichment Analysis (GSEA). a) GSEA method overview which shows an expression data set sorted by correlation with phenotype, the location of genes from a gene set (S) within the sorted list and a plot of the running sum for S in the data set including the location of the maximum ES and the leading-edge genes (Image modified from Subramanian, Tamayo et al. 2005). **b**) Example of an upregulated (top) and a downregulated signaling pathway (bottom) (Image from Results article III).

GSEA interprets data at the level of gene sets, which contain genes belonging to a certain signaling pathway, cell type, disease phenotype, etc. This tool considers the expression profiles from two classes (e.g. A vs B) and ranks the genes according to the correlation of their expression and the class distinction. From here, the goal of GSEA is to determine which genes from a distinct gene set are up or downregulated in the studied condition compared to the control sample. This is represented by an enrichment score (ES) which is calculated by walking down the ranked list and increasing a running-sum statistic when encountering a gene present in the gene set or decreasing it if not present. The ES corresponds to the

maximum deviation from zero encountered in the random walk and is composed of the ''*leading-edge genes*'' which are the core of a gene set and account for the enrichment signal. In order to adjust for multiple hypothesis testing, the ES is normalized to account for the size of the gene set which yields a normalized enrichment score (NES) and a false discovery rate (FDR). An example of this type of results is presented above showing up or downregulated pathways (**Fig. 13b**).

2.- Results

2.1.- Impaired expression of PTPRD during HCV infection and HCC development

2.1.1.- Aims and summary

With the aim of having a deeper understanding of the host factors implicated in HCV entry, our team had previously performed a functional siRNA screen targeting protein kinases which could potentially have an impact in this process. This work led to a milestone in the molecular characterization of the HCV life cycle since it uncovered the receptor tyrosine kinases EGFR and Eph2A as key factors during viral entry (Lupberger, Zeisel et al. 2011).

This work shed light on a possible role of negative regulators of kinase-mediated protein phosphorylation by protein phosphatases. Therefore, the host laboratory analyzed liver biopsies of patients with chronic HCV infection and compared them to non-infected patients by quantifying the expression of 84 disease-relevant protein phosphatases. This highlighted 24 phosphatases that were significantly deregulated in HCV-infected tissues. Among them, there were several potentially relevant in the context of liver disease, including the one which would become the center of our subsequent studies, PTPRD.

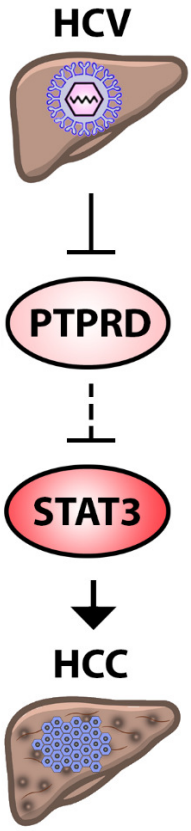
The rationale behind the selection of PTPRD was based on several factors: **1**) Its expression was not correlated with the degree of liver inflammation or fibrosis which suggested that its downregulation was directly HCV-related. **2**) As mentioned before, PTPRD had been shown to regulate the activity of STAT3 which is itself a host factor for HCV (McCartney, Helbig et al. 2013). **3**) *PTPRD* was found to be frequently mutated in HCC patients, suggesting a potential role as tumor suppressor in the liver (Acun, Demir et al. 2015).

In order to understand the mechanism implicated in the HCV-induced downregulation of PTPRD, our main hypothesis revolved around the miRNA machinery and more in particular by the action of miR-135a-5p. This was based on a combination of bioinformatic predictions, cell culture- and patient-derived data pointing to a model in which the HCV-induced upregulation of miR-135a-5p leads to the downregulation of its target PTPRD. My contribution for this part of the project involved firstly the quantification of miR-135a-5p expression in the liver and compare it to different tissues in order to assess if its level was relevant enough to induce an effect. My results showed that indeed miR-135a-5p is expressed in the human liver at levels that are similar to high-expressing tissues such as mouse brain and cerebellum.
Secondly, I performed the validation of miR-135a-5p as negative regulator of *PTPRD* expression. This consisted on the subcloning of the 3'UTR of *PTPRD* mRNA in a Renilla luciferase reporter construct (Luc-3'UTR) and the mutation of two predicted miR-135a-5p binding sites on this sequence to validate specificity. My results showed that cotransfection of miR-135a-5p with the Luc-3'UTR reporter significantly impaired luciferase activity, while this repression was lost when the reporter with mutated miR-135a-5p binding sites was used. This demonstrated that miR-135a-5p can silence *PTPRD* expression.

Similar to the case of glioblastoma (Veeriah, Brennan et al. 2009), our analysis of publicly available data sets from HCC tissues demonstrated that patients with low *PTPRD* expression presented an increased activity of the oncogenic STAT3 signaling pathway. Moreover, this impaired *PTPRD* expression was associated with a lower survival and a higher tumor recurrence. My contribution for this part was to validate these observations in two independent cohorts of HCC patients from the University hospitals of Strasbourg and Reims (*n*=44). Although the association of low *PTPRD* expression with a poor HCC prognosis was not significant due to the short follow up of these patients, we observed a trend similar to the main cohorts with longer follow-up. These results strengthened the case for a potential tumor suppressor role of PTPRD in the liver.

In summary, my results in this project contributed to the validation of a model in which HCV induces the downregulation of PTPRD via miR-135a-5p, leading to an increased activity of the STAT3 signaling pathway and a lower patient survival from HCC.

2.1.2.- Results article I



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.; De Blasi, V.; Pessaux, P.; Piardi, T.; Sommacale, D.; Ono, A.; Chayama, K.; Fujita, M.; Nakagawa, H.; Hoshida, Y.; Zeisel, M. B.; Heim, M. H.; Baumert, T. F.; Lupberger, J., miR-135a-5p-mediated downregulation of protein tyrosine phosphatase receptor delta is a candidate driver of HCV-associated hepatocarcinogenesis. *Gut* **2018,** *67* (5), 953-962.

OPEN ACCESS

ORIGINAL ARTICLE

miR-135a-5p-mediated downregulation of protein tyrosine phosphatase receptor delta is a candidate driver of HCV-associated hepatocarcinogenesis

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► Additional material is published online only. To view please visit the journal online (http://dx.doi.org/10.1136/gutjnl-2016-312270).

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Received 19 May 2016
Accepted 12 January 2017
Published Online First 3 February 2017

ABSTRACT

Background and aims HCV infection is a leading risk factor of hepatocellular carcinoma (HCC). However, even after viral clearance, HCC risk remains elevated. HCV perturbs host cell signalling to maintain infection, and derailed signalling circuitry is a key driver of carcinogenesis. Since protein phosphatases are regulators of signalling events, we aimed to identify phosphatases that respond to HCV infection with relevance for hepatocarcinogenesis.

Methods We assessed mRNA and microRNA (miRNA) expression profiles in primary human hepatocytes, liver biopsies and resections of patients with HCC, and analysed microarray and RNA-seq data from paired liver biopsies of patients with HCC. We revealed changes in transcriptional networks through gene set enrichment analysis and correlated phosphatase expression levels to patient survival and tumour recurrence.

Results We demonstrate that tumour suppressor protein tyrosine phosphatase receptor delta (PTPRD) is impaired by HCV infection in vivo and in HCC lesions of paired liver biopsies independent from tissue inflammation or fibrosis. In liver tissue adjacent to tumour, high PTPRD levels are associated with a dampened transcriptional activity of STAT3, an increase of patient survival from HCC and reduced tumour recurrence after surgical resection. We identified miR-135a-5p as a mechanistic regulator of hepatic PTPRD expression in patients with HCV.

Conclusions We previously demonstrated that STAT3 is required for HCV infection. We conclude that HCV promotes a STAT3 transcriptional programme in the liver of patients by suppressing its regulator PTPRD via upregulation of miR-135a-5p. Our results show the existence of a perturbed PTPRD–STAT3 axis potentially driving malignant progression of HCV-associated liver disease.

Significance of this study

What is already known on this subject?

- Chronic HCV infection is a leading cause of hepatocellular carcinoma (HCC); HCC risk remains elevated even after viral clearance.
- The mechanisms contributing to HCC development are poorly understood.
- HCV requires host cell signalling including the STAT3 pathway to maintain infection.
- Protein tyrosine phosphatase receptor delta (PTPRD) suppresses tumour by controlling STAT3 activity.

What are the new findings?

- PTPRD expression is impaired in hepatocytes of HCV-infected liver tissues and in HCC lesions.
- PTPRD expression in liver tissue of patients with HCC is associated with STAT3 transcriptional activity, patient survival and tumour recurrence after surgical resection.
- STAT3-mediated transcriptional programme is enriched in HCV-infected livers.
- miR-135a-5p is strongly upregulated in livers of patients with HCV and is a regulator of PTPRD mRNA.

How might it impact on clinical practice in the foreseeable future?

- HCV infection promotes the activity of its cofactor STAT3 by suppressing its negative regulator PTPRD via miR-135a-5p. Our model suggests the existence of a perturbed PTPRD–STAT3 axis driving malignant progression of liver disease. This finding is of further clinical relevance since it provides a target for urgently needed HCC chemoprevention.

Check for updates

To cite: Van Renne N, Roca Suarez AA, Duong FHT, *et al*. *Gut* 2018;**67**:953–962.

INTRODUCTION

More than 150 million people worldwide are infected by HCV,[1] which is a leading cause of liver cirrhosis and hepatocellular carcinoma (HCC).[2] The arrival of highly effective new therapies consisting of direct-acting antivirals can cure the vast majority of patients,[3] but those with advanced liver disease remain at risk for developing HCC even after viral clearance.[4] Moreover, the exact mechanisms responsible for this increased

susceptibility remain only partly understood. Traditionally, carcinogenesis has been attributed to a multistep accumulation of genetic damage resulting in gain of function by proto-oncogenes and inactivation of tumour suppressor genes.[5] Only a minority of these driver genes is frequently involved in cell transformation, while the majority is only occasionally affected.[6] In other words, a myriad of combinations in genetic and epigenetic alterations can lead to cancer, and as a consequence, every tumour displays a heterogeneous molecular profile, further clouding understanding of cancer development. In chronic hepatotropic virus infections such as hepatitis C and B, the picture is even more complicated as both viral and non-viral factors are the drivers of hepatocarcinogenesis.[7 8] Nonetheless, the complexity of malignant transformation can be reduced to a handful of logical underlying principles, of which derailed signalling circuitry is a key component.[9] We have previously demonstrated that cellular receptor tyrosine kinase (RTK) signalling is involved in regulating HCV entry into hepatocytes and that the virus binding to the hepatocytes triggers RTK activation.[10 11 12] This indicates that chronic HCV infection modulates host signalling patterns that may contribute to the development of virus-induced liver disease. Since these signalling processes are tightly regulated by protein phosphatases and aberrant phosphatase expression is involved in various syndromes and diseases,[13] we screened for disease-relevant phosphatase expression in liver biopsies of chronic patients with HCV.

MATERIALS AND METHODS

Liver tissue, cells and virus

Human needle liver biopsies and liver tissue from patients undergoing surgical resection were collected at the Gastroenterology and Hepatology outpatient clinic of the Basel University Hospital, Switzerland, the Centre Hospitalier Universitaire de Reims, France and the Hôpitaux Universitaires de Strasbourg, France. Protocols for patient tissue collection were reviewed and approved by the ethics committees of the respective university hospitals. Written informed consent was obtained from all patients. Eligible patients were identified by a systematic review of patient charts at the hepatology centres of the university hospitals of Basel, Reims and Strasbourg. Histopathological grading and staging of the HCV liver biopsies, according to the METAVIR classification system, were performed at the pathology institutes of the respective university hospitals. All the patients that donated liver tissue are summarised in online supplementary table S1. Liver biopsy tissues were analysed as described.[14] Mouse tissue was obtained from C57BL/6J mice. Tissues were lysed and subjected to quantitative PCR (qPCR) and immunoblot analysis (described below). Primary human hepatocytes (PHH) were isolated and cultured as previously described.[11] Huh7.5.1, HEK293T cells and cell culture derived HCV (HCVcc) strains Luc-Jc1 and Jc1E2^{FLAG} have been described.[11 12 15] Jc1E2^{FLAG} was affinity-purified as described.[15] Huh7.5.1 were infected with Jc1E2^{FLAG} as described.[16]

Patient cohorts

Protein tyrosine phosphatase receptor delta (PTPRD) expression was correlated with corresponding clinical data in three independent patient cohorts from Icahn School of Medicine at Mount Sinai, New York, USA (National Centre for Biotechnology Information Gene Expression Omnibus GSE10140) (cohort A),[17] from the Hiroshima University Hospital, Hiroshima, Japan (European Genome-phenome Archive, https://ega.crg.eu, accession number, EGAD00001001880) (cohort B)[18] and from the University Hospitals of Strasbourg and Reims, France (cohort C).

Antibodies and western blotting

P-STAT1 mAb (58D6) was obtained from Cell Signaling Technology, β-Actin mAb (AC-15) was obtained from Invitrogen and PTPRD pAb (C-18) from Santa Cruz Biotechnology. Western blots were performed using Hybond-P membranes (GE Healthcare), visualised using ECF substrate (GE Healthcare) and quantified with a fluorescence scanner (Typhoon Trio, GE Healthcare).

Analysis of mRNA and miRNA expression

mRNA expression of 84 disease-relevant protein phosphatases was assessed in six liver biopsies from patients with chronic HCV infection and in six non-infected biopsies using qPCR (Human Protein Phosphatases RT^2 Profiler PCR Array, Qiagen). Total RNA from Huh7.5.1 cells, PHHs and liver tissue was extracted using RNeasy Mini Kit (Qiagen) or Trizol (Life Technologies). Gene expression in the total RNA extracts was assessed using two-step qPCR. The reverse transcription on total RNA extract was made using Maxima reverse transcriptase (Thermo Scientific). qPCR for detecting PTPRD, radical S-adenosyl methionine domain containing 2 (RSAD2), ubiquitin-specific peptidase 18 (USP18) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was performed with RT^2 qPCR Primer Assays (Qiagen) using Real-Time PCR ABI Prism 7500 (Thermo Scientific) or Corbett Rotor Gene 6000 (Qiagen). All qPCRs were performed following manufacturers' instructions. Specificities of phosphatase PCR products were validated by melting curve analysis and PCR product sequencing. Differential gene expression of patient biopsies was calculated after $2^{-\Delta CT}$ transformation into individual data points. The transcriptome of Jc1E2^{FLAG}-infected Huh7.5.1 cells was measured by RNA-seq at the Broad Institute as previously described.[19] All microRNA (miRNA) indicated in this study are human (hsa-miR) if not indicated differently. miRNA expression was measured using miScript (Qiagen) with forward DNA primers derived from the miRNA sequence implemented in Sanger miRBase database (V.21.0) and referenced with a MIMAT accession number (see online supplementary table S2). miRNA target sites were predicted using miRSystem.[20] Synthetic miRNAs (mimics) of miR-135a-5p and the non-targeting negative control miRNA cel-miR-67 (miR-CTRL) were obtained from GE Healthcare.

Luciferase reporter assay

The 3′-untranslated region (3′UTR) of PTPRD was amplified with 5′-TTT CTC GAG CTT TGA CCA CTA TGC AAC GTA G-3′ and 5′-TTT CTC GAG CTG TCC TCG CCG TTT TCT AA-3′, and subcloned in psiCheck-2 (Promega) using *Xho*I. As specificity control, the seed sequence AGCCAT of two miR-135a-5p target sites on the 3′UTR of PTPRD was replaced by AAAAAA using site-directed mutagenesis. An amount of 150 ng luciferase reporter plasmid was cotransfected with 10 nmol/L miRNA in HEK293T cells using Lipofectamine 2000 (Thermo Fisher Scientific). Activity of *Renilla* and firefly luciferase was assessed 48 hours post transfection using Dual-Luciferase Reporter Assay (Promega) and a Mithras LB940 plate reader (Berthold Technologies).

FISH analysis

Human needle liver biopsies were collected, immediately embedded in optimal cutting temperature compound and frozen

in liquid nitrogen chilled 2-methylbutane. Tissues were then stored at −80°C until use. Sections (10 µm) were cut at cryostat (Leica) and mounted onto Superfrost Plus Gold glass slides (K5800AMNZ72, Thermo Fisher Scientific), fixed overnight in 4% formaldehyde at 4°C and hybridised, as previously described,[21] with the following modifications: tissue sections were pretreated by boiling (90°C–95°C) in pretreatment solution (Panomics, Affymetrix) for 1 min, followed by a protease digestion for 10 min at 40°C. Hybridisation was performed using probe sets against patient-specific HCV RNA sequence (type 1) and against human PTPRD mRNA (NM_002839, target region 4754-5835). Preamplification, amplification and detection were performed according to provider's protocol. Images were acquired with a laser scanning confocal microscope (LSM710, Carl Zeiss Microscopy) and Zen2 software, using the same settings for all the tissues analysed. Five random fields were acquired from each section. Image analysis was performed using ImageJ and CellProfiler software, with a customised pipeline. Total number of cells, frequency of HCV-positive and PTPRD-positive cells, and signal intensity were then evaluated and exported for statistical analysis (two-way analysis of variance (ANOVA), $p \leq 0.05$).

Bioinformatics of gene expression database

Paired gene expression of 62 formalin-fixed paraffin-embedded tumour and adjacent liver tissues was explored from cohort A[17]). Downregulation of PTPRD expression in tumour tissue was analysed *in silico* for 46 patients with chronic HCV. Values of p were calculated with Wilcoxon signed-rank test (two-tailed). Probability of survival and tumour recurrence were evaluated by the log-rank test available on the GenePattern Survival Analysis module (http://www.broad.mit.edu/cancer/software/genepattern).[22] Gene Set Enrichment Analysis (GSEA) was performed as described.[23 24] The normalised enrichment score (NES) is a measure expressing to what extent the members of a gene set shift towards the top or bottom in the gene list ranked on differences in expression between two phenotypes. A false discovery rate (FDR) smaller than 0.25 is generally considered an appropriate cut-off. The GSE10143 database comprised 78 of the 87 response genes designated as the Hallmark_IL6 JAK STAT3 signalling gene set.[25]

RESULTS

Chronic HCV infection impairs PTPRD expression in vivo

To study the impact of chronic HCV infection on phosphatase expression in patient liver tissue, we extracted RNA from six biopsies of patients with chronic hepatitis C (CHC) and six non-infected biopsies (see online supplementary table S1), and quantified expression of 84 disease-relevant protein phosphatases using qPCR. We identified 24 phosphatases that were significantly ($p<0.01$, U-test) deregulated in HCV-infected tissue compared with non-infected biopsies (figure 1A). Interestingly, among the phosphatases with most deregulated expression levels in HCV biopsies, we observed an enrichment of candidates with potential relevance for the development of HCC. More particularly, some of these phosphatases act as tumour suppressors in various cancers including PTPRD. PTPRD is frequently inactivated and mutated in human cancers[26 27] including HCC.[28] In order to validate HCV-induced PTPRD downregulation, we measured mRNA expression in a second sample series of 24 liver biopsies of patients with chronic HCV and validated that PTPRD mRNA expression is significantly ($p=0.0003$, U-test) downregulated compared with 11 additional non-viral control biopsies (figure 1B).

PTPRD expression in patients with HCV is independent from fibrosis or inflammation

Since biological circuits in liver tissues from patients with CHC reflect a complex interaction of different cell types integrating an inflammatory immune response, we first compared phosphatase expression levels with METAVIR grading and staging data (see online supplementary table S1). No significant relationship between METAVIR score and PTPRD expression was observed in 30 liver biopsies (figure 1C, D), suggesting that PTPRD expression is independent from the degree of inflammation and fibrosis of the studied biopsies and rather susceptible to a more direct virus-induced mechanism within infected hepatocytes.

PTPRD expression is impaired in HCV-infected hepatocytes

To identify the cell type displaying impaired PTPRD expression levels upon HCV infection, we applied an established protocol for multiplex fluorescent in situ hybridisation (FISH) analysis of liver biopsies at single cell resolution.[21] This strategy enabled us to distinguish HCV-infected cells from uninfected cells in liver biopsies. Simultaneous hybridisation with specific probe set for PTPRD mRNA demonstrated that PTPRD was significantly and specifically ($p<0.05$, two-way ANOVA) impaired in infected cells compared with uninfected cells in three biopsies from different patients infected with HCV genotype 3a (see figure 1E, online supplementary table S1 and figure S1). Differential PTPRD expression was detected in cells with hepatocyte morphology. The analysed fields did not show an abnormal level of infiltrating non-parenchymal cells, and the level of PTPRD expression in those cells was marginal compared with hepatocytes (data not shown). This indicates that HCV impairs PTPRD expression directly in hepatocytes. To validate this observation, we measured PTPRD expression levels in isolated PHH infected in vitro with HCV. We observed that PHH robustly express PTPRD both at the mRNA and the protein level, providing a competent model system to study PTPRD. Two days after isolation, PHH were infected for 5 days with HCVcc strain JFH1, after which cells were lysed, and both mRNA and protein were isolated. With this established protocol, up to 10%–30% of hepatocytes can be infected by HCVcc (strain JFH1) as shown[29] (see online supplementary figure S2) using a reporter red fluorescence protein–nuclear localisation sequence–interferon-β promoter stimulator lentivirus (RFP-NLS-IPS).[30] HCV-infected PHH from different donors showed a significant decrease ($p=0.015$, U-test) in PTPRD mRNA expression (figure 2A), which also translated into a decreased PTPRD protein level as detected by immunoblot ($p=0.016$, U-test) (figure 2B, C). To exclude that PTPRD downregulation was caused by an antiviral response mounted by immunocompetent PHH, we treated PHH for 5 days with interferon-α (IFN-α) (figure 2D). No change in PTPRD protein levels was observed by western blot for different concentrations of IFN-α, while STAT1 phosphorylation was properly induced. In parallel, interferon response genes *USP18* and *RSAD2* displayed an increase in mRNA transcription after IFN-α stimulus, while PTPRD remained at baseline levels (figure 2E). These data confirm that PTPRD downregulation is independent from the innate immune response.

miR-135a-5p is a mechanistic regulator of PTPRD expression in HCV-infected hepatocytes

The miRNA machinery is a regulator of gene expression and is exploited by HCV to maintain its replication.[31 32] We applied multiple bioinformatical algorithms[20] scanning for human miRNAs potentially targeting PTPRD mRNA. Combining

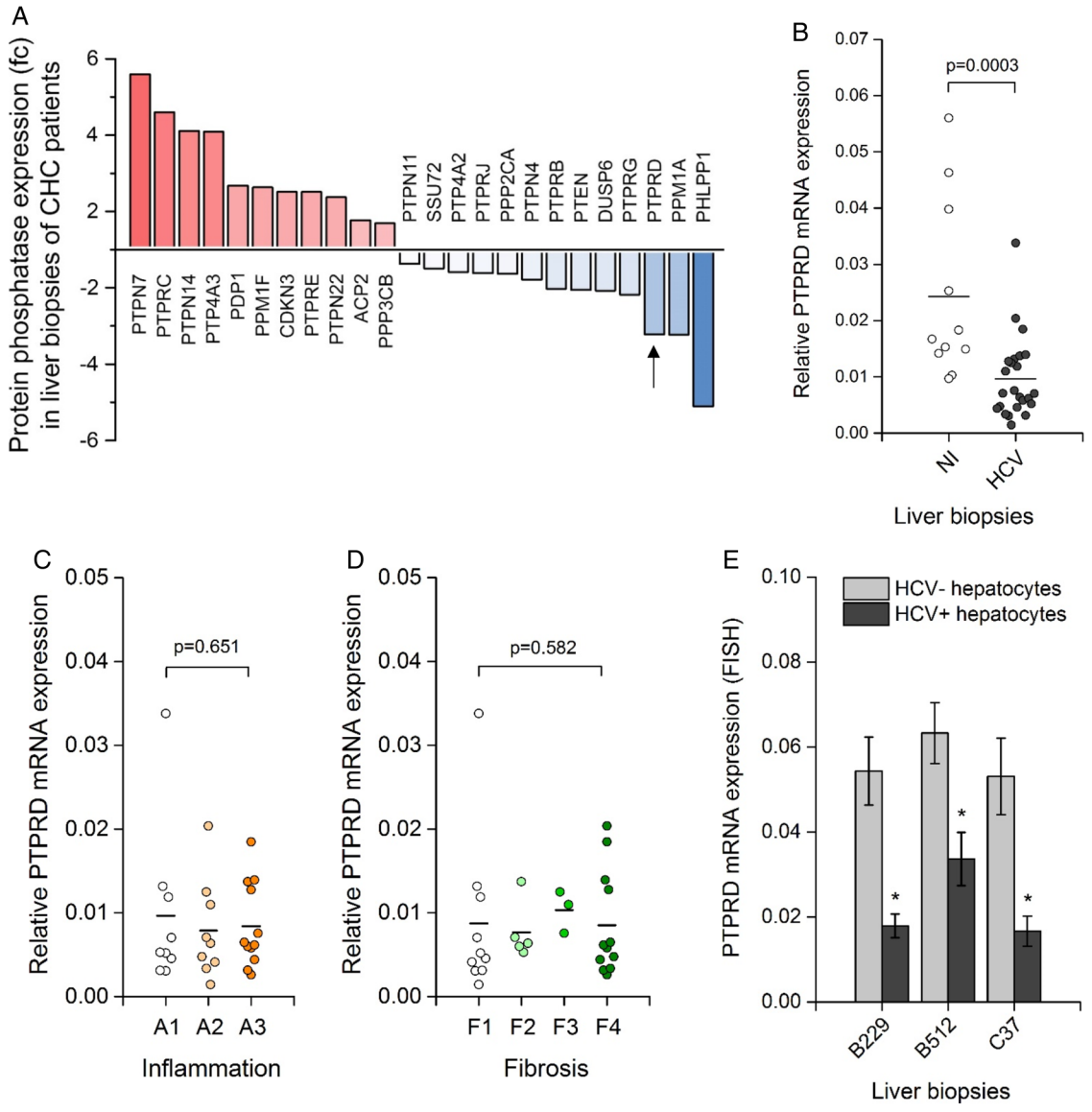

Figure 1 Protein tyrosine phosphatase receptor delta (PTPRD) expression is significantly impaired in livers of patients with chronic hepatitis C (CHC). Expression of 84 protein phosphatases associated with diseases and syndromes was quantified in needle stick biopsies using qPCR and in situ hybridisation of RNA probes. (A) mRNA expression of 11 phosphatases was significantly ($p<0.01$, U-test) increased (red), and expression of 13 phosphatases was significantly ($p<0.01$, U-test) decreased (blue). Data are expressed as fold change (fc) phosphatase expression of non-HCV (n=6) and HCV-infected biopsies (n=6) relative to the average expression levels of five housekeeping genes (*actin, B2M, GAPDH, HPRT1, RPLP0*). Arrow indicates tumour suppressor PTPRD. (B) Expression of PTPRD is significantly impaired in liver biopsies of patients with chronic HCV ($p=0.0003$, U-test). Validation of HCV-induced downregulation of PTPRD compared with non-infected biopsies (NI) observed in (A) in additional liver biopsies from patients infected with HCV (see online supplementary table S1). Data are expressed as PTPRD mRNA expression relative to GAPDH and visualised as individual data points and median (line). (C and D) Expression of PTPRD is independent from fibrosis and inflammation in liver tissues. PTPRD expression was correlated with METAVIR score of the HCV-infected liver biopsies, but no significant correlation ($p>0.5$, U-test) between METAVIR grading (C) or staging (D) of the studied biopsies in (A) and (B) (see online supplementary table S1) was observed. Data are expressed as PTPRD mRNA expression relative to GAPDH and visualised as individual data points and median (line). (E) PTPRD expression is impaired in HCV-infected hepatocytes in liver biopsies. Fluorescent in situ hybridisation (FISH) analysis of liver biopsies infected with HCV genotype 3 by simultaneous hybridisation with HCV-specific and PTPRD-specific probe sets. PTPRD labelling was assessed in five random fields from each section. Data are expressed as mean of PTPRD fluorescence intensities in HCV-positive and HCV-negative cells per patient (±SD, $p<0.05$, two-way analysis of variance).

computational miRNA prediction with observations in HCV-infected Huh7.5.1 cells, we defined a panel of eight miRNAs with two essential characteristics: they potentially target PTPRD and they are upregulated by HCVcc in Huh7.5.1 cells (see online supplementary table S2). To validate the relevance of these eight miRNAs in a real-life setting, we screened for their expression in liver biopsies of patients infected with HCV (figure 3A). This uncovered a striking twofold upregulation of miR-135a-5p in liver specimens of patients with HCV ($p=0.0006$, U-test) and establishes it as a possible PTPRD regulator (figure 3B). Moreover, similar to PTPRD, miR-135a-5p expression is independent from the host antiviral response, as shown by a kinetics study measuring miR-135a-5p in IFN-α stimulated PHH. miR-135a-5p expression remained level ($p=0.5$, U-test), while the interferon response gene *RSAD2* was significantly ($p=0.02$, U-test) upregulated (figure 3C). This suggests that miR-135a-5p does not take part in the innate immune response, but is rather relevant to the HCV life-cycle itself. To

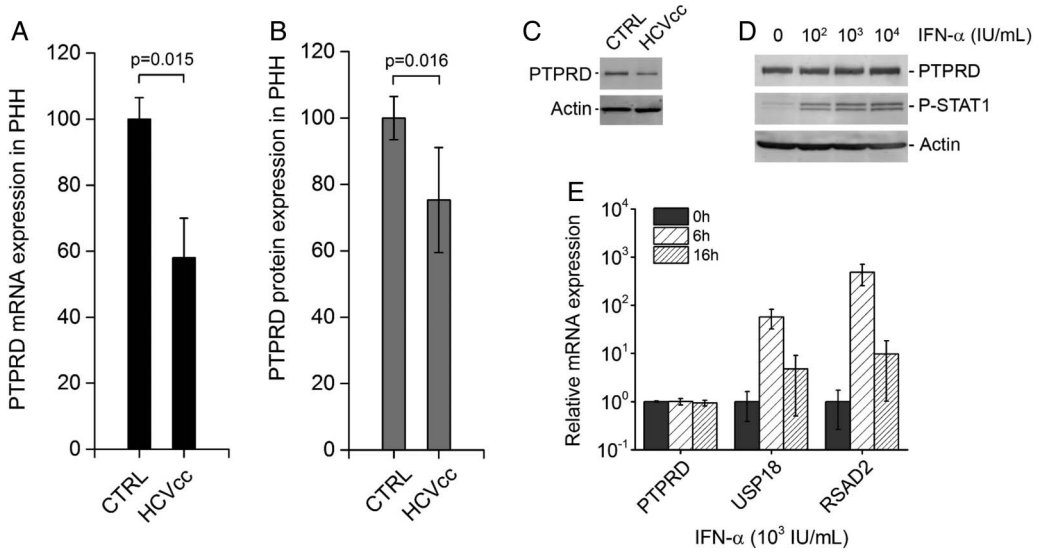

Figure 2 Protein tyrosine phosphatase receptor delta (PTPRD) is significantly impaired by HCV in hepatocytes independent from the innate immune response. (A) PTPRD mRNA expression is significantly impaired in primary human hepatocytes (PHH) after 5 days of infection with HCVcc (strain JFH1). Data are expressed as mean PTPRD expression relative to GAPDH±SEM (p=0.015, U-test; six independent infections of PHH from three donors). (B) PTPRD protein expression is significantly impaired by HCV in PHH. PTPRD and actin expression were assessed by western blotting 5 days after infection of PHH with HCVcc (strain JFH1) compared with non-infected PHH (NI). Band intensities were quantified using Image Quant and are expressed as mean PTPRD expression relative to actin±SEM (p=0.016, U-test; five independent infections of PHH from two different donors). (C) A representative western blot quantified in (B) is shown. (D) PTPRD protein expression is independent from innate immune response. Host antiviral response was simulated by incubation of PHH with interferon-α (IFN-α). Cell culture medium with IFN-α was replaced every day. Cells were lysed after 5 days of incubation, and PTPRD, phospho-STAT1 (p-STAT1) and actin were measured by western blotting. (E) PTPRD is not an IFN-response gene. PHH were incubated for 10 min with 10^3 IU/mL IFN-α, and mRNA expression of PTPRD and the known interferon-response genes *USP18* and *RSAD2* was assessed by RT-qPCR relative to GAPDH 6 and 16 hours post IFN stimulation. CTRL, control.

prove that miR-135a-5p targets PTPRD mRNA, we subcloned the 3′UTR of PTPRD mRNA in the *Renilla* luciferase expression cassette (Luc-3′UTR) of a bicistronic *Renilla*/firefly luciferase reporter construct (psiCheck-2, Promega). The 3′UTR of PTPRD harbours two predicted highly conserved miR-135a-5p target sites (see online supplementary table S2). Cotransfection of a miR-135a-5p mimic with the Luc-3′UTR reporter significantly ($p=3.29\times10^{-4}$, U-test) impaired normalised luciferase activity compared with empty vector, while the repression of luciferase expression was lost when the Luc-ΔmiR-135a-5p reporter with mutated miR-135a-5p binding sites was used (figure 3D). This demonstrates that miR-135a-5p is able to silence PTPRD expression, which is also reflected in vivo where a significant (p=0.04, r=−0.03, one-tailed Spearman's correlation test) inverse correlation of PTPRD mRNA and miR-135a-5p levels could be discerned in liver biopsies studied in figure 3B. Remarkably, high levels of miR-135a-5p always corresponded with low PTPRD expression, while low amounts of miR-135a-5p give PTPRD expression more leeway to vary. Interestingly, some of the studied liver biopsies did neither exhibit high PTPRD mRNA nor miR-135a-5p levels (figure 3E), suggesting the presence of an additional more general regulatory mechanism of PTPRD expression that is independent from HCV. Taken together, these data establish miR-135a-5p as a potent HCV-driven regulatory element for PTPRD expression.

STAT3 transcriptional activity is induced in HCV-infected Huh7.5.1 cells in vitro and associated with attenuated PTPRD expression in vivo

PTPRD is a signal transducer and activator of transcription 3 (STAT3) phosphatase,[27] and therefore dampened PTPRD expression should promote the interleukin 6-Janus Kinase–STAT3 (IL6-JAK–STAT3) signalling axis. STAT3 signalling is essential for liver regeneration[33] and an integral part of the regulation of the host interferon response.[10] Moreover, STAT3 is a cofactor for HCV infection, suggesting an accumulation of STAT3 activity in host cells during HCV infection.[10] Indeed, a temporal GSEA of HCVcc-infected Huh7.5.1 transcriptomes revealed an accumulation of a STAT3 transcriptional signature (Hallmark_IL6 STAT3 signalling gene set[25]) over the first 7 days of infection (figure 4A). However, PTPRD is not expressed in hepatoma cells and cannot be rescued in our hands (data not shown), precluding the in vitro study of PTPRD on the induction of the IL6–JAK–STAT3 signalling cascade. Therefore, we analysed a publicly accessible gene expression database of 82 adjacent non-tumour liver tissue specimens of patients with HCC (cohort A).[17] Those patients with the lowest 20 percentile of hepatic PTPRD expression in adjacent tissue showed a strong and marked enrichment (NES=1.75, FDR=0.009) of the STAT3 transcriptional programme compared with patients with the highest 20 percentile (see figure 4B, C and online supplementary figure S3), which is testimony of a PTPRD-mediated STAT3 deactivation in vivo. For 62 of these 82 patients, HCV infection status was available. We could thus confirm PTPRD expression downregulation in 46 HCV(+) biopsies compared with 16 HCV(−) biopsies (figure 4D) and show the enhanced STAT3 activity in HCV(+) through GSEA (NES=1.96, FDR=0.001) (see figure 4E and online supplementary figure S4). Taken together, these findings demonstrate that transcriptional activity of the oncogene STAT3 is clearly linked to PTPRD in the livers of patients. Because STAT3 activity is associated with HCCs with poor prognosis,[34 35 36] our findings suggest that HCV infection accumulates STAT3 signalling via suppression of its negative regulator PTPRD and as such may contribute to the exacerbation of chronic liver disease.

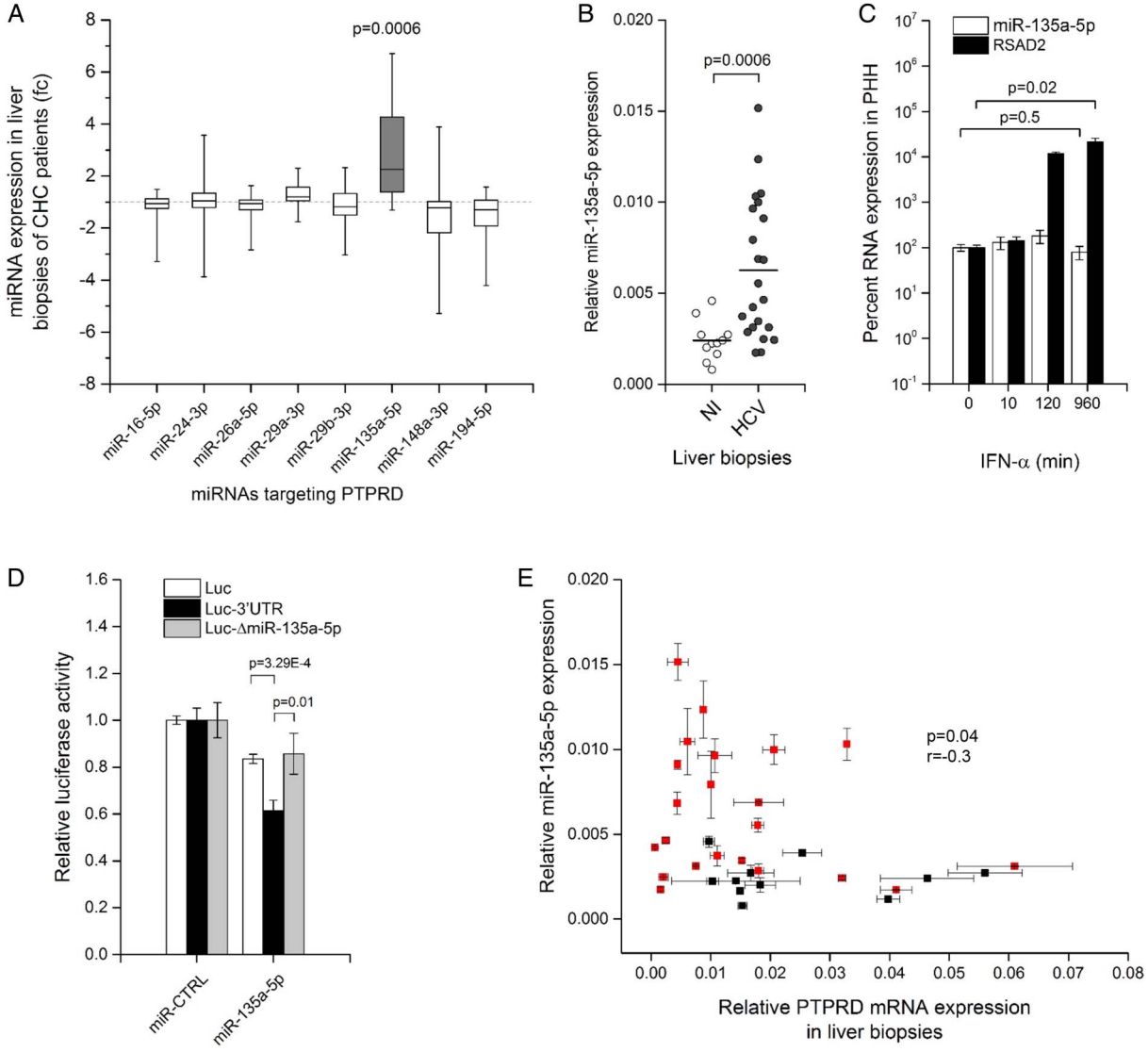

Figure 3 HCV-induced expression of miR-135a-5p mediates protein tyrosine phosphatase receptor delta (PTPRD) silencing. (A) miRNA expression screening of potential regulators of PTPRD mRNA in livers of patients with chronic hepatitis C (CHC). Predicted miRNAs potentially regulating the PTPRD mRNA and that are upregulated by HCV in Huh7.5.1 cells (see online supplementary table S2) were screened in liver biopsies using qPCR. Data are expressed as fold change (fc) miRNA expression relative to SNORD61 and visualised as box-and-whisker plot (box=50% of biopsies, line=median, whiskers=minimal and maximal values) centred to the median expression levels of the miRNA in the corresponding non-infected (NI) liver biopsies. Grey bar highlights miR-135a-5p that is upregulated more than twofold in patients with HCV. (B) miR-135a-5p expression is significantly (p=0.0006, U-test) upregulated in livers of patients with HCV (dark grey circles) compared with NI tissues (open circles). (C) miR-135a-5p is not induced by the antiviral response to HCV infection. Isolated primary human hepatocytes (PHH) were treated over 16 hours with 10^3 IU/mL interferon-α (IFN-α) prior to qPCR analysis of miR-135a-5p and the interferon-stimulated gene *RSAD2*. While interferon-response gene *RSAD2* is significantly (p=0.02, U-test) induced by IFN-α, miR-135a-5p remains level (p=0.5, U-test). (D) miR-135a-5p induces silencing of PTPRD expression. 3′UTR of PTPRD harbouring two miR-135a-5p target sites was subcloned in the *Renilla* luciferase expression cassette of a bicistronic *Renilla*/firefly luciferase reporter plasmid (Luc-3′UTR). As a control (Luc-ΔmiR-135a-5p), the seed sequence AGCCAT in Luc-3′UTR was replaced by AAAAAA. Cotransfection of Luc-3′UTR but not Luc-ΔmiR-135a-5p with a miR-135a-5p mimic significantly ($p=3.29\times10^{-4}$, U-test) impairs *Renilla* luciferase expression in HEK293T cells compared with empty vector (Luc). A minor unspecific effect of miR-135a-5p on empty vector (Luc) was observed. Data are expressed as mean *Renilla* luciferase activity±SEM normalised to firefly luciferase (five independent experiments in triplicate) relative to cotransfection of the vectors with non-targeting miRNA derived from *Caenorhabditis elegans* (miR-CTRL). (E) PTPRD mRNA levels significantly and inversely correlate (p=0.04, r=−0.03, one-tailed Spearman's correlation test) with miR-135a-5p levels in liver biopsies. PTPRD mRNA and miR-135a-5p expression in NI and HCV-infected liver biopsies analysed in (B) were compared. All liver biopsies with high miR-135a-5p levels exhibited low PTPRD mRNA expression and *vice versa*. Liver biopsies from patients infected with HCV are highlighted in red.

PTPRD expression is impaired in HCC and associated with survival of patients with HCC and decreased tumour recurrence after surgical resection

We observed that PTPRD mRNA is only marginally expressed in hepatoma cell lines such as Huh7.5.1. Moreover, it does not translate into detectable protein levels as assessed by western blot in a panel of hepatic cell lines tested including HepG2, Huh7, Huh7.5.1, differentiated HepaRG and non-differentiated HepaRG (data not shown), which suggests that impaired PTPRD is a hallmark of cell transformation. To ascertain whether PTPRD is also downregulated in HCC, we assessed PTPRD protein expression in six paired biopsies of patients

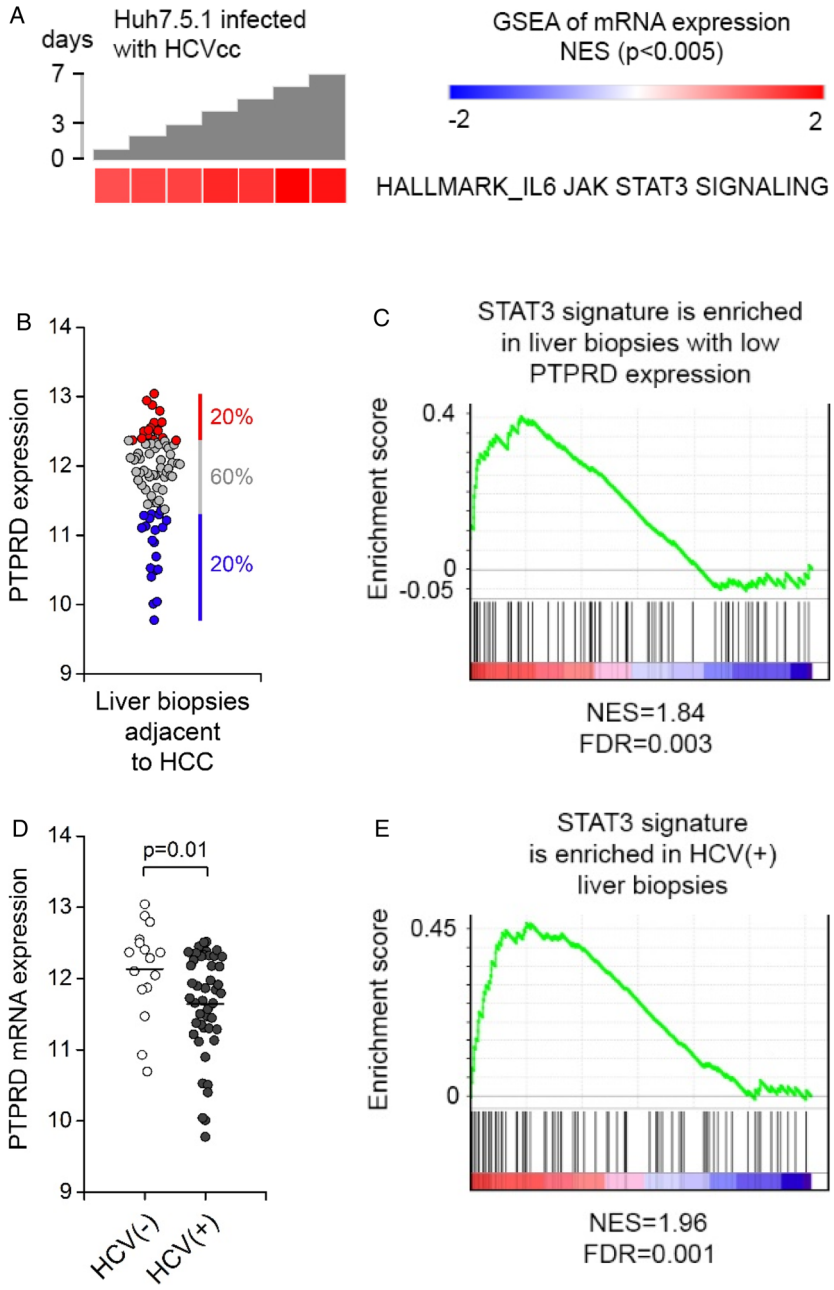

Figure 4 Dampened protein tyrosine phosphatase receptor delta (PTPRD) expression in liver biopsies is associated with an accumulated STAT3 transcriptional programme. (A) HCV infection induces STAT3 transcriptional activity. Huh7.5.1 cells were subjected to infection with affinity-purified HCVcc (Jc1E2^{FLAG}) for up to 7 days. Control cells were mock-infected using FLAG peptide. Cell transcriptome was profiled every day by RNA-seq as previously described,[19] and the modulation of the Hallmark_IL6 STAT3 signalling gene set was assessed by Gene Set Enrichment Analysis (GSEA) in HCV-infected cells as compared with controls. Significant (p<0.005) normalised enrichment scores (NES) of the gene set are indicated as red (positive enrichment) for each day of infection (grey stairs). (B) Patients were classified according to PTPRD expression in tissue adjacent to hepatocellular carcinoma (HCC) into the highest 20 percentile (red), lowest 20 percentile (blue) and intermediate (grey). GSEA of these 82 adjacent liver biopsies (cohort A)[17] revealed (C) a significant enrichment (NES=1.75, false discovery rate (FDR)=0.001) of the Hallmark_IL6 STAT3 signalling gene set (STAT3 signature) in biopsies with 20 percentile lowest PTPRD expression (B, blue). (D) In the adjacent tissues described in (B), PTPRD expression is significantly (p=0.01, U-Test) impaired in the HCV-infected (HCV(+), grey circles, n=46) versus confirmed HCV-negative (HCV(−), empty circles, n=16) biopsies and (E) associated with a significant enrichment (NES=1.96, FDR=0.001) of the STAT3 signature. Gene expression patterns of the leading edge genes of the STAT3 signatures in liver tissues are provided as online supplementary figures S3 and S4.

infected and uninfected with HCC (see online supplementary table S1). In four out of six donors, PTPRD expression was downregulated or even completely absent in tumour lesions compared with paired adjacent tissue samples (figure 5A). To verify whether this downregulation was also specifically present in HCV-infected patients with HCC, we analysed PTPRD expression in paired tumour and adjacent liver tissue specimens.[17] In this data set of patients undergoing surgical resection of liver tumours, information for 46 patients with HCV-associated HCCs could be retrieved. In 30 out of 46 paired biopsies was PTPRD expression downregulated in tumour lesions compared with adjacent non-tumoural tissue (figure 5B). This 30/46 ratio echoes the western blot results of paired HCC biopsies (figure 5A), and the observed downregulation in tumour tissue was statistically significant (p=0.01, two-sided Wilcoxon signed-rank test) (figure 5B). Next, we took advantage of the long-term clinical follow-up information provided in the database. Strikingly, when looking at all patients with HCC (n=82), those ranking in the highest 20 percentile levels of PTPRD expression in adjacent tissue (figure 4B) had a higher long-term survival rate (p=0.048, log-rank test) (figure 5C) and had less chance of recurrent liver cancer (p=0.02, log-rank test) (figure 5D), irrespective of cancer aetiology. We confirmed the same trend of prognostic association in two additional independent cohorts with shorter patient follow-up (see online supplementary figures S5 and S6).

DISCUSSION

HCC is the second largest cause of death from cancer worldwide and poses an increasing burden on global health.[37] Chronic HCV infection is a main cause of HCC, and even though recent pharmacological breakthroughs can efficiently eradicate HCV infection, the risk of developing HCC in patients after sustained virological response remains elevated. Moreover, given the epidemiological history of the virus, HCV-associated

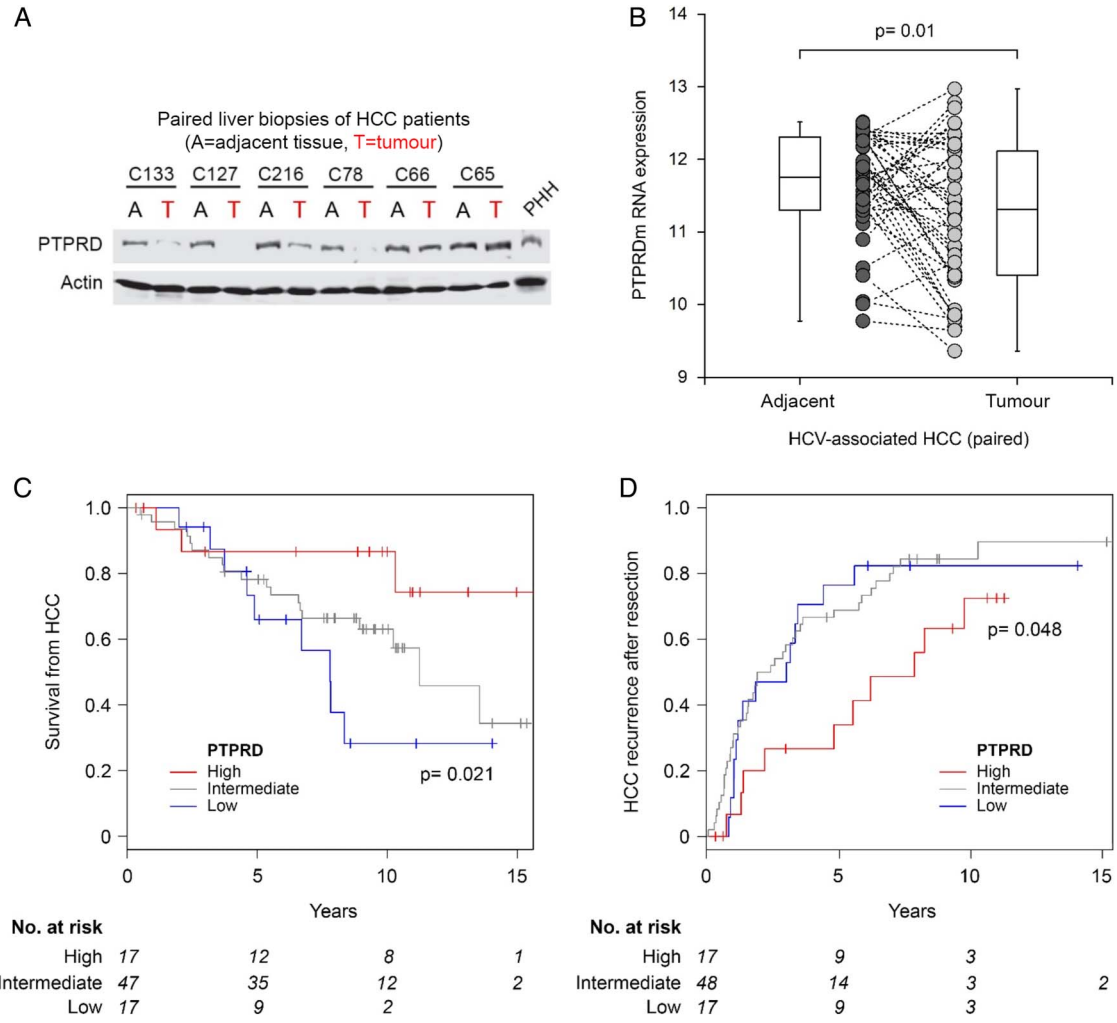

Figure 5 Dampened protein tyrosine phosphatase receptor delta (PTPRD) expression in liver biopsies is associated with a decreased patient survival and increased tumour recurrence after surgical resection. (A) PTPRD is impaired in tumour lesions of patients with hepatocellular carcinoma (HCC). Expression of PTPRD and actin was assessed in HCC lesions (*T*), and the corresponding paired adjacent tissue (*A*) of six patients with HCC by western blotting using specific antibodies. Patient codes are indicated. A random primary human hepatocytes (PHH) lysate served as positive control. (B) PTPRD is significantly (p=0.01, two-sided Wilcoxon signed-rank test) impaired in HCV-associated HCCs. The gene expression database of cohort A was analysed for PTPRD expression in paired liver biopsies from 46 patients infected with HCV.[17] Pairs of tumour lesions (dark grey) with the corresponding adjacent non-tumour tissue (light grey) are connected by a dashed line. Same data are summarised side by side as box-and-whisker plot (box=50% of biopsies, line=median, whiskers=minimal and maximal values) in the same panel. (C) Patients from cohort A with high PTPRD expression in adjacent non-tumour tissues (red) exhibit a significantly (p=0.021, log-rank test) higher survival rate from HCC and (D) a significantly (p=0.048, log-rank test) decreased tumour recurrence after surgical resection of the tumours. Product-limit estimation of PTPRD expression in adjacent non-tumour tissue of 82 patients compared with disease progression data from patients. Patient survival from HCC (C) and tumour recurrence after surgical resection (D) were compared between biopsies with highest PTPRD expression (figure 4B) (top 20 percentile; red) and biopsies exhibiting low PTPRD expression (lowest 20 percentile; blue) using Kaplan-Meier estimator. Number of patients at risk (No. at risk) is indicated.

complications are set to peak over the next decade, and novel preventive strategies are urgently needed. By studying the impact of HCV infection on protein phosphatase expression patterns in liver biopsies, we aimed to identify drivers for HCV-associated disease development. Here, we show that tumour suppressor PTPRD is consistently downregulated upon HCV infection, both in vivo, in chronically infected patient biopsies, and in vitro in infected PHH. PTPRD is a well-established tumour suppressor[27] whose chromosomal locus on 9p23–24.1 is regularly subject to genetic deletion or epigenetic inactivation in a broad spectrum of human malignancies including neuroblastoma, glioblastoma,[27] lung cancer, cutaneous squamous cell carcinoma,[38] laryngeal squamous cell carcinoma,[39] melanoma[40] and also HCC.[28] In addition, PTPRD copy number loss associates with a poor prognosis in breast cancer, colon cancer[41] and gastric adenocarcinoma.[42] Here, we demonstrate for the first time that PTPRD protein expression is downregulated in patients chronically infected with HCV. Moreover, we show that this downregulation is even more pronounced in tumour lesions of paired liver biopsies compared with adjacent non-tumour tissue of non-infected patients. Taken together, the evidence suggests that a gradual loss of PTPRD expression is a common event in liver disease progression, and it highlights the role of PTPRD as a potential suppressor of hepatocarcinogenesis, irrespective of aetiology.

In this study, we identified a HCV-specific regulatory mechanism that silences PTPRD expression in vivo. We demonstrate that PTPRD mRNA is targeted by miR-135a-5p, a miRNA that

is expressed in the liver (see online supplementary figure S7) and that was described to be markedly elevated in HCC lesions from patients with poor prognosis.[43 44] We demonstrate that miR-135a-5p expression is also significantly elevated in liver biopsies of patients with HCV (figure 3A, B). Interestingly, miR-135a has been recently suggested as a cofactor for HCV replication by interacting with the 5′UTR of the viral genome.[45] Our data demonstrate HCV-induced upregulation of miR-135a-5p expression is a cause of PTPRD silencing (figure 3D) and that high miR-135a-5p levels in liver tissues significantly ($p=0.04$, one-tailed Spearman's correlation test) correlate with low PTPRD levels and *vice versa*. The existence of a population of samples where both miR-135a-5p and PTPRD are weakly expressed (figure 3E) suggests the existence of additional regulatory mechanisms of PTPRD expression that may as well involve HCV-dependent or independent epigenetic modification of the PTPRD promoter or histones. These findings support the view that miR-135a-5p controls PTPRD in HCV-infected livers, potentially together with additional so far unidentified mechanisms for PTPRD mitigation.

Interestingly, the transcription factor STAT3 is a confirmed target for PTPRD and a key player during liver regeneration. Loss of PTPRD function leads to aberrant STAT3 phosphorylation in glioblastoma.[27 46] Here we demonstrate a significant association of PTPRD levels with STAT3 transcriptional activity in the livers of patients (figure 4). We have previously demonstrated that STAT3 is an indispensable host factor for HCV infection and that the viral infection is promoted by STAT3 activation.[10] Thus, it is conceivable that HCV downregulates PTPRD in order to benefit from a STAT3-driven transcriptional programme. Since STAT3 activity also plays a role in liver disease progression and HCC development,[34 35 36 47] this may contribute to the protumourigenic environment during chronic HCV infection. In other words, our model suggests the existence of a perturbed PTPRD–STAT3 axis driving malignant progression of liver disease. We believe that although not associated to other hallmarks of HCC development like inflammation and fibrosis (figure 1), a perturbed PTPRD–STAT3 axis adds additional oncogenic pressure to the liver as STAT3 is associated to HCCs with poor prognosis.[34 35 36] This finding may also be of further clinical relevance since it provides a target for HCC chemoprevention. Indeed, therapeutic intervention on signalling events constitutes a new chemopreventive strategy, as proof of concept has been demonstrated using the clinical epidermal growth factor receptor inhibitor erlotinib to attenuate liver fibrosis and the development of HCC in an animal model.[48] Given that PTPRD expression is suppressed by chronic HCV infection and associated with HCC and patient survival, our data suggest a PTPRD-centred signalling network as a potential target for novel chemopreventive strategies for HCV-induced HCC.

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Acknowledgements The authors thank Dr Sébastien Pfeffer (UPR 9002, IBMC, University of Strasbourg, France) for providing small RNA sequencing data of HCVcc-infected Huh7.5.1 cells, Dr Michel Tremblay (McGill University, Montreal, Canada), Dr Catherine Schuster (Inserm U1110, University of Strasbourg, France) and Luc Dupuis (Inserm U1118, University of Strasbourg, France) for helpful discussions; Sarah C. Durand (Inserm U1110, University of Strasbourg, France) for isolation of primary human hepatocytes and excellent technical assistance. Moreover, they thank Emilie Heuillard, Dr Eric Robinet (Inserm U1110, IHU, University Hospital Strasbourg) and the Biological Resource Centre (Centre de Ressources Biologiques (CRB) of the University Hospital Strasbourg, France) for managing the biobank; Dr Frank Chisari (The Scripps Research Institute, La Jolla, California, USA) for the Huh7.5.1 cells; Dr Takaji Wakita (National Institute of Infectious Diseases, Tokyo, Japan) for the HCV JFH1 strain; Charles M. Rice (The Rockefeller University, New York City, New York, USA) and François Helle (Université de Picardie Jules Verne, Amiens, France) for the RFP–NLS-IPS lentivirus. The small RNA-seq analysis of HCV-infected Huh7.5.1 cells was performed at the IGBMC Microarray and Sequencing Platform (Illkirch, France), member of the France Genomique programme.

Contributors JL initiated and supervised the study. JL and TFB obtained funding. NVR, AARS, FHTD, JL, CG, DC, NF and AA designed and conducted experiments and analysed data. TP, DS, VD, PP and MHH provided patient liver tissue and corresponding clinical data. AO, KC, MF, HN and YH provided gene expression data from patient cohorts and performed patient survival analysis. SB and MBZ analysed HCV-induced miRNA expression in Huh7.5.1 cells and designed miRNA reporter assays. TC, NP and NVR performed GSEA analysis. NVR and JL wrote the manuscript. MHH, YH, MBZ and TFB critically reviewed the manuscript.

Funding This work was supported by grants from the Wilhelm Sander-Stiftung (Förderantrag 2010.023.1 to TFB), the European Union (Interreg IV-Rhin Supérieur-FEDER-Hepato-Regio-Net to TFB and MBZ; ERC-AdG-2014 HEPCIR to TFB; EU H2020 HEPCAR to TFB), the French Cancer Agency (ARC IHU201301187 to TFB), the University of Strasbourg (IdEx, Projet Attractivité 2014, ANR, to JL) and from the French ANRS (ECTZ4236, ECTZ4446 to JL and AARS). NVR was funded by an IdEx fellowship from the University of Strasbourg (IdEx, Contrat Doctoral 2012, ANR, to MBZ and TFB). This work has been published under the framework of the LABEX ANR-10-LAB-28 and benefits from a funding from the state managed by the French National Research Agency as part of the investments for the future programme.

Competing interests None declared.

Ethics approval Ethics Committees of the University Hospital of Basel (Switzerland), the Centre Hospitalier Universitaire de Reims (France) and the Hôpitaux Universitaires de Strasbourg (France).

Provenance and peer review Not commissioned; externally peer reviewed.

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SUPPLEMENTARY INFORMATION

miR-135a-5p-mediated downregulation of protein-tyrosine phosphatase delta is a candidate driver of HCV-associated hepatocarcinogenesis

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Supplementary table S1: Human liver biopsies used in this study. NET= neuroendocrine tumor, gGT= gamma-glutamyl transferase, HCC= hepatocellular carcinoma, ASH= alcoholic steatohepatitis, FNH= focal nodular hyperplasia, PSC= primary sclerosing cholangitis, alcohol= alcohol abuse, AHT= arterial hypertension, SA= sleep apnea, COPD= chronic obstructive pulmonary disease, f= female, m= male, NA= not assessed.

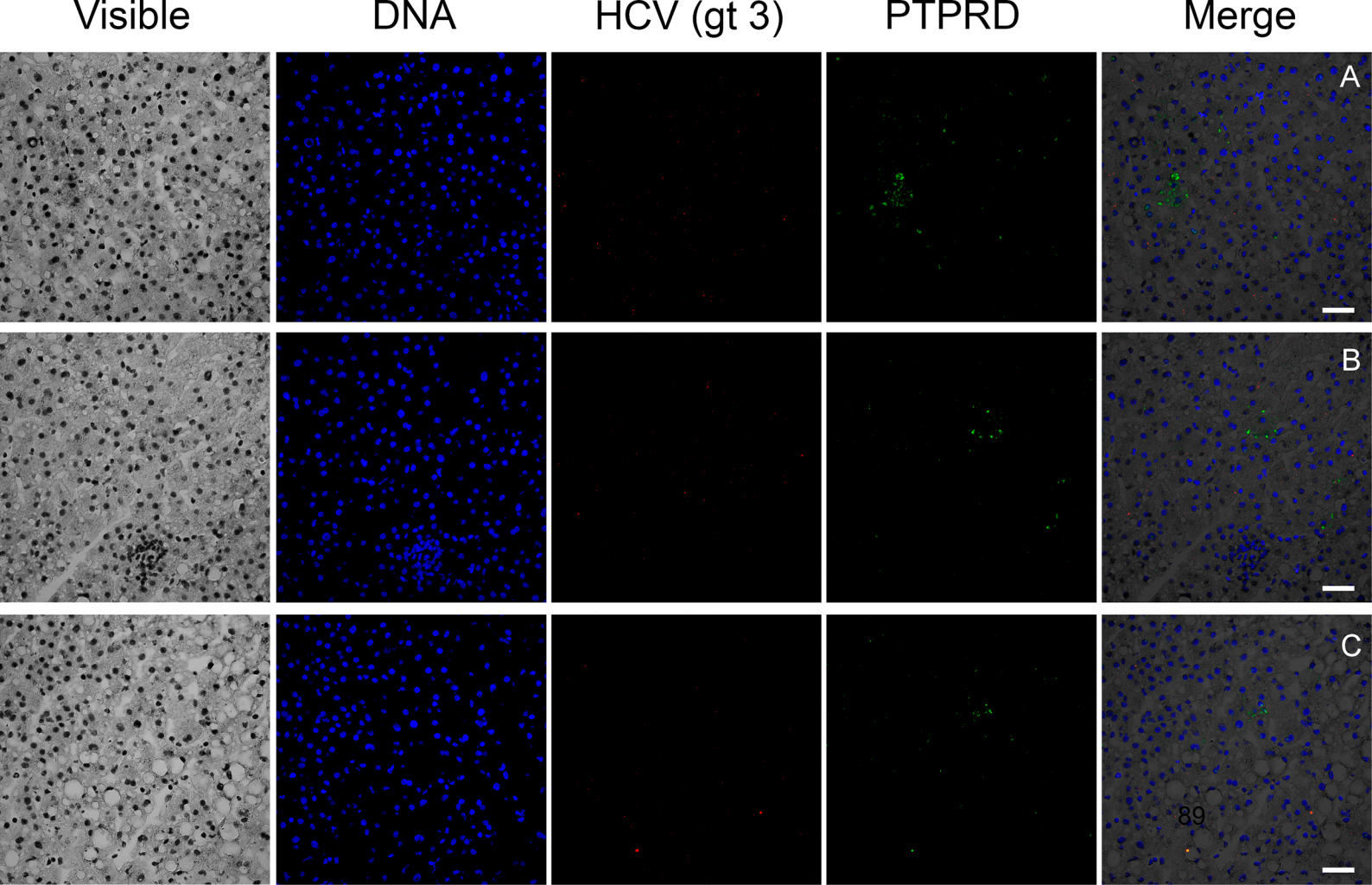
Figure	Code	Sex	Age	HCV	Viral load	METAVIR	Diagnosis
				Genotype	*IU/mL*		
1A	B318	f	78				NET, sample shows normal histology
1A	B724	m	66				Transaminase elevations
1A	B748	f	53				gGT and transaminase elevations
1A	B803a	m	69				Adenocarcinoma, sample shows normal histology
1A	B831	f	46				gGT elevations
1A	B895a	f	29				FNH, sample shows normal histology
1A,C-D	C32	m	59	1b	8.57E+05	A3/F2	HCV
1A,C-D	C12	m	53	4	3.31E+06	A2/F1	HCV
1A,C-D	C20	f	51	1a	NA	A1/F1	HCV
1A,C-D	C21	m	40	1a	NA	A1/F2	HCV
1A,C-D	C4	m	58	1b	3.21E+05	A3/F4	HCV
1A,C-D	C7	m	46	1a	4.33E+06	A3/F4	HCV
1B-D	C114	m	45	1a	1.17E+06	A1/F1	HCV
1B-D, 3	C121	m	65	2	5.89E+06	A2/F2	HCV

1B-D, 3	C124	f	48	3a	1.51E+06	A3/F4	HCV
1B-D, 3	C146	m	42	1a	NA	A2/F4	HCV
1B-D, 3	C149	m	34	3a	2.29E+04	A3/F4	HCV
1B-D, 3	C172	m	51	3a	3.47E+06	A2/F3	HCV
1B-D, 3	C221	m	34	3c	5.28E+05	A2/F4	HCV
1B-D, 3	C238	f	54	1b	NA	A1/F1	HCV
1B-D, 3	C239	m	52	NA	2.79E+05	A3/F4	HCV
1B-D, 3	C257	m	30	1a	1.08E+05	A1/F1	HCV
1B-D, 3	C263	f	62	3a	1.93E+05	A2/F1	HCV
1B-D, 3	C269	m	46	1a	1.62E+06	A2/F2	HCV
1B-D, 3	C270	f	76	1b	1.75E+06	A3/F3	HCV
1B-D, 3	C281	f	40	3a	NA	A1/F1	HCV
1B-D	C285	f	42	1a	2.80E+06	A1/F1	HCV
1B-D, 3	C291	m	53	3a	1.42E+05	A3/F2	HCV
1B-D, 3	C293	m	57	NA	NA	A3/F4	HCV
1B-D, 3	C300	m	55	3a	1.95E+06	A3/F4	HCV
1B-D, 3	C304	f	38	1a	1.55E+06	A2/F3	HCV
1B-D, 3	C44	f	41	1b	2.91E+05	A3/F4	HCV
1B-D, 3	C53	m	55	1b	2.17E+06	A2/F4	HCV
1B-D, 3	C58	m	54	4c/d	1.31E+06	A1/F1	HCV
1B-D, 3	C73	m	55	1b	2.62E+06	A3/F4	HCV
1B-D, 3	C89	m	29	3a	3.22E+04	A1/F1	HCV
1B-D, 3	C145	f	43				Normal
1B-D, 3	C187	f	31				Normal
1B-D, 3	C28A	f	51				Normal
1B-D, 3	C29	m	62				Normal
1B-D, 3	C305	m	46				Minimal unspecific hepatitis
1B-D, 3	C330	m	47				Minimal steatosis
1B-D, 3	C366A	f	56				Normal
1B-D, 3	C369	m	37				Normal
1B-D, 3	C442A	m	69				Minimal reactive hepatitis, 10 % steatosis
1B-D, 3	C445	m	33				Normal
1B-D, 3	C51A	f	55				Normal
1E	B229	m	37	3a	8.3E+05	A2/F3	HCV
1E	B512	m	44	3a	2.4E+04	A2/F2	HCV
1E	C37	f	51	3a	8.5E+06	A2/F1	HCV
5A	C126	m	81				HCC, ASH, cirrhosis
5A	C127	m	66				HCC, ASH, cirrhosis
5A	C133	m	67				HCC
5A	C65	m	77				HCC, ASH, cirrhosis
5A	C66	m	72				HCC, ASH, cirrhosis
5A	C78	f	57	3a	2.8E+04	A2/F4	HCV, HCC, cirrhosis
S6-7	14B04331	m	60			F3	Diabetes, alcohol
S6-7	14B04479	m	80			F3	Alcohol
S6-7	14B04674	m	65			F4	Diabetes, alcohol
S6-7	14B05087	m	63			F4	Diabetes, alcohol, HBV

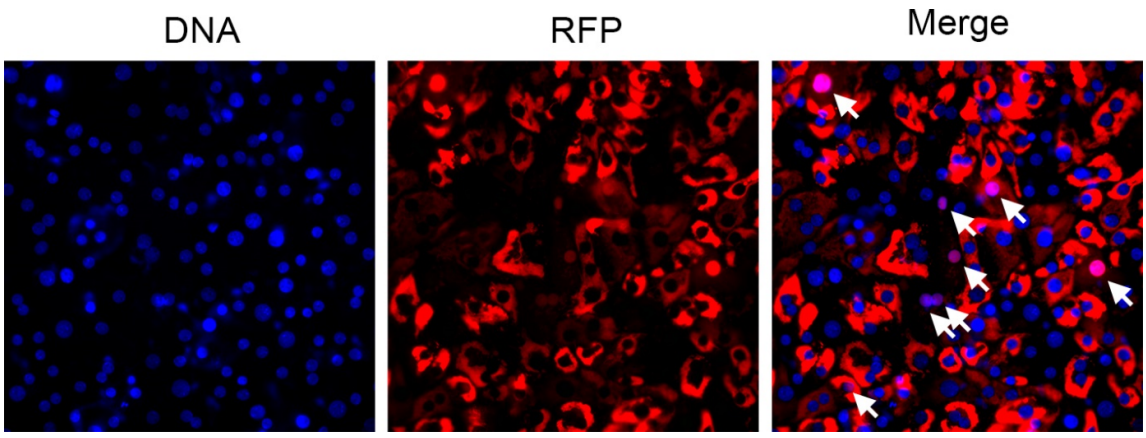
S6-7	14B05224	m	63			F4	Alcohol
S6-7	14B05345	m	72			F4	Alcohol
S6-7	14B05732	m	54	1b		F4	HCV, alcohol
S6-7	14B06994	m	66			F0	
S6-7	14B07954	m	74			F3	Diabetes
S6-7	14B08723	f	68			F2	
S6-7	15B00027	m	65			F4	HBV
S6-7	15B00395	m	64			F3/4	Alcohol, HBV
S6-7	15B00147	m	58			F4	Diabetes, alcohol
S6-7	15B00738	m	64			F3/4	Alcohol
S6-7	15B00971	m	81			F4	
S6-7	15B01490	m	54	1b		F4	HCV, diabetes
S6-7	15B01592	f	54	1b		F4	HCV
S6-7	15B02140	f	71			F0	
S6-7	15B02690	m	70	1b		F3	HCV
S6-7	15B03066	f	57			F4	Diabetes, alcohol
S6-7	15B04170	m	72			F4	Alcohol
S6-7	15B05646	m	75			F2	Diabetes
S6-7	15B05842	m	67			F4	Alcohol
S6-7	15B06370	m	66			F0	
S6-7	15B06514	m	65			F4	Alcohol
S6-7	16B00429	m	60			F0	Alcohol
S7	2065512	m	75				Alcohol, AHT, diabetes, dyslipidemia, obesity
S6-7	1369075	m	69				Alcohol, AHT, diabetes, dyslipidemia, obesity
S6-7	0020219	m	67				AHT, PSC
S6-7	1066280	m	70				AHT, arteriopathy
S6-7	1501765	m	80	1b		A2/F2	HCV
S6-7	2099524	m	71				Alcohol, obesity, AHT, dyslipidemia
S6-7	0099746	f	56	3a		A0/F2	HCV, obesity, AHT
S6-7	0644778	m	46	3a		A0/F2	HCV, AHT, alcohol
S6-7	1111249	m	57				Obesity, alcohol
S6-7	0320724	m	64				Obesity, diabetes, AHT
S6-7	1514711	m	57				AHT, diabetes
S6-7	1830303	m	50	3a		A2/F4	HCV, alcohol
S6-7	1164689	m	58	1a		A0/F2	HCV
S6-7	0211312	m	54	1a		A0/F4	HCV
S6-7	1450114	m	72				COPD, diabetes, AHT, SA, dyslipidemia
S6-7	1932776	f	65	1b		A2/F2	HCV, AHT, depression, vasculopathy
S6-7	0659735	f	70	1b		A1/F1	HCV, AHT, alcohol, vascular injury, COPD, osteoporosis
S6-7	0730036	m	60				Diabetes, AHT

Supplementary table S2: miRNA expression in Huh7.5.1 cells infected with HCVcc and in human liver biopsies from HCV infected patients. miRNAs that are upregulated by HCVcc (strain Jc1) in undifferentiated Huh7.5.1 cells and that are predicted to target the 3' untranslated region (3'UTR) of the PTPRD mRNA. miRNA targeting prediction was performed using the following tools incorporated in the miRSystem database [1]: a= DIANA, b= MIRANDA, c= MIRBRIDGE, d= PICTAR, e= PITA, f= TARGETSCAN. fc= fold change, p-values (U-Test, n=33) correspond to miRNA expression levels in liver biopsies.

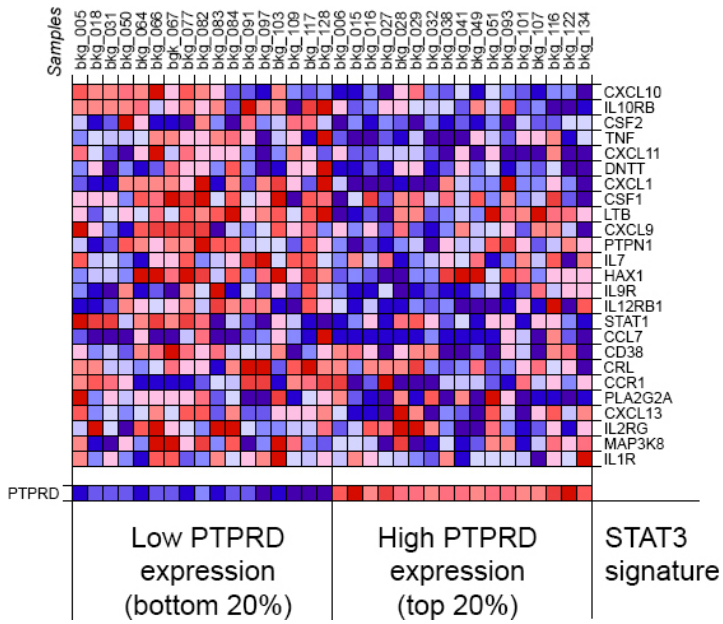
miRNA	ID *(MIMAT)*	Target sites on 3'UTR	Predicted by *(tools)*	Expression in HCVcc-infected Huh7.5.1 *(fc)*	Expression in HCV(+) liver biopsies *(median fc)*	U-test *(p)*
miR-16-5p	0000069	1	a,b,c,e,f	1.62	1.03	0.90
miR-24-3p	0000080	1	a,b,c,d,e,f	1.34	1.33	0.59
miR-26a-5p	0000082	1	a,b,c,e,f	2.77	1.03	0.64
miR-29a-3p	0000086	1	a,c,f	1.70	1.28	0.19
miR-29b-3p	0000100	1	a,c,f	1.49	1.13	0.94
miR-135a-5p	0000428	2	a,b,d,e,f	2.09	2.51	6E-04
miR-148a-3p	0000243	1	a,b,c,e,f	2.00	-1.34	0.04
miR-194-5p	0000460	1	a,b,c,e,f	6.39	-1.05	0.40


Visible
DNA
HCV (gt 3)
PTPRD
Merge
A
B
C
89

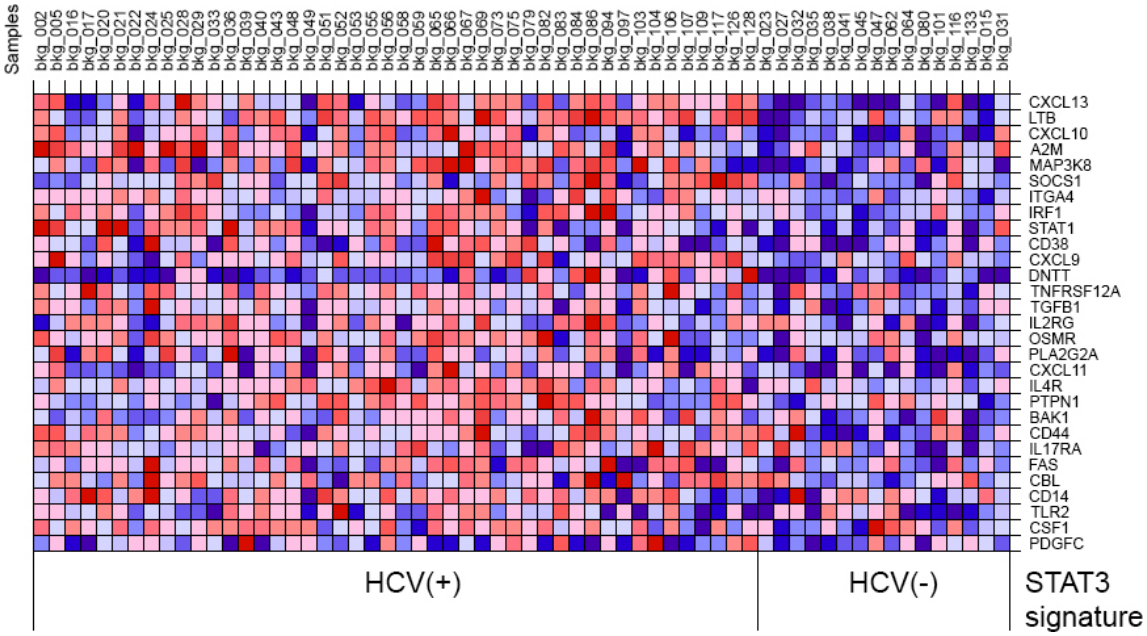
Supplementary figure S1: PTPRD expression is impaired in HCV-infected hepatocytes in liver biopsies. Representative images from FISH analysis of three different liver biopsies infected with HCV by simultaneous hybridization with HCV-specific and PTPRD-specific probe sets. (**A**) Liver biopsy B229, (**B**) liver biopsy B512, (**C**) liver biopsy C37; Grey= visible light channel, blue= genomic DNA, red= HCV RNA genotype 3, green= PTPRD RNA, scale bar= 100 µm.



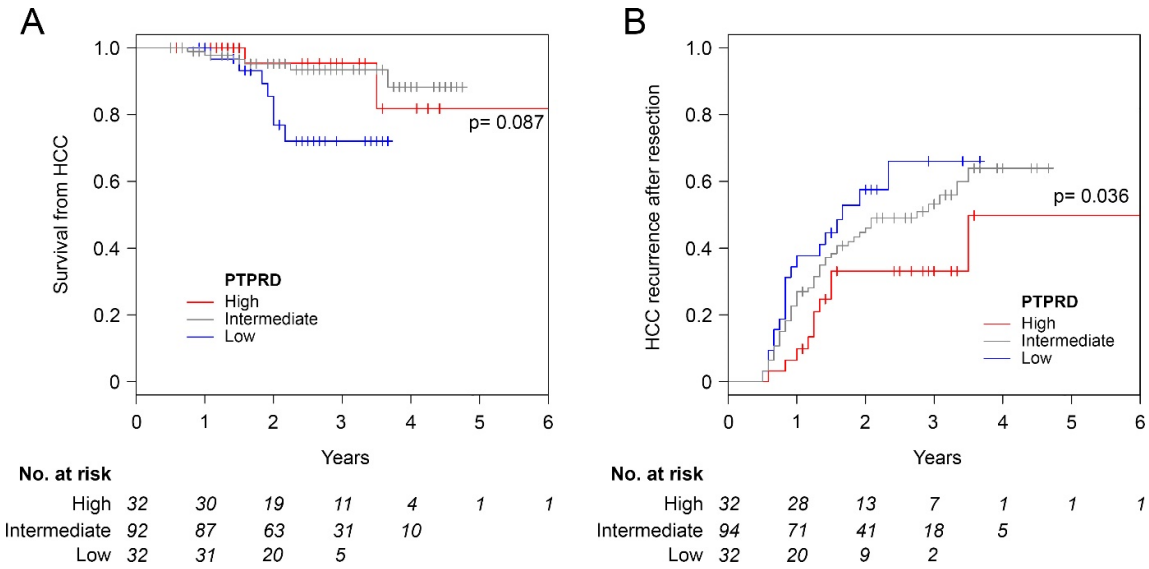
Supplementary figure S2: HCV infection of primary human hepatocytes *ex vivo*. Representative image of a RFP-NLS-IPS HCV infection reporter assay [2] from an independent experiment. Primary human hepatocytes were transduced with lentiviruses expressing RFP-NLS-IPS at day 1 post-seeding and then were inoculated with HCVcc (strain JFH1). HCV-infected cells were identified by translocation of the cleavage product RFP-NLS to the nucleus (pink nuclei indicated by arrows) 72 h post-infection. Image magnification 40x.



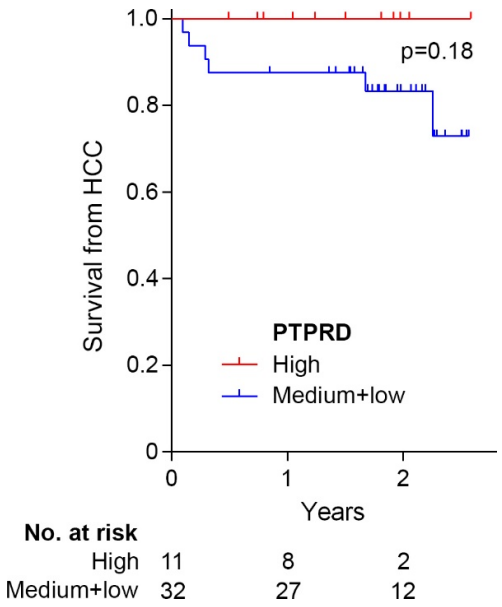
Supplementary figure S3: A gene expression signature responsive to STAT3 transcriptional activity is enhanced in liver biopsies with intermediate/low PTPRD expression. *In silico* analysis of mRNA expression in liver biopsies adjacent to HCC lesions [3]. Enrichment of the Hallmark_IL6 JAK STAT3 signaling transcriptional program (STAT3 signature) [4] clustered with the 20 % liver biopsies with lowest PTPRD mRNA expression. 25 of 78 leading edge genes of the STAT3 signature contributing to the enrichment score shown in Fig. 4C. High PTPRD expression corresponds to the top 20 % percentile of biopsies measured, low PTPRD expression corresponds to the 20 % percentile with lowest PTPRD expression of all assessed liver biopsies in Fig. 4B (Supplementary table S1). Red= high mRNA expression, blue= low mRNA expression.



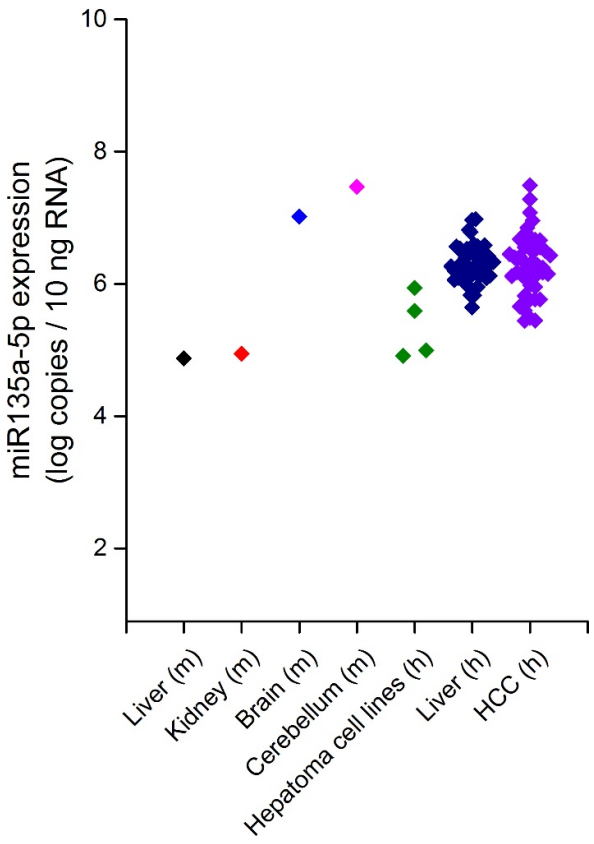
Supplementary figure S4: A gene expression signature responsive to STAT3 transcriptional activity is enhanced in liver biopsies of HCV-infected patients. *In silico* analysis of an mRNA expression database [3]. Enrichment of the STAT3 transcriptional program clusters in HCV-infected biopsies (HCV(+), n=46) vs. confirmed HCV negative biopsies (HCV(-), n=16). 29 of 78 leading edge genes from the Hallmark_IL6 JAK STAT3 signaling gene set (STAT3 signature) [4] were contributing to the enrichment score shown in Fig. 4E. Red= high mRNA expression, blue= low mRNA expression.



Supplementary figure S5: Low PTPRD expression in liver biopsies is associated with a decreased patient survival from HCC and elevated HCC recurrence after surgical resection in cohort B. Patients with low PTPRD expression in adjacent non-tumor tissues are associated with decreased survival from HCC (p=0.087, log-rank test) after a follow-up of 6 years. Cohort B comprises 158 patients with HCC from the Hiroshima University Hospital, Japan. Product-limit estimation of PTPRD expression in adjacent non-tumor tissue compared with disease progression data from patients. (**A**) Probability of overall survival after surgical resection according to PTPRD expression levels (**B**) Probability of HCC recurrence after surgical resection according to PTPRD expression levels. Data were analyzed using Kaplan-Meier estimator. Number of patients at risk (No. at risk) are indicated.



Supplementary figure S6: High PTPRD expression in liver biopsies is potentially associated with an elevated patient survival from HCC in cohort C. Patients with high PTPRD expression in adjacent non-tumor tissues exhibit a trend of increased survival from HCC (p=0.18, log-rank test) after only 2 ½ years of follow-up. Cohort C comprises 44 patients with HCC from the University Hospitals of Strasbourg and Reims, France. One patient was omitted from the analysis due to perioperative mortality. Product-limit estimation of PTPRD expression in adjacent non-tumor tissue of 43 patients compared with disease progression data from patients. Because of the short follow-up period patient survival from HCC was compared between biopsies with highest PTPRD expression (top 25 %) and biopsies exhibiting medium to low PTPRD expression (75 %) using Kaplan-Meier estimator (Software GraphPad Prism 6). Number of patients at risk (No. at risk) are indicated.



Supplementary figure S7: Tissue expression of miR-135a-5p. miR-135a-5p is expressed in liver tissue from both mouse and human. The sequences of miR-135a-5p in mouse (mmu-miR-135a-5p) and human (hsa-miR-135a-5p) are identical. miR-135a-5p expression was analyzed by RT-qPCR in mouse-derived tissues (liver, kidney, brain and cerebellum) and human liver specimens from cohort C (n=44) including HCC resections and adjacent liver tissues (Supplementary table S1, Fig. S6). Dilutions of synthetic miR-135a-5p mimic RNA served as standard for the miR-135a-5p quantification.

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2.2.- Combined multi-omics analysis for the characterization of HCV infection

2.2.1.- Aims and summary

Our previous results pointed towards an important role of HCV in signaling-mediated proteogenomic changes and its associated alterations. Therefore, we performed a combined multi-omics analysis of the transcriptome, proteome and metabolome, which allowed us to have a complete overview of the interactions between HCV and the infected hepatocyte.

As a starting point, our team characterized the molecular alterations induced by HCV in a cell-culture model. DMSO-differentiation of HCV-permissive Huh7.5.1 cells renders proliferating hepatoma cells into quiescent hepatocyte-like cells during a 7-day differentiation phase (Bauhofer, Ruggieri et al. 2012). This model overcomes the different growth rates of mock- and HCV-infected cells, allowing an accurate temporal characterization of longer infection periods. In order to ensure the reliability of our model, I performed a series of validation experiments. My immunofluoresce results showed that using a multiplicity of infection (MOI) of 8 was >10x the minimal required to infect 100% of the cells at the later timepoints of the experiment.

Following GSEA of the transcriptomics and proteomics from our cell-culture model, a detail that caught our attention was the downregulation of multiple pathways associated to peroxisomal function and lipid metabolism. I contributed to the validation of this observation by the quantification of the top leading-edge genes from the peroxisome gene set that were downregulated following HCV infection. In the same line, I demonstrated an HCV-induced inhibition of PPAR-α expression, which is a central regulator of peroxisomal function, and the downregulation of its target gene catalase (CAT). Moreover, I performed GSEA on proteomic data obtained from HCV-infected chimeric mice. My results validated the HCV-induced impairment of peroxisome pathways in this second experimental model. These findings were further strengthened by the analysis of human liver samples where similar results were obtained.

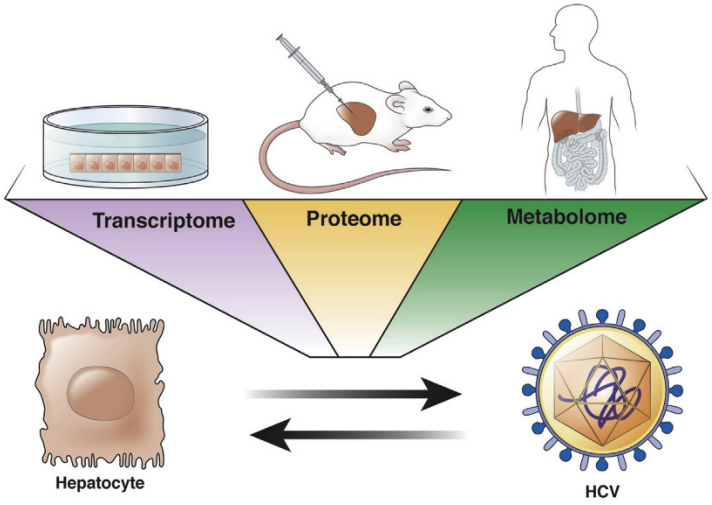
In order to identify the mechanism implicated in the impairment of peroxisomal function we analyzed the transcriptomic data from our HCV time course experiment using the AMARETTO algorithm (Champion, Brennan et al. 2018). This bioinformatic tool connects known regulatory driver genes with their co-expressed targets. This analysis highlighted the interleukin 6 receptor (IL-6R) as a potential regulator of

signaling pathways associated with peroxisomal function. Since the action of IL-6 pathway is mediated by STAT3, we hypothesized that its inhibition could restore the impaired peroxisomal function observed during HCV infection. Therefore, I conducted experiments employing the FDA-approved STAT3-inhibitor niclosamide. My results demonstrated that niclosamide treatment in the context of HCV infection rescued the virus-induced inhibition of peroxisomal genes, suggesting a regulatory link between STAT3 activity and peroxisomes.

In order to evaluate the potential clinical relevance of our findings, we analyzed the gene expression in liver biopsies from patients with HCV-related early-stage liver cirrhosis and in paired liver biopsy specimens from patients with HCV-associated HCC. By comparing the clinical data from these patients to the enrichment of a peroxisomal gene set, we showed a significant association of impaired peroxisomal gene expression with liver cirrhosis, HCC development and patient survival.

In summary, our multi-omics analysis of HCV infection allowed an in-depth characterization of the virus-induced signaling perturbations driving disease progression. Moreover, my data highlighted potential new strategies for the management of liver disease such as the use of clinical STAT3 inhibitors.

2.2.2. – Results article II



* Image modified from Gal-Tanamy 2019

Lupberger, J.; Croonenborghs, T.; **Roca Suarez, A. A.**; 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 HCV-infected Cells and Liver to Identify Pathways Associated With Disease Development, *Gastroenterology* **2019**. doi: 10.1053/j.gastro.2019.04.003.

Gastroenterology 2019;157:537–551

BASIC AND TRANSLATIONAL—LIVER

Combined Analysis of Metabolomes, Proteomes, and Transcriptomes of Hepatitis C Virus–Infected Cells and Liver to Identify Pathways Associated With Disease Development

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See Covering the Cover synopsis on 271. See editorial on page 300.

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^{dif} (hepatocyte-like cells) were infected with culture-derived HCV and used in RNA sequencing, proteomic, metabolomic, and integrative genomic analyses. uPA/SCID (urokinase-type plasminogen activator/severe combined immunodeficiency) mice were injected with serum from HCV-infected patients; 8 weeks later, liver tissues were collected and analyzed by RNA sequencing 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 biopsy specimens 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 messenger RNAs (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 the 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 β-oxidation of very long-chain fatty acids; we found intracellular accumulation of very long-chain fatty acids 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 the

mRNAs and proteins associated with peroxisome function, indicating perturbation of peroxisomes. We found that defects in peroxisome function were associated 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 the expression levels 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.

Keywords: HCC; signal transduction; metabolic disease; immune regulation.

WHAT YOU NEED TO KNOW

BACKGROUND AND CONTEXT

Hepatitis C virus (HCV) infection is a major cause of liver disease including steatosis, fibrosis and liver cancer. However, the mechanisms promoting liver disease progression are only poorly understood.

NEW FINDINGS

HCV infection accumulates very long-chain fatty acids due to virus-induced STAT3 signaling, accelerates glucose metabolism, and perturbs peroxisomal function, which correlates with steatohepatitis and poor prognosis in patients.

LIMITATIONS

Neither HCV-infected hepatocyte-like cells nor chimeric mice possess a functional immune system, which contributes to liver disease progression.

IMPACT

This proteogenomic and metabolic atlas is useful to identify drivers of liver disease-relevant pathways as potential therapeutic targets. HCV is a suitable model to study viral and non-viral liver disease.

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 coexistence with the host through either chronic or latent infection. Viral and cellular gene expression levels 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 not only increases 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 its refined strategies to evade antiviral responses and the alteration of metabolic pathways and regulatory cell circuits, including signaling pathways, translation machinery, and RNA interference.[2–5] This may have a 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 showed similar genetic profiles between HCV-associated HCCs and HCCs of other causes.[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 available for only 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 shown 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 on 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 and cancer.

* Author share co-first authorship; § Authors share co-senior authorship.

Abbreviations used in this paper: **DAPI, 4′,6-diamidino-2-phenylindole; DMSO, dimethyl sulfoxide; GSEA, gene set enrichment analysis; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HCVcc, cell culture–derived hepatitis C virus; IL, interleukin; MOI, multiplicity of infection; mRNA, messenger RNA; NASH, nonalcoholic steatohepatitis; PPAR, peroxisomal proliferator-activated receptor; RNA-seq, RNA sequencing; STAT3, signal transducer and activator of transcription 3; VLCFA, very long-chain fatty acid.**

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0016-5085/$36.00
https://doi.org/10.1053/j.gastro.2019.04.003

Materials and Methods

Hepatitis C Virus Infection of Human Hepatocyte Chimeric Mice

Mouse uPA$^{+/+}$/SCID$^{+/+}$ (urokinase-type plasminogen activator/severe combined immunodeficiency) liver samples used, including ethical approval and informed consent details, have been described.[11] Mice with human albumin levels >10 mg/mL (~80%–90% human hepatocyte 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 5 control mice were killed at week 8. RNA-seq data on a subset of these mice (3 HCV-infected and 3 noninfected mice) have been described.[11] All liver samples were snap frozen and stored at −80°C before analysis.

Immunofluorescence Microscopy

Cells were fixed with 4% paraformaldehyde for 20 minutes before permeabilization (15 minutes) with 0.1% Triton X-100, 0.5% bovine serum albumin. Antibody incubation was performed as recommended. Slides were mounted with Fluoroshield (Sigma-Aldrich, St Louis, MO) including 4′,6-diamidino-2-phenylindole (DAPI). 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 Dulbecco's modified Eagle medium containing 1% dimethyl sulfoxide (DMSO)[15] for 10 days before infection. A total of 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 multiplicity of infection (MOI) of 8 using affinity-purified cell-culture derived HCV (HCVcc) (50% tissue culture infective dose, 6.7×10^5/mL) or mock-inoculated with FLAG-peptide elution buffer. After 6 hours of incubation, the inoculum was replaced by fresh medium supplemented with 1% DMSO. This MOI is >10 times higher than the minimal MOI (0.12) required to infect 100% of Huh7.5.1^{dif} cells after 7 days (Supplementary Figure 1*C*).

Proteome Analysis

Protein lysates were harvested (duplicates) at days 0, 3, 7 and 10 after infection for both HCV-infected and mock-infected Huh7.5.1^{dif} cells. Cells were lysed for 15 minutes at room temperature in 8.0 mol/L urea buffer containing 75 mmol/L NaCl, 50 mmol/L Tris pH 8, 1 mmol/L EDTA, aprotinin 2 μg/mL (Sigma-Aldrich), leupeptin 10 μg/mL (Roche, Basel, Switzerland), phenylmethylsulfonyl fluoride 1 mmol/L (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 minutes, 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) and the tissue lysates were analyzed at the Max Delbrück Center for Molecular Medicine/Berlin Institute of Health (Berlin, Germany) using 10-plex tandem mass tag 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, Hilden, Germany) supplemented with 1% 2-mercaptoethanol. Cellular messenger RNA (mRNA) was isolated and analyzed as described.[17] RNA-seq was performed at the Broad Institute of MIT and Harvard (Cambridge, MA). 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, version 2.0.14. HCV RNA sequencing paired-end reads were aligned to the HCV Jc1E2^{FLAG} genome using Bowtie2, version 2.2.5, software. Gene expression levels for 21,950 human genes were estimated using Cufflinks, version 2.2.1, 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- and extracellular metabolites were analyzed by mass spectrometry as described[20] by the Barteesy lab at the Massachusetts General Hospital (Boston, MA). Methyl derivatives of very long-chain fatty acids (VLCFAs) were extracted in the presence of internal standards by the Ricci and Dali-Youcef laboratories and analyzed by gas chromatography-mass spectrometry at the Hôpitaux Universitaires de Strasbourg (France) as described.[21]

Bioinformatics Analyses

Proteins and transcripts were mapped despite possible many-to-many relationships (ie, isoforms) by constructing analysis groups.[22] Host responses to HCV infection were assessed over a time course on a temporal population level. Preranked 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 2 different sources, an indirect comparison was applied. Each time point for both sources was 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 ($P < .005$) for the transcriptional and proteomic data sets in this metaspace. The following gene sets from the Molecular Signatures Database,[25] version 4.0, were included for the analysis: BIOCARTA, BIOPLEX, KEGG, NABA, PID, REACTOME, SA, SIG, ST, CORUM, HCVpro, HALLMARK, E1, E2, E3, and DUB. Leading-edge genes were extracted from all previous GSEA analyses, followed by overlap analyses

using the hypergeometric test. The data set was compared with HCVpro database signatures identifying significant overlaps with the current knowledge in HCV–host interactions. Raw reads of patients' samples with low interferon-stimulated genes (ISG)[26] were trimmed with cutadapt[27] and mapped to the human genome hg19 with HISAT2 (Johns Hopkins University, Baltimore, MD). Reads mapping to GENCODE, version 19, genes were counted with htseq-count. Differentially expressed genes were analyzed with 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 exact test] and *z*-score) of the protein–protein interaction database and those profiled by DNA sequencing after chromatin immunoprecipitation in mammalian cells. These were then successively compared with 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 Shows the Spatiotemporal Map of Hepatitis C Virus–Hepatocyte Interactions During Infection

We used a 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 to primary human hepatocytes in cell culture.[15,29] Indeed, Huh7.5.1^{dif} are quiescent and display enhanced hepatocyte-specific marker expression compared with undifferentiated Huh7.5.1 cells (Supplementary Figure 1*A* and *B*). Huh7.5.1^{dif} were infected with HCVcc (Jc1E2^{FLAG}) prior sampling during a 10-day culture period (Figure 1*A*). HCV protein expression was visualized by an HCV peptide time course analysis (Figure 1*B*) and 59 peptides from 8 viral proteins were quantified (Figure 1*C*). During infection, all HCV peptides increased in abundance until day 7 after infection (Figure 1*C*) simultaneously ($P < .0001$, chi-squared test), with indifferent expression levels or kinetics ($P > .05$, *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 8297 proteins providing a multidimensional atlas of persistent HCV infection. The atlas shows a time-resolved proteogenomic state of HCV-infected cells. Overall, 7,416 proteins (90.8%) were uniquely mapped to overlapping mRNAs by using integrative functional genomic analyses (Figure 2*A* and Supplementary Table 1). The replicates (n = 2) showed excellent sensitivity and technical quality of the approach, superior to recent studies for other viral infections[22] (Supplementary Figure 2).

Disparate Dynamics Between Host Cell messenger RNA and Proteins Upon Hepatitis C Virus Infection

We then analyzed 2006 predefined gene sets representing specific host pathways, cellular functions (The Molecular Signatures 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 predefined gene set associated with a phenotype. We found that 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 after infection (Figure 2*B*). Most of these gene sets were generally down-regulated (negatively enriched). In gene set collections with >50 gene sets (BIOCARTA, BIOPLEX, KEGG, PID, REACTOME, CORUM, and HALLMARK) a median of 62% and 79% of RNA and protein was negatively enriched, respectively. Moreover, 83% of these down-regulated gene sets on the RNA level were also significantly impaired on the protein level. In contrast, only 17% of the up-regulated pathways on the RNA level matched with the corresponding protein trends (Figure 2*C*). This suggests that HCV infection shuts down most of the nonvital processes on the transcriptional and/or at the posttranscriptional level to divert the resources toward viral replication and persistence. Up-regulated pathways include the antiviral response and inflammation, as well as proviral signaling pathways, as discussed in the supplementary information (Supplementary Figure 3 and Supplementary Table 2).

Persistent Hepatitis C Virus 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 (Figure 3*A*). Peroxisomes are involved in lipid synthesis, signaling, β-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 (Figure 3*A*). We confirmed these findings in a transcriptomic database comprising liver biopsy specimens from 25 patients with chronic HCV infection and 6 noninfected individuals[26] and in the livers of HCV-infected chimeric mice (n = 3) (Figure 3*A* and Supplementary Table 3). 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 the liver tissue of HCV-infected patients. The expression of 11 of these leading-edge genes changed significantly ($P < .05$, Wald test) in HCV-infected Huh7.5.1^{dif} cells and in the livers of HCV-infected patients (Figure 3*B* and Supplementary Figure 4). We further validated this finding by showing impaired catalase expression in infected cells (Figure 3*C* and

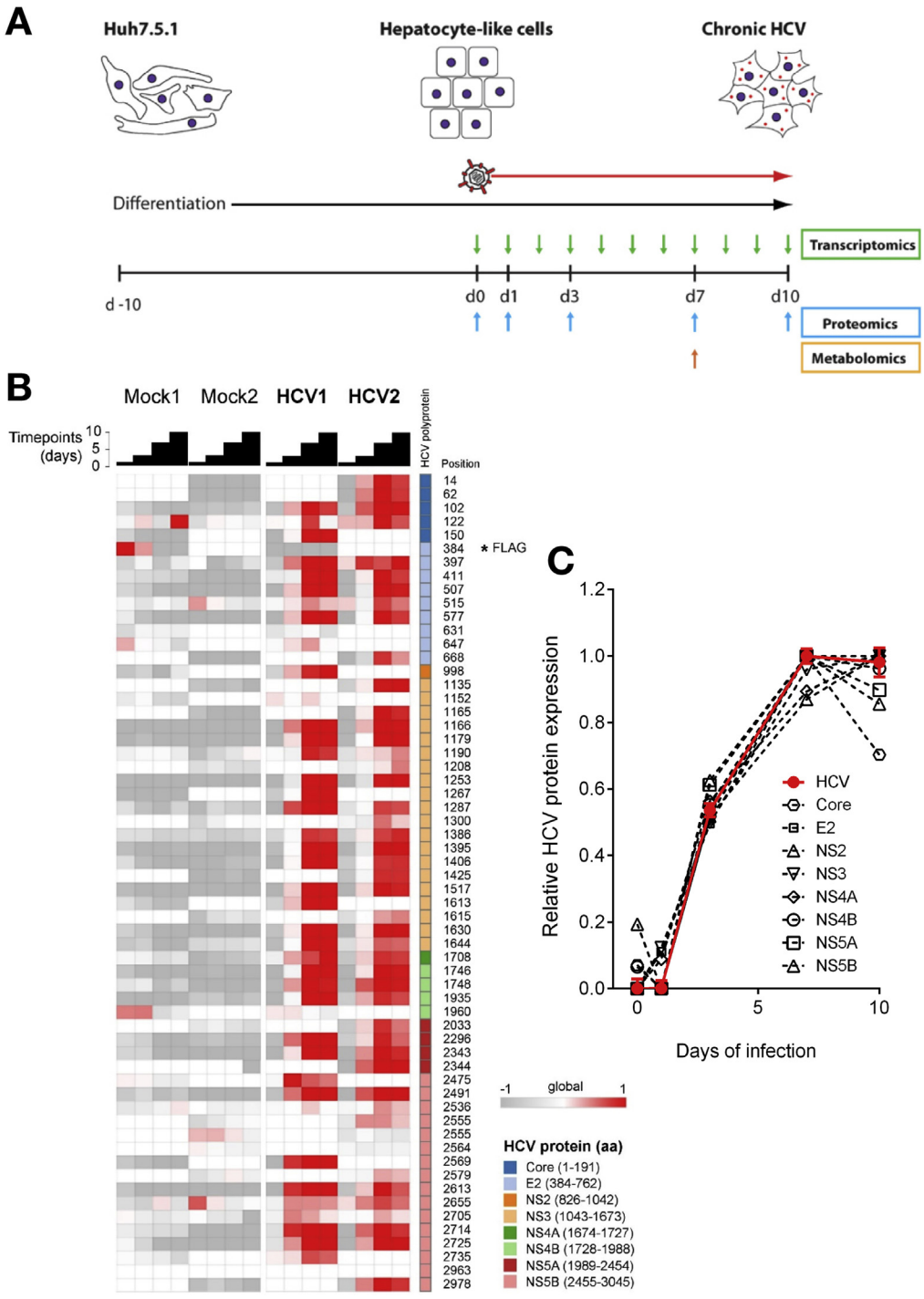


Figure 1. Mapping of HCV protein expression by time-resolved proteogenomics. (*A*) Hepatocyte-like Huh7.5.1^{dif} cells were infected with HCVcc (Jc1E2^{FLAG}) over 10 days. Sampling intervals for transcriptomics, metabolomics, and proteomics are indicated. (*B*) Quantification of HCV-specific peptides over the infection time course relative to noninfected and mock-infected (Mock) cells. The data represent tandem mass tag 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 an asterisk. (*C*) Increase of HCV protein abundance between 3 and 7 days after infection. Data are displayed as relative expression levels (mean) of all peptides corresponding to a given HCV protein over the time course. HCV replication is depicted in red as the median of total HCV peptide abundance ± standard error of the median.

Supplementary Figure 5), which is a peroxisome-specific enzyme. Consistently, quantification of catalase-stained peroxisomes showed a significantly ($P < .005$, t test) lower number of catalase puncta formed in HCV-infected cells compared with uninfected hepatocytes (Figure 3*C*), suggesting an impaired metabolic function of these organelles and an accumulation of fatty acids in infected cells. Whether fewer 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 show that HCV infection of Huh7.5.1^{dif} increases intracellular concentrations of VLCFAs with a chain length of 20–26 carbons, whereas shorter fatty acids (C16–C18) are less accumulated (Figure 4*A*). 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

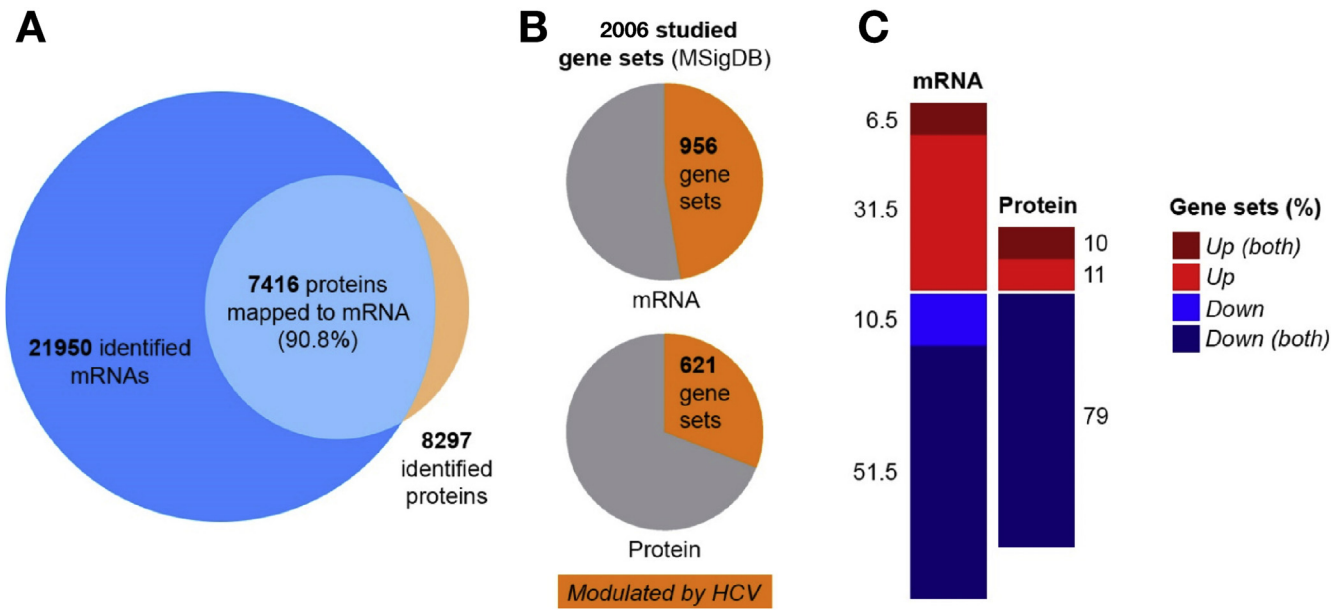


Figure 2. Persistent HCV infection manipulates host pathways and triggers an attenuated innate immune response. HCV-infected Huh7.5.1^{dif} cells relative to mock-infected cells until 7 days after infection. (*A*) Proteogenomic mapping of HCV infection. (*B*) Gene sets obtained from the MSigDB that are modulated by HCV infection on the RNA and protein level. (*C*) Stimulation (*red*) and suppression (*blue*) of host pathways (gene sets) by HCV infection. MSigDB, The Molecular Signatures Database.

pressure on infected cells. In contrast to HCV, persistent hepatitis B virus (HBV) infection increased peroxisomal function and its associated metabolic processes (Figure 3*D*), 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 Peroxisomal Proliferator-Activated Receptor Signaling Is a Regulator of Peroxisomal Function During Hepatitis C Virus 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-α,[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 the RNA and the protein levels (Figure 3*A*), suggesting a perturbed switch between fatty acid and glucose metabolism in HCV-infected hepatocytes. This is consistent with suppressed PPAR-α 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 showed elevated concentrations of the glucose metabolites succinate, pyruvate, 3-phosphoglycerate, and citrate in HCV-infected Huh7.5.1^{dif} cells (Figure 4 and Supplementary Table 4), consistent with the high glucose dependence of the HCV replication.[38] Indeed, our analyses showed increased glucose consumption (Figure 4*C*), an accumulation of lactate, and a decrease of malate, arguing for impaired gluconeogenesis in infected cells (Figure 4*B*). 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 Figure 6, HCV infection induces hypoxia, presumably contributing to lactate production. When measuring the hepatocellular lactate flux, a significant accumulation inside the cells was observed (Figure 4*D*). Collectively, these data show 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. To do this, 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 5) by using Enrichr.[28] Furthermore, we added to the 11 leading-edge genes described in Figure 3*B* 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 5). Among these HCV-sensitive regulators were the peroxisome proliferator-activated receptor-α (*PPARA*) and its functional partner retinoid X receptor β (*RXRB*). Moreover, a

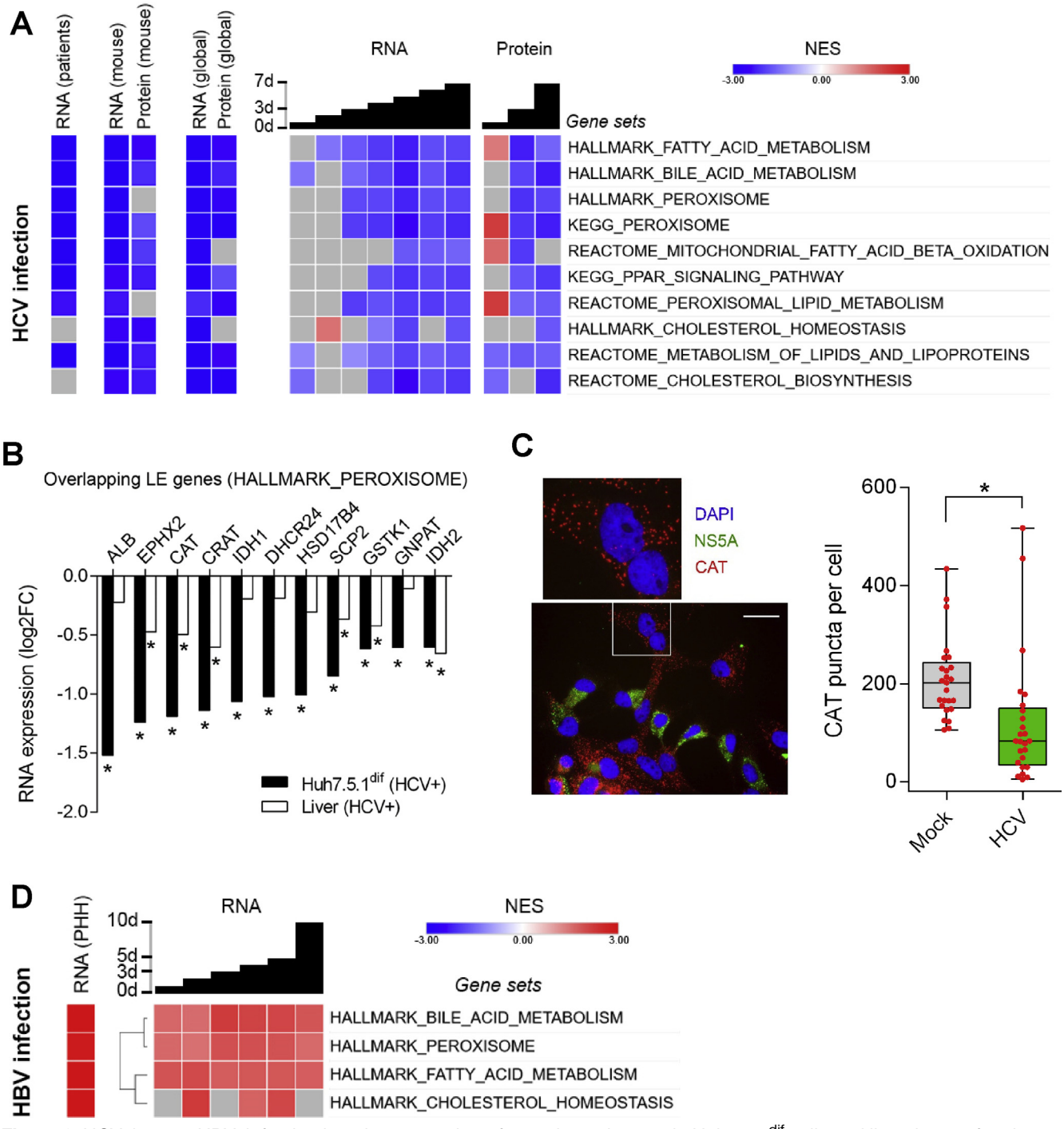


Figure 3. HCV, but not HBV, infection impairs expression of peroxisomal genes in Huh7.5.1^{dif} cells and liver tissue of patients. (*A*) HCV infection impairs metabolic pathways associated with peroxisomal function and lipid homeostasis. GSEA of RNA-seq data from liver tissue of 25 chronic HCV-infected patients vs 6 noninfected 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 ($P < .05$, Wald test) suppressed by HCV in Huh7.5.1^{dif} and in the liver tissue of patients with chronic HCV infection.[26] Log fold change of leading-edge gene expression of the HALLMARK_PEROXISOME gene set in *A*. (*C*) Peroxisome marker expression is significantly ($P = .0048$, *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 Figure 5). Quantification of catalase-stained peroxisomes in 25 random HCV-infected cells and 25 mock cells is shown in the boxes/whiskers; scale 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). NESs are displayed in red (increased), blue (decreased), and gray (no significant change). Temporal analysis of infected Huh7.5.1^{dif} are presented as global trend (global) and individual time points. The statistical cutoff for GSEA of liver tissues was a false discovery rate of $Q < .05$ and for Huh7.5.1^{dif} was $P < .005$. FC, fold change; NES, normalized enrichment score; * significant, as stated in the respective panel legends.

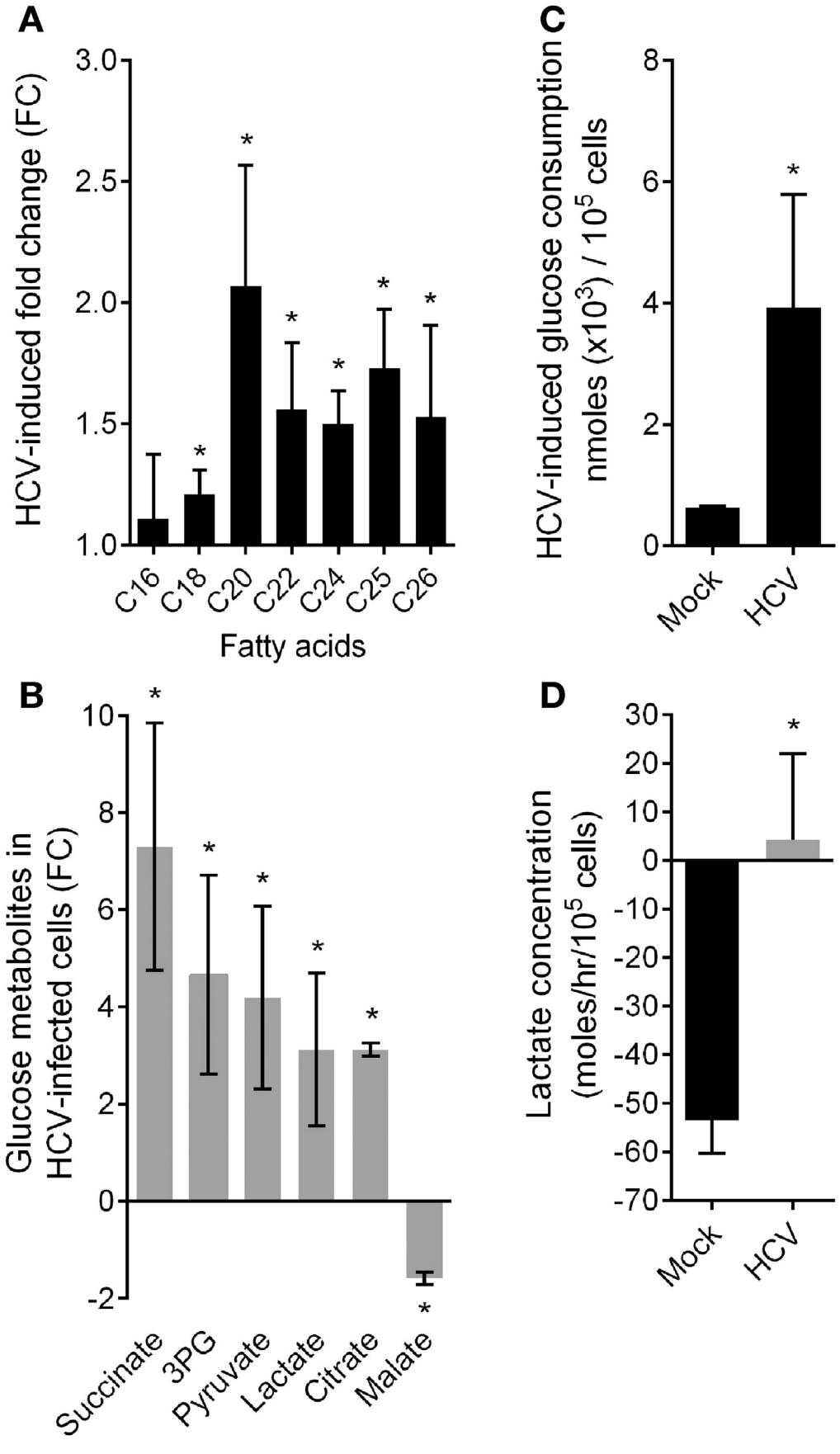


Figure 4. Persistent HCV infection enhances metabolism of intracellular VLCFAs and glucose and creates a Warburg-like shift of the lactate flux. (*A*) HCV infection induces intracellular accumulation of VLCFAs. Data are expressed as mean fold change of intracellular C16 (palmitic acid), C18 (oleic acid), C20 (arachidic acid), C22 (behenic acid), C24 (lignoceric acid), C25 (pentacosanoic acid), and C26 (cerotic acid) fatty acids relative to mock-infected cells ± standard error of the mean (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 ± standard deviation (4 independent experiments in triplicate). (*C*) HCV infection of Huh7.5.1^{dif} increases glucose consumption approximately 6-fold. Glucose consumption of HCV- and mock-infected (control) cells was measured at day 7 after infection in supernatants. Mean glucose consumption per 100,000 cells ± standard deviation (2 independent experiments in triplicate). (*D*) HCV creates a Warburg-like shift in infected cells. Lactate uptake (negative value) from culture supernatant inverts to lactate secretion (positive value) in infected Huh7.5.1^{dif} cells. Mean lactate flux ± standard error of the mean (mol/h/100,000 cells; 4 independent experiments in triplicate). *$P < .05$, *t* test.

comparison of the identified transcription factors with the HCVpro protein interaction database of HCV–host interactions,[40] showed that HCV core protein associates to PPAR-α and RXR-β. In line with these computational analyses, we observed that HCV infection strongly inhibits PPAR-α expression in Huh7.5.1^{dif} (Figure 5*A*). Finally, we analyzed the RNA-seq profiles of the HCV infection and noninfected control time courses using the AMARETTO algorithm.[41] This regulatory network inference tool learns regulatory modules by connecting known regulatory driver genes with the coexpressed target genes that they control. Interestingly, AMARETTO highlighted interleukin (IL) 6 receptor (IR-6R) as a driver candidate of a module comprising a significant (false discovery rate, <0.001) functional negative enrichment of pathways associated with peroxisome function (7 genes) and fatty acid (12 genes) and lipid metabolism (20 genes) (Supplementary Figure 7 and Supplementary Tables 6 and 7). This suggests a functional role for IL6/signal activator and transducer of transcription (STAT) 3 signaling in the regulation of peroxisome function in HCV infection. Indeed, infection induces a STAT3 transcriptional signature[5] (Supplementary Figure 6), and STAT3 activation causes a rapid inhibition of IL-6R receptor transcripts (Figure 5*B*) as part of a negative feedback regulation. Moreover, inhibition of STAT3 activity by niclosamide (Figure 5*C*) rescued the virus-induced inhibition of peroxisomal genes (Figure 5*D*), suggesting a regulatory link between STAT3 signaling and peroxisomes. Indeed, inflammatory pathways regulate PPAR family members.[42]

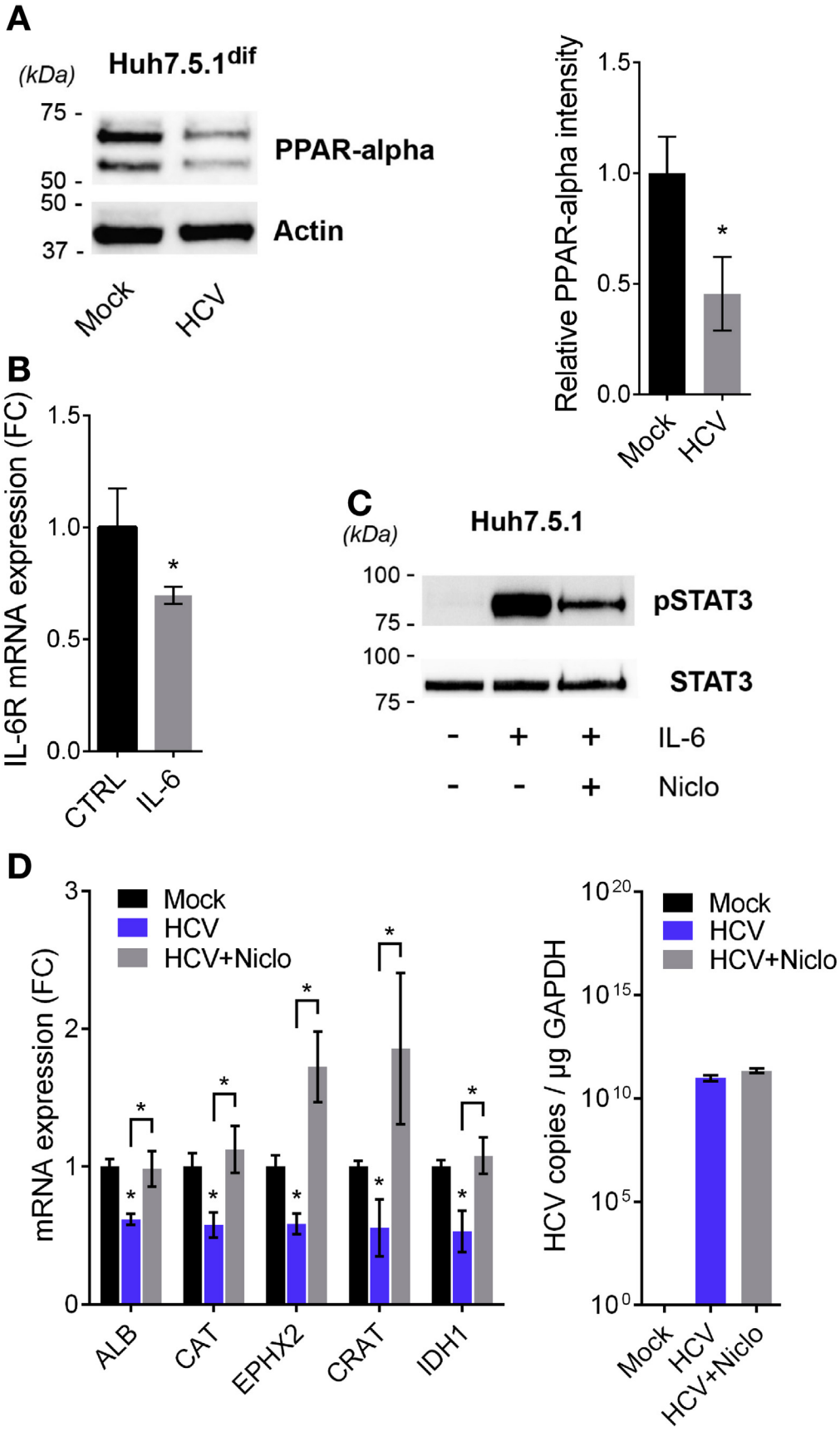
Taken together, our data suggest that HCV infection suppresses peroxisomal function at different levels by interfering with PPAR-α expression and function. This affects the metabolic switch between fatty acid and glucose metabolism and, thus, contributes to the glucose dependence and lipid accumulations in infected hepatocytes that are hallmarks of chronic liver disease and HCC development.

Impaired Peroxisomal Function Is Associated With Hepatitis C Virus Clinical Liver Disease, Including Cirrhosis and Hepatocellular Carcinoma

Because perturbed peroxisome function might be relevant for outcome of HCV-associated liver disease, we analyzed the gene expression in liver biopsy specimens from 216 patients with HCV-related early-stage liver cirrhosis[43] and in paired liver biopsy specimens from patients with HCV-associated HCC[44] (Supplementary Tables 8 and 9). Comparing the enrichment of the HALLMARK_PEROXISOME gene set in each individual sample with the clinical outcome of the patients, we showed a significant association of peroxisomal gene expression with liver cirrhosis (P = .001, log-rank test), HCC development (P = .03, log-rank test) and patient survival from HCC (P = .006, log-rank test) (Figure 6*A*). 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-α1 expression (*COL1A1*) (R = –0.16, P = .02, Spearman correlation test) and with α-smooth muscle actin (*ACTA2*) (R = –0.19, P = .004, Spearman correlation test) (Figure 6*B*), 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 Figure 6*A*. Interestingly, peroxisomal gene expression was reduced in tumor and adjacent tissue of HCC patients with steatohepatitis (Figure 6*C*), suggesting that an impaired peroxisome is not only associated with fatty liver development but also a hallmark of tumors reflecting their glucose dependence and the shutting down of β-oxidation.[45,46] We validated key findings in Figure 6 using *CAT* expression (Supplementary Figure 8), thus confirming the reliability of *CAT* as a 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 and inflammation to fibrosis, cirrhosis, and, ultimately, 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 on 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 double-stranded RNA sensors RIG-I, MDA5, and TLR3 and their downstream effector IRF3. This is consistent with reports showing that MDA5 is more critical for HCV sensing than RIG-I.[47,48] HCV replication intermediates are also partially recognized by the TLR3 protein but are not sufficient to mount a fully effective host response against HCV.[49] However, all of these antiviral gene expression patterns do not translate into protein in Huh7.5.1^{dif}. These findings support a model in which HCV infection overcomes the antiviral defense in hepatocytes mainly by inhibiting the translation of interferon-stimulated gene 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 after infection (Supplementary Table 1). Why this evasion strategy seems to be distinctly different in HCV-infected chimeric mice versus HCV-infected patients and Huh7.5.1^{dif} is an interesting observation that may be useful in refining our understanding of viral immune evasion. This shows that the Huh7.5.1^{dif} is a useful model for studying and understanding the innate immune responses to HCV infection and serves as an example of how this data


A
Huh7.5.1dif
(kDa)
75 -
50 -
50 -
37 -
PPAR-alpha
Actin
Mock
HCV
Relative PPAR-alpha intensity
1.5
1.0
0.5
0.0
*
Mock
HCV
B
IL-6R mRNA expression (FC)
1.5
1.0
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*
CTRL
IL-6
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Huh7.5.1
(kDa)
100 -
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100 -
75 -
pSTAT3
STAT3
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- - + Niclo
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mRNA expression (FC)
3
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Mock
HCV
HCV+Niclo
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ALB
CAT
EPHX2
CRAT
IDH1
HCV copies / µg GAPDH
10^20
10^15
10^10
10^5
10^0
Mock
HCV
HCV+Niclo

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 to a thus far unparalleled depth and comprehension (Supplementary Figure 6) and correlated it with metabolic features resembling cancer, that is, 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 the liver tissue of patients and HCV-infected human liver chimeric mice (Figure 3*A* and Supplementary Figures 3 and 6), further emphasizing the suitability of this atlas to identify previously unrecognized disease-relevant processes in vivo.

Peroxisomes are key organelles for VLCFA metabolism and the detoxification of membrane-permeable peroxides and oxidative stress. We observed a marked decrease of 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, in whom peroxisomal function is increased (Figure 3). Consistently, HCV, but rarely HBV, infection is associated with fat accumulations in the liver, leading to nonalcoholic fatty liver disease and nonalcoholic steatohepatitis (NASH).[51] Both HBV and HCV induce nuclear factor 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 showed that HCV causes a phenotype of VLCFA accumulation, which corresponds to impaired peroxisomal β-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 because 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, in whom 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 cause. Moreover, high levels of VLCFA in serum provoked hepatic steatosis, NASH, and HCC in an animal model.[58] Our data suggest that reactivation of HCV-impaired nuclear receptors like PPAR-α has a potential clinical relevance for HCC prevention. Furthermore, targeting of PPAR-α is in development for NASH, where treatment options are currently limited. Indeed, PPAR stimulation improves steatosis, inflammation, and fibrosis in preclinical models of nonalcoholic fatty liver disease,[59,60] although 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 the livers of HCV-infected chimeric mice 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 that showed 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 Table 7). The multi-omics approach in this study integrated transcriptome, proteome, and polar metabolites, and lipid analysis showed 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 the 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.

Figure 5. HCV inhibits peroxisomal gene expression by suppressing PPAR-α function via STAT3 signaling. (*A*) HCV infection of Huh7.5.1^{dif} significantly ($P < .05$, *U* test) inhibits PPAR-α expression. Western blotting for PPAR-α and actin after 7 days of infection with HCVcc (Jc1). Quantification of band intensities of PPAR-α and actin. Mean relative PPAR-α intensity ± standard deviation (2 independent experiments in triplicate). (*B*) Activation of the IL6/STAT3 pathway rapidly down-regulates IL6 receptor (IL6R). Huh7.5.1 cells were incubated for 6 hours with 10 ng/mL IL6 before RNA extraction and quantitative polymerase chain reaction analysis. Mean fold change ± standard error of the mean in triplicate. *$P < .05$ (*t* test). (*C*) Niclosamide inhibits IL6-induced STAT3 phosphorylation. Incubation of Huh7.5.1 cells with 2 μmol/L niclosamide for 24 hours and/or 10 ng IL6 (30 minutes). Western blotting for phosphorylated STAT3 (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 after infection, cells were treated with solvent control or 2 μmol/L niclosamide. Niclosamide reversed the HCV-induced inhibition of 5 top leading-edge genes of the HALLMARK_PEROXISOME gene set (Figure 3*B* and Supplementary Figure 4), but it had no effect on HCV replication. Mean fold change of copy number normalized to glyceraldehyde-3-phosphate dehydrogenase (phosphorylating) GAPDH from triplicate ± standard deviation. *$P < .05$ *t* test. CTRL, control; FC, fold change.

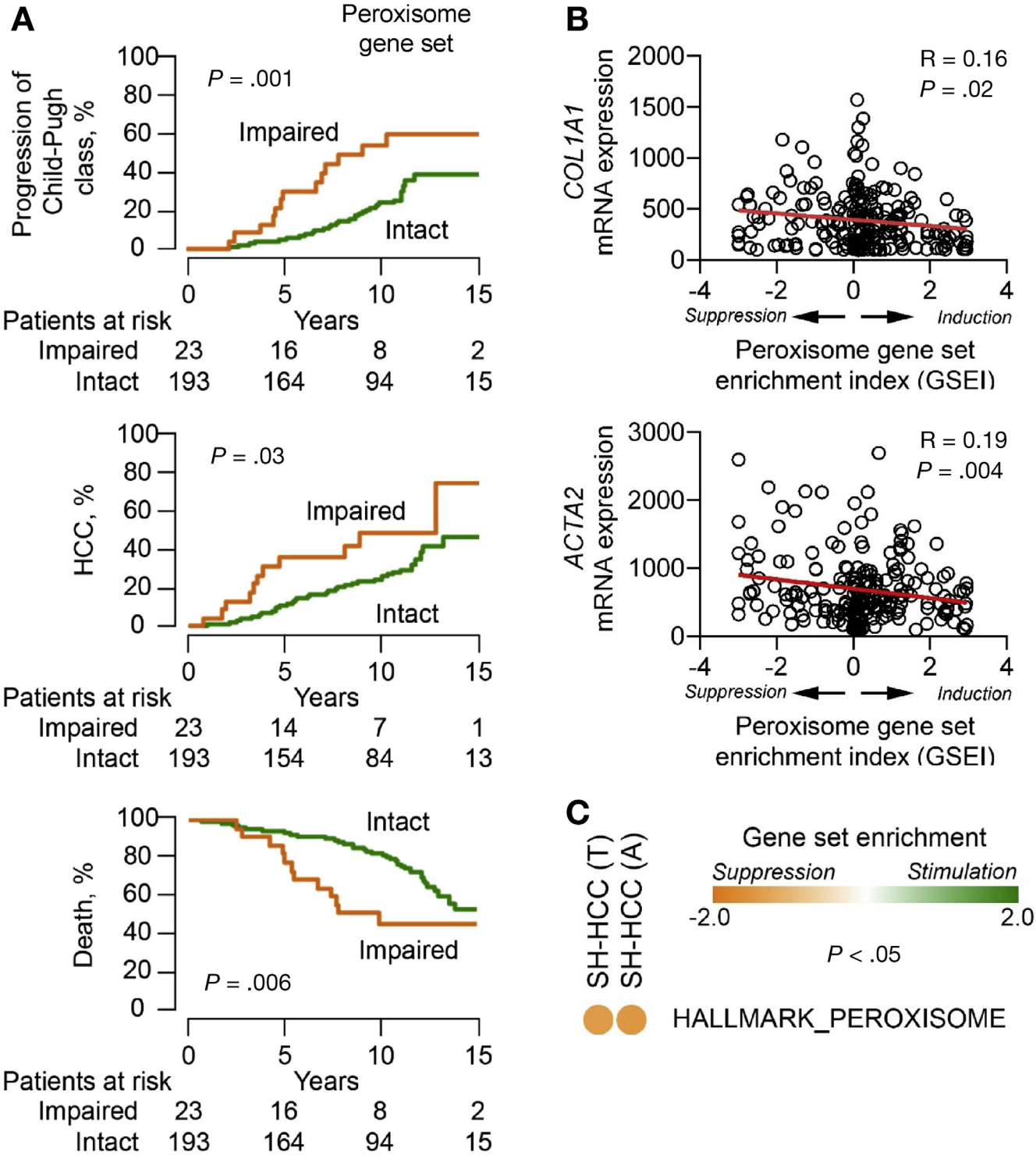

Figure 6. Significant association of hepatic peroxisome expression with clinical outcomes and phenotypes in viral and metabolic liver disease. (*A*) Patients with impaired peroxisomal function showed worse outcomes compared with those with intact function (log-rank test). Early-stage HCV cirrhosis patients (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, <.10).[6] (*B*) Expression levels of fibrosis-related genes, *COL1A1* and *ACTA2*, tend to be higher in livers with a suppressed peroxisome pathway (Spearman correlation test). Induction or suppression of the HALLMARK_PEROXISOME gene set were measured by the gene set enrichment index (GSEI).[6] GSEI was calculated from the gene set enrichment *P* value based on iterative random gene permutations (1000 times). GSEI of +3 indicates induction at enrichment *P* = .001, GSEI of –3 indicates suppression at enrichment *P* = .001, and GSEI of 0 indicates no modulation at enrichment *P* = 1.0. (*C*) The peroxisome is significantly (*P* < .05) suppressed in histologic steatohepatitis-associated HCC (SH-HCC) (n = 17) compared with other histologic types (n = 82) in tumor samples (NES = –1.38) and in tissue adjacent to tumors (NES = –1.43) of paired liver biopsy specimens.[44] GSEA using the HALLMARK_PEROXISOME gene set. NES, normalized enrichment score.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at https://doi.org/10.1053/j.gastro.2019.04.003.

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Received August 3, 2018. Accepted April 4, 2019.

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Acknowledgements
The authors thank Francis V. Chisari (The Scripps Research Institute) for providing Huh7.5.1 cells, Charles M. Rice (Rockefeller University) for providing JFH1-based sequences, and Arvind Patel (MRC Virology Unit, Glasgow) for monoclonal antibody AP33. We thank Jim Boccachio (Broad Institute) for performing RNA-seq analyses, and we thank Drs Aviv Regev and Orit Rozenblatt-Rosen for helpful discussions and all members of the AMARETTO/Community-AMARETTO algorithm and software development team, including Drs Vincent Carey (BWH, HMS, Broad), Jill Mesirov, Michael Reich, Ted Liefeld, Thorin Tabor (UCSD), Jayendra Ravindra Shinde, and Shaimaa Hesham Bakr (Stanford).

Author contributions: Joachim Lupberger, Nathalie Pochet, and Thomas F. Baumert designed experiments; Armando Andres Roca Suarez, Atish Mukherji, Alessia Virzi, Carole Jamey, Daniel Brumaru, Evelyn Ramberger, Eloi R. Verrier, Gergö Meszaros, Hussein El Saghire, Laura Heydmann, Marine A. Oudot, Mirjam B. Zeisel, Marko Jovanovic, Nassim Dali-Youcef, Naoto Fujiwara, Nourdine Hamdane, Nabeel M. Bardeesy, Nicolaas Van Renne, Philipp Mertins, Romeo Ricci, Steven A. Carr, Simonetta Bandiera, Sarah C. Durand, Shaunt Fereshetian, and Yujin Hoshida conducted experiments; Alessia Virzi, Celine Everaert, Frank Jühling, Naoto Fujuwara, Mohsen Nabian, Nathalie Pochet., Rileen Sinha, Tom Croonenborghs, and Yujin Hoshida performed computational analyses; Nathalie Pochet, and Olivier Gevaert, developed software; Joachim Lupberger, Nathalie Pochet, Nicolaas Van Renne, and Thomas F. Baumert wrote the manuscript; Ralf Bartenschlager and Kazuaki Chayama provided material and revised the manuscript; Thomas F. Baumert initiated the study.

Conflicts of interest
The authors disclose no conflicts.

Funding
This work was supported by the European Union (ERC-AdG-2014 HEPCIR to Thomas F. Baumert and Yujin Hoshida and EU H2020 HEPCAR 667273 to Thomas F. Baumert and Joachim Lupberger), the Agence nationale de recherche sur le sida et les hépatites virales (ECTZ4236 to Joachim Lupberger and ECTZ4446 to Armando Andres Roca Suarez), the French Cancer Agency (ARC IHU201301187 to Thomas F. Baumert), the US Department of Defense (W81XWH-16-1-0363 to Thomas F. Baumert and Yujin Hoshida), the National Institutes of Health (National Institute of Allergy and Infectious Diseases R03AI131066 to Nathalie Pochet and Thomas F. Baumert; National Cancer Institute 1R21CA209940 to Nathalie Pochet, Thomas F. Baumert, and Olivier Gevaert; National Institute of Allergy and Infectious Diseases 5U19AI123862-02 to Thomas F. Baumert; National Cancer Institute/Informatics Technology for Cancer Research (ITCR) U01 CA214846 to Nathalie Pochet and Olivier Gevaert), the Fondation de l'Université de Strasbourg (HEPKIN) (TBA-DON-0002) and the INSERM Plan Cancer 2019-2023 to Thomas F. Baumert. This work has benefitted from support by the Initiative of Excellence IDEX-Unistra (ANR-10-IDEX-0002-02 to Joachim Lupberger) and has been published under the framework of the LABEX ANR-10-LAB-28 (HEPSYS). INSERM Plan Cancer, IDEX, and LABEX are initiatives from the French program "Investments for the Future." The work of Ralf Bartenschlager was supported by the Deutsche Forschungsgemeinschaft (TRR179, TP9). Kazuaki Chayama was supported by the Research Program on Hepatitis from the Japanese Agency for Medical Research and Development (AMED) Japan (JP18fk0210020h0002). The work of Romeo Ricci and Gergö Meszaros was supported by a European Research Council (ERC) starting grant (ERC-2011-StG, 281271-STRESS METABOL); by the European Foundation for the Study of Diabetes (EFSD)/Lilly European Diabetes Research Program grant; and by the ANR-10-LABX-0030-INRT grant, a French State fund managed by the ANR under the frame program Investissements d'Avenir ANR-10-IDEX-0002-02.

Supplementary Materials and Methods

Reagents, Antibodies, and Quantitative Polymerase Chain Reaction Primers

All chemicals, the enhanced chemoluminescence reagent, Hyperfilms, and Fluoroshield with DAPI were purchased from Sigma-Aldrich. HBV surface antigen (HBsAg)–specific monoclonal antibody (mAb) (NCL-HBsAg-2, clone 1044/341) was obtained from Leica Biosystems (Buffalo Grove, IL); recombinant human interleukin–6 (IL6) from Gibco (Waltham, MA); anti-catalase rabbit monoclonal antibody (mAb) (D4P7B), anti-STAT3 (9132), and anti-phosphorylated STAT3 Y705 (D3A7) from Cell Signaling Technology (Danvers, MA); anti-PPARA (H2) from Santa Cruz (Dallas, TX); anti-NS5A mouse mAb (1827) from ViroStat (Westbrook, MD); anti-β actin (AC-15) from Sigma-Aldrich; and anti-rabbit IgG polyclonal antibody (AF647) and anti-mouse immunoglobulin G polyclonal antibody (AF488) were obtained from Thermo Fisher Scientific (Waltham, MA). Generally, 20 μg of protein was loaded for Western blotting. Complementary DNA was generated using the Maxima first strand complementary DNA synthesis kit (Thermo Fisher Scientific). Quantitative polymerase chain reaction (PCR) was performed using a CFX96 real-time PCR system (Bio-Rad, Hercules, CA) and iTaq Universal SYBR Green Supermix (Bio-Rad) as recommended. If not indicated otherwise, all quantitative PCR primers were synthetized by Sigma-Aldrich. HCV genomes were quantified with the SensiFAST Probe No-ROX One-Step Kit (Bioline, London, UK) using JFH1 probes from Sigma (A221 and S147). Primer sequences to quantify albumin (*ALB*), hepatocyte nuclear factor 4 α (*HNF4A*), and α-1-antitrypsin (*A1AT*) have been described[1]; CRAT (5′-GTA CCA CAG TGA CGG GAC AC-3′, 5′-CCG GTT CAC CTT GTC TTT GAT-3′), EPHX2 (5′-GAC ATC GGG GCT AAT CTG AAG-3′, 5′-GGC TTT ACT GTC ACG TAC CCA-3′), glyceraldehyde-3-phosphate dehydrogenase (phosphorylating) (GAPDH) (5′-TGC ACC ACC AAC TGC TTA-3′, 5′-GGA TGC AGG GAT GAT GTT C-3′), IDH1 (5′-TGT GGT AGA GAT GCA AGG AGA-3′, 5′-TTG GTG ACT TGG TCG TTG GTG-3′), and IL6-R (5′-CCC CTC AGC AAT GTT GTT TGT-3′, 5′-CTC CGG GAC TGC TAA CTG G-3′) were also used. Catalase quantitative PCR primer assay was obtained from Qiagen.

Virus Strains, Purification, and Titration

Cell culture-derived HCV (HCVcc) strains Jc1[2] and Jc1E2^{FLAG} [3] have been described. To generate a high-titer virus stock for proteomics experiments, culture medium containing FLAG-tagged HCVcc particles were collected daily starting 2 days after electroporation. In addition, 500 mL of cell culture supernatant were concentrated by ultracentrifugation (Vivaspin 20, molecular weight cutoff 100,000 Dalton; GE Healthcare, Little Chalfont, UK), purified using anti-FLAG M2 affinity gel (Sigma-Aldrich), and eluted with FLAG-peptide (Sigma-Aldrich). HCVcc infectivity was determined by calculating the 50% tissue culture infective dose as described.[4] MOI was derived from the 50% tissue culture infective dose as described by the American Type Culture Collection. HBV was produced by the cell line HepAD38 secreting infectious HBV (genotype D) into the culture medium. HBV enrichment and infection are described.[5]

Inference of Transcriptional Regulatory Networks

To infer the regulatory networks underlying the HCV-infected and noninfected Huh7.5.1^{dif} time courses, we analyzed the 21,950 annotated genes from the RNA-seq time course data (Gene Expression Omnibus (GEO) data set GSE126831) using the AMARETTO algorithm.[6–8] First, AMARETTO starts by selecting the top 50% most varying genes across the samples in an unsupervised manner, which resulted in 10,975 genes out of the 21,950 annotated reference genes to be included in the analysis. From a predefined list of 4906 candidate regulators (Supplementary Table 7/Input_regulators), 2720 candidate regulators were included in the analysis after the 50% variation filtering. The AMARETTO algorithm subsequently identified 722 regulators (Supplementary Table 7/Regulator_list_AMARETTO) of these candidate regulators as those putatively controlling the target genes in 150 modules of coexpressed target genes genome-wide using regularized regression. These modules were assessed for their enrichments in known functional categories from the Molecular Signatures Database (MSigDB) Hallmark and C2CP Collections.[9] The top scoring regulatory module 111 for enrichments in the HALLMARK_PEROXISOME signature genes is shown in Supplementary Figure 7. AMARETTO's source code in R is available from GitHub (https://github.com/gevaertlab/AMARETTO and https://github.com/broadinstitute/CommunityAMARETTO), and user-friendly analysis modules are available from GenePattern (https://cloud.genepattern.org/ analysis modules 00378 and 00380).

Proteogenomic Profiling

The complete RNA-seq time-course profiling of HCV-infected Huh7.5.1^{dif} including baseline expression levels are accessible in GEO (GSE126831). Transcriptomic profiling of HCV-infected chimeric mice can be retrieved from the Sequence Read Archive data set SRP170244 (biosamples: SAMN10465389, SAMN10465390, SAMN10465391, SAMN10465395, SAMN10465396, and SAMN10465397). Proteomic profiling of HCV-infected Huh7.5.1^{dif} and chimeric mice are accessible at MassIVE (MSV000083382). RNA-seq from infected vs control patients was obtained from GEO data set GSE84346 (low interferon-stimulated gene [ISG] samples).

Supplementary Results

HCV Induces a Hepatic Inflammatory Response in Quiescent Huh7.5.1 Cells

Chronic inflammation of infected tissue is an important hallmark of HCV-induced pathogenesis contributing to fibrosis, cirrhosis, and HCC. The innate antiviral response is triggered by double-stranded RNA sensors RIG-I and MDA5

recognizing the viral genome and activating an antiviral program mediated by interferons and expression of ISGs Only the cell line Huh7.5.1 and its progenitor Huh7.5 can be efficiently infected by HCV in cell culture. This is due, at least in part, to an impaired capacity to activate interferon response.[10,11] Despite these obeservations, in HCV-infected Huh7.5.1^{dif}, we observe a robust induction of gene sets involved in interferon alfa response and inflammation (Supplementary Figure 3) at the RNA level. Leading-edge genes involve the ISG *Mx1*, the expression of which is associated with HCV infection in vivo. Also, gene expression patterns of cytokines are strongly enriched in HCV-infected cells, including the ISGs *IFIT1*, *IFIT3*, and *IFIT5* as leading-edge genes witnessing at least partially functional virus sensing and induction of an antiviral response (Supplementary Table 2/all GSEA_anno [Huh7.5.1dif]). Consistently, we observe a significant enrichment of toll-like receptor signaling and RIG-I/MDA5 pathways that are involved in double-stranded RNA sensing. Leading-edge genes driving the enrichment include up-regulated RIG-I (*DDX58*), MDA5 (*IFIH1*), and *TLR3*, suggesting a partial rescue of the impaired RIG-I function by alternative sensors. A functional sensing of HCV in hepatocyte-like cells is also reflected by a strong *IRF3* gene expression on the RNA level (Supplementary Table 1), which, however, did not translate into protein. This is consistent with general strong and significant enrichment of the interferon response pathways observed at the RNA level that is not found for the corresponding proteins and a strongly impaired expression of the RNA translation machinery in HCV-infected cells (Supplementary Figure 3). Interestingly, although genes associated with translation are strongly impaired in the livers of HCV-infected patients and in Huh7.5.1^{dif}, the same gene set is positively enriched in the livers of the HCV-infected chimeric mice (Supplementary Figure 3 and Supplementary Table 3). This correlates with a more pronounced enrichment of interferon-response genes on the protein level in these chimeric livers. This further supports the conclusion that HCV infection overcomes the antiviral defense in hepatocytes, mainly by inhibiting the translation of ISG mRNAs.

HCV-Infected Hepatocytes Display a Proteogenomic Pattern Resembling Hallmarks for Cancer Development

Chronic HCV infection is a major risk factor of HCC, suggesting virus-induced alterations of cancer-relevant pathways. Host signaling is required not only for HCV infection,[12–14] but persistent signals are also transmitted by HCV infection that are involved in carcinogenesis.[15] Simultaneously, chronic HCV infection impairs cellular defense mechanisms of tumor development.[16] These data suggest a persistent mitogen-activated protein kinase and IL6/STAT3 signaling exerting a proliferative pressure onto the infected cell. Indeed, our proteogenomic atlas described here confirms this hypothesis and shows a profound temporal activation of mitogen-activated protein kinase and STAT3 transcriptional signature in infected hepatocytes (Supplementary Figure 6). At the same time, we observe an induction of hypoxia in infected cells (Supplementary Figure 6) that likely contributes to an amplification of HCV-induced epidermal growth factor receptor activation.[15] Moreover, overactivations of both pathways are hallmarks of carcinogenesis and are functionally linked in human cancers.[17] As expected, we also observed a significant induction of epithelial-to-mesenchymal transition in infected cells, as has been previously linked to epidermal growth factor receptor signaling in cancer[18] and HCV infection.[19] We found significantly decreased expression of components of the DNA repair machinery in infected cells (Supplementary Figure 6), suggesting an increased genetic instability. Taken together, our proteogenomic data outline the oncogenic pressure in infected hepatocytes. Like an emergency break, the infected cell seems to arrest the cell cycle to counteract virus-induced proliferative signals and increased oxidative stress. However, repair mechanisms maintaining the genetic stability of the infected cell are impaired at the same time, creating an imbalance that might promote carcinogenesis.

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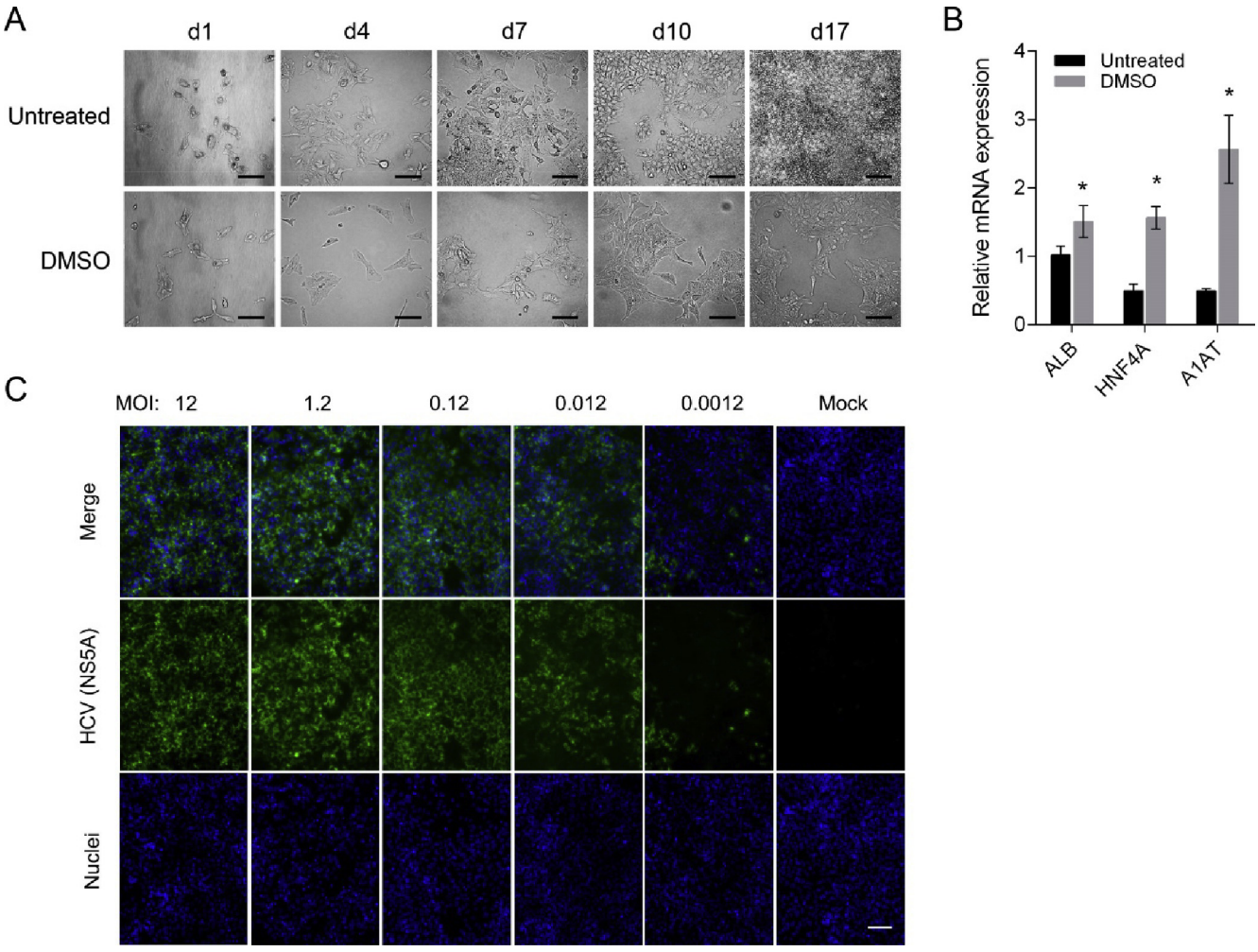
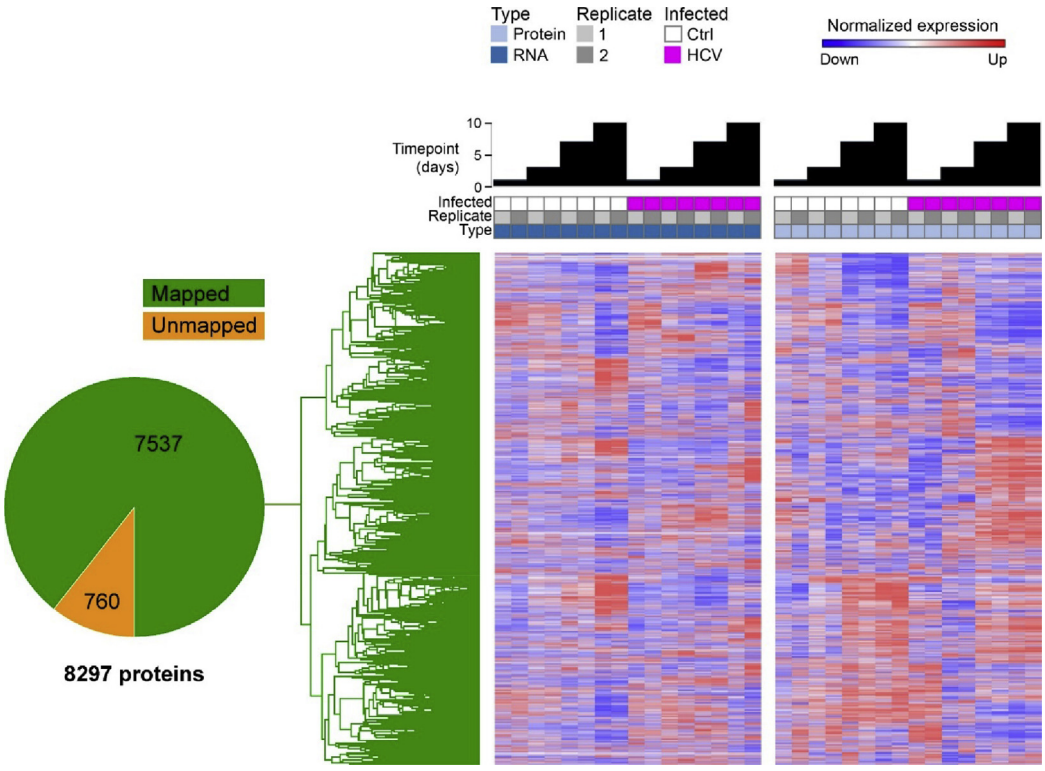
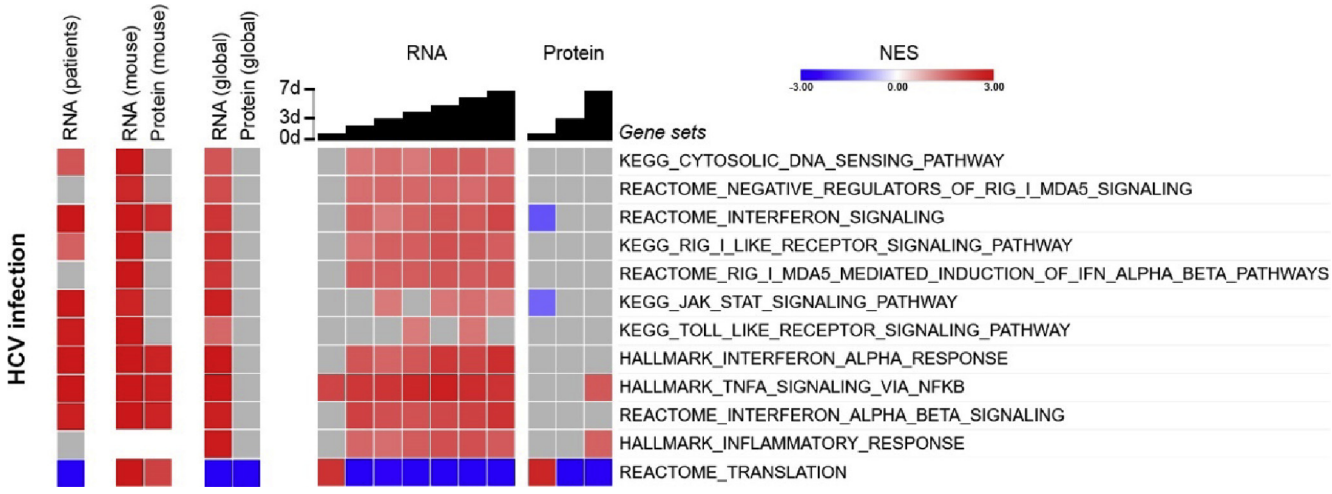


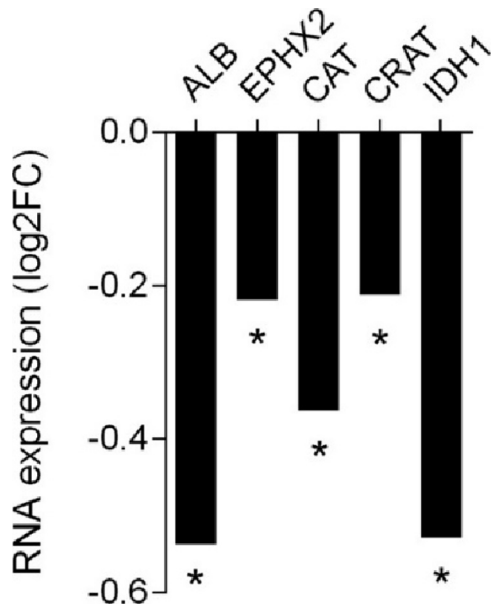
Supplementary Figure 1. Characterization and infection of DMSO-differentiated hepatocyte-like cells. (*A*) Cell culture with 1% DMSO induces a redifferentiation of Huh7.5.1 cells into quiescent hepatocyte-like cells (Huh7.5.1^{dif}). (*B*) Huh7.5.1^{dif} cells display higher levels of hepatocyte-specific differentiation marker expression compared with untreated Huh7.5.1 cells. Scale bars represent relative mRNA expression compared with GAPDH measured by quantitative PCR after 17 days of treatment with 1% DMSO (mean ± standard deviation, 3 independent experiments in duplicates). (*C*) Huh7.5.1 cells were incubated with 1% DMSO for 10 days before infection with HCVcc (strain Jc1E2^{FLAG}) and for an additional 7 days in the presence of 1% DMSO. Cells were infected at a MOI of 12 or dilutions of the same virus preparation. Nuclei were visualized by DAPI staining of cellular DNA, and HCV infection was visualized by NS5A staining. Scale bar represents 200 μm. d, day.



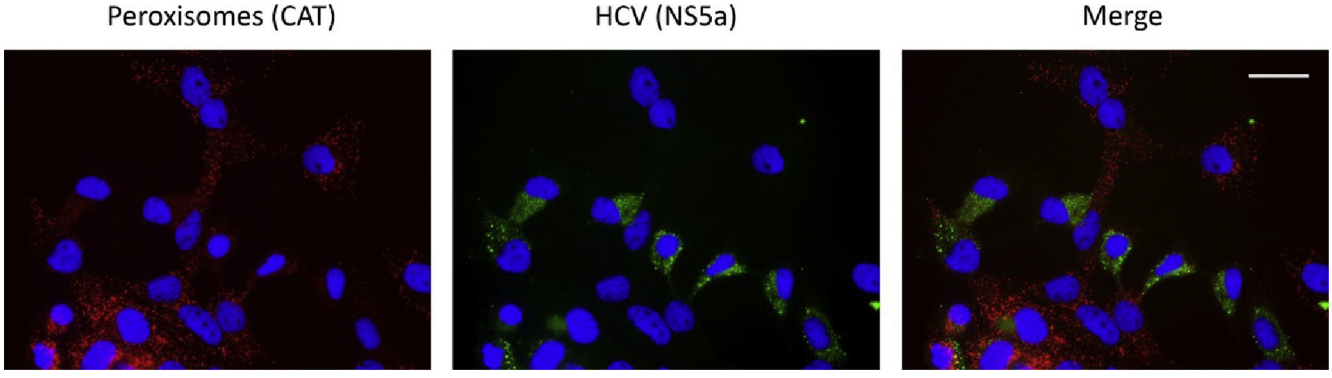
Supplementary Figure 2. Highly reproducible mapping of proteomics to mRNA transcripts in HCV-infected liver cells. Overall, 8,297 proteins were altered during HCV infection, and 7,537 (*green*) were mapped to corresponding mRNAs of the same experiment. The heatmap clustering of the mapped and normalized mRNA and protein expression levels emphasizes the high reproducibility of the 2 biological replicates. Ctrl, control.



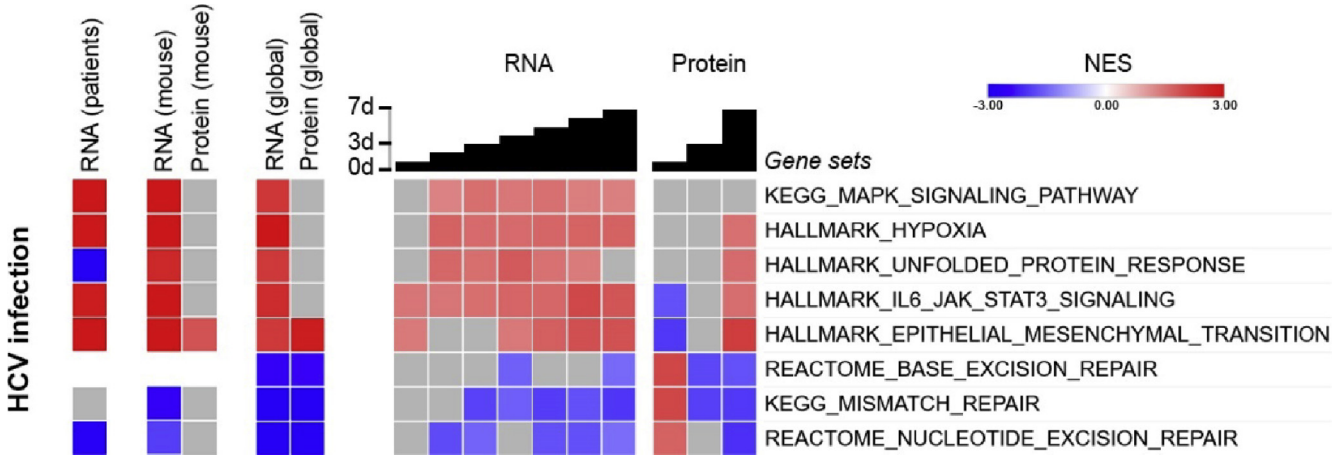
Supplementary Figure 3. Persistent HCV infection triggers an attenuated innate immune response. HCV-infected Huh7.5.1^{dif} cells relative to mock-infected cells until 7 days after infection. GSEA of proteomics and/or transcriptomics of livers of HCV-infected patients, human liver chimeric uPA/SCID mice, and Huh7.5.1^{dif}. HCV infection induces RNA expression of genes associated with innate immunity but suppresses their translation into proteins. NESs are displayed in red (increased), blue (decreased), gray (no significant change), and white (below analysis cutoff). Temporal analysis of infected Huh7.5.1^{dif} are presented as global trend (global) and individual time points. Statistical cutoff for GSEA of liver tissues was a false discovery rate of $Q < .05$ and for infected Huh7.5.1^{dif} ($P < .005$).



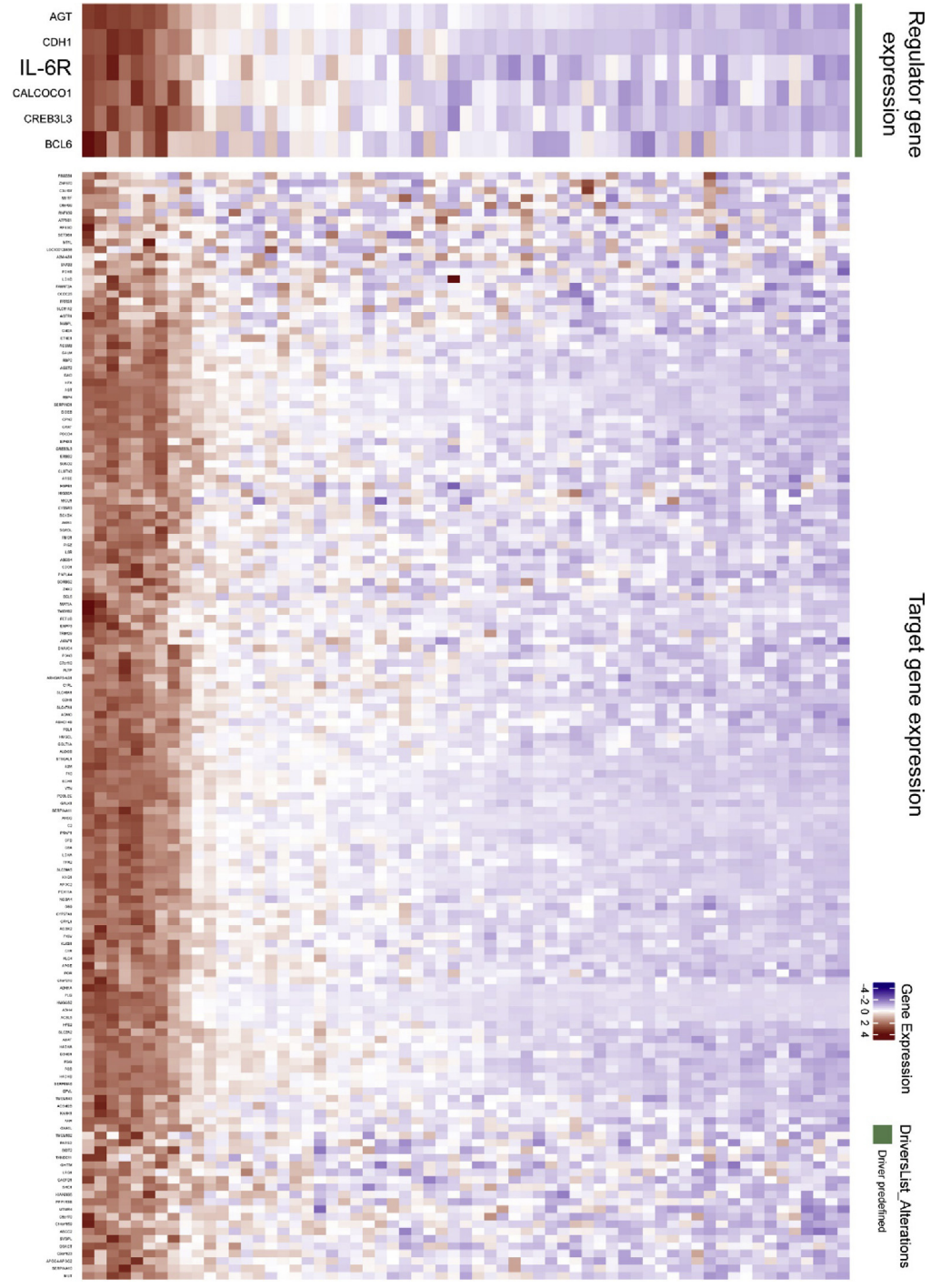
Supplementary Figure 4. Expression of leading-edge genes of the HALLMARK_PEROXISOME gene set are impaired by HCV infection. Validation of the top 5 genes (Huh7.5.1^{dif}, RNA-seq) shown in Figure 3*B* using quantitative PCR. Data are displayed as log2FC RNA expression in HCV-infected Huh7.5.1^{dif} cells. GAPDH expression was used as the housekeeping gene. *$P < .05$, 1-tailed t test (n = 3 in duplicate).



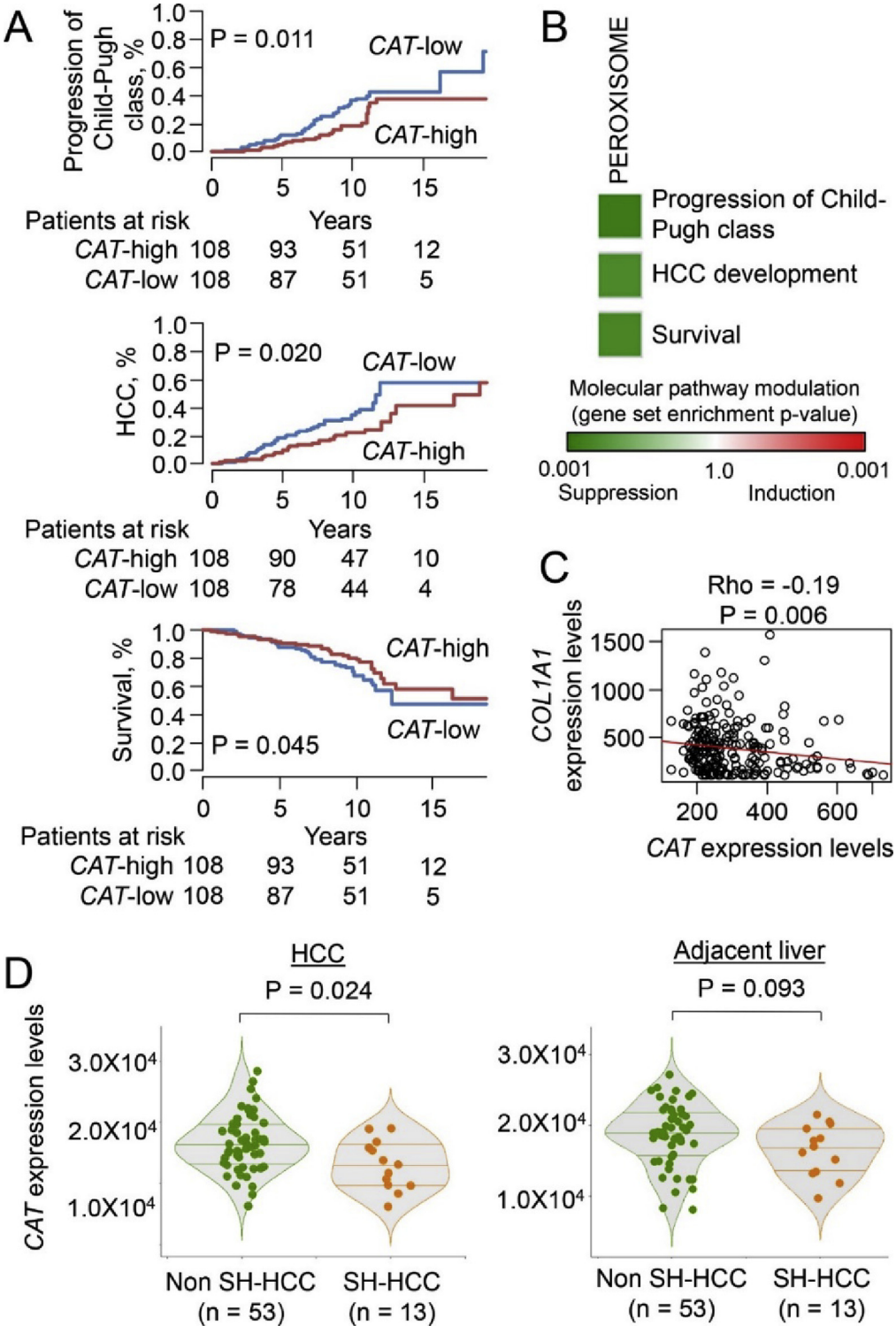
Supplementary Figure 5. Peroxisome marker expression is perturbed in HCV-infected hepatocyte-like cells. Immunofluorescence microscopy of Huh7.5.1^{dif} cells infected for 3 days with HCVcc. The peroxisomal marker catalase (CAT) is stained in red, nuclear DNA (DAPI) in blue, and HCV (NS5A) in green. Scale bar represents 200 μm.



Supplementary Figure 6. Persistent HCV infection perturbs cell circuits driving liver disease and cancer. GSEA of transcriptomics and proteomics of HCV-infected Huh7.5.1^{dif} cells relative to mock-infected cells until 7 days after infection. HCV infection perturbs pathways that are known hallmarks of cancer development. NESs are displayed in red (increased), blue (decreased), gray (no significant change), and white (below analysis cutoff). Temporal analysis of infected Huh7.5.1^{dif} are presented as a global trend (global) and individual time points. Statistical cutoff for GSEA of liver tissues was a false discovery rate of $Q < .05$ and for infected Huh7.5.1^{dif} was of $P < .005$.



Supplementary Figure 7. IL6 receptor is a predicted regulator of peroxisomal function. The top-scoring regulatory module (no. 111) was inferred by the AMARETTO algorithm for enrichments in peroxisomal function (HALLMARK_PEROXISOME signature genes). The heatmap of the regulatory module shows the regulators that act together in a regulatory program (*top heatmap*) and the coexpressed target genes (*bottom heatmap*) that they putatively control. IL6R is 1 of 6 regulators that together best predict the target genes' expression in this regulatory module. Enrichments in known functional categories from the MSigDB Hallmark and C2CP Collections are summarized in Supplementary Table 6.



Supplementary Figure 8. Significant association of hepatic catalase expression with clinical outcomes and phenotypes in viral and metabolic liver disease. (*A*) Early-stage HCV cirrhosis patients (N = 216)[20] are classified into 2 groups, *CAT*-high and *CAT*-low groups, based on median expression level. Association of the grouping with overall survival (*bottom*), HCC development (*middle*), and progression of Child-Pugh class[21] from A to B or C (*top*). Patients with lower *CAT* expression levels show worse clinical outcomes (log-rank test). (*B*) Association of peroxisome pathway modulation with the clinical outcomes in the 216 HCV cirrhosis patients determined by GSEA. (*C*) *CAT* expression levels were negatively correlated (Spearman correlation test) with hepatic *COL1A1* expression levels in the 216 HCV cirrhosis patients. (*D*) Distribution and probability density of *CAT* expression in HCC tumor (*left*) and adjacent non-tumor liver tissues (right) in 66 HCC patients[22] according to presence of histologic steatohepatitic HCC (SH-HCC) variant[23] (violin plot, Wilcoxon rank-sum test).

2.3.- Impaired expression of PTPRD is associated with glucose metabolic alterations *in vivo*

2.3.1.- Aims and summary

In the previously discussed articles, I described how as a result of our systems approach we were able **1**) to identify PTPRD as a phosphatase implicated in the negative regulation of the STAT3 signaling pathway in the liver, **2**) to characterize the effects of a prolonged STAT3 activation leading to an impaired peroxisomal function and **3**) to show the potential application of STAT3 inhibitors in the management of chronic liver disease.

Although the regulatory role of PTPRD over additional targets such as Aurora Kinase A (AURKA) (Meehan, Parthasarathi et al. 2012) and the β-catenin pathway (Funato, Yamazumi et al. 2011) has been shown in neuroblastoma and colon cancer respectively, other than STAT3 little is known regarding the function of PTPRD in the liver. Therefore, the work that we are currently focused on, involves the identification of additional signaling pathways associated to the impaired expression of PTPRD. In particular, we were interested in the possible role of PTPRD over glucoregulatory pathways, since it was previously reported that single nucleotide polymorphisms (SNPs) in the *PTPRD* gene are associated with the development of type 2 diabetes (Tsai, Yang et al. 2010) and the treatment response in these patients (Pei, Huang et al. 2013).

As a first step, we performed GSEA on a publicly available data set containing hepatic gene expression from healthy patients (Horvath, Erhart et al. 2014). My results showed that in the human liver, signaling pathways associated with low *PTPRD* expression fall mainly into three categories which are the alteration of glucose metabolic pathways, upregulation of the inflammatory response and the impairment of peroxisomal function.

Given that Akt is one of the central components implicated in the regulation of glucose metabolism (Haeusler, McGraw et al. 2018), I investigated the potential regulatory role of PTPRD expression on Akt activation using an RNAi approach. My results showed that in PTPRD-silenced PHH, insulin-induced Akt phosphorylation is significantly impaired. These results suggest the functional relevance of PTPRD as regulator of hepatic glucose homeostasis by maintaining insulin signaling.

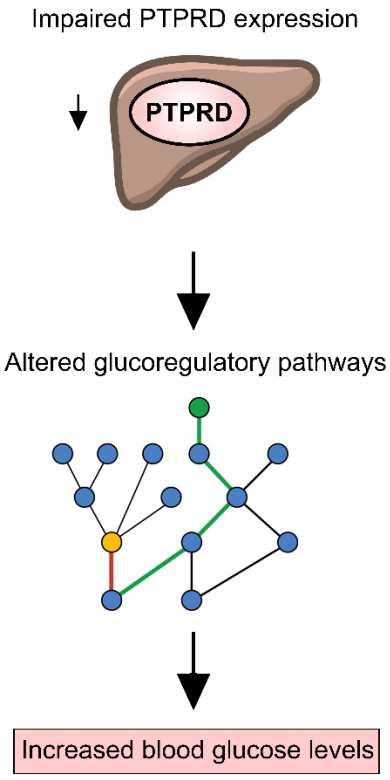
To validate our *in vitro* findings we utilized a PTPRD-deficient mouse model (Uetani, Kato et al. 2000). As, PTPRD KO (*Ptprd*-/-) leads to perturbed brain development and affects feeding behavior, our study was

restricted to the WT (*Ptprd*+/+) and PTPRD heterozygous (*Ptprd*+/-) mice. By the analysis of liver transcriptomic data from the *Ptprd*+/- mice, I was able to observe the upregulation of signaling processes associated with diabetes and downregulation of the insulin pathway. Moreover, when WT and *Ptprd*+/- mice were subjected to a choline-deficient high fat diet (CD-HFD), we observed significantly increased fasting blood glucose levels after 8 weeks of this regimen.

Given the observed diabetic phenotype in *Ptprd*+/- mice under a high-fat diet, I validated these findings by the analysis of a large cohort of obese patients. My analysis showed that patients with low *PTPRD* expression exhibit significantly higher levels of diabetic markers including fasting blood glucose, glycated hemoglobin (Hba1c) and HOMA2 scores.

In summary, our results support the role of hepatic PTPRD in the regulation of glucose homeostasis, as its perturbed expression is associated with the clinical manifestations of hepatic metabolic disease.

2.3.2.- Results article III



Roca Suarez A. A.; Mukherji A.; Brignon N.; Mailly L.; Jühling F.; Obringer C.; Oudot M.; Durand, SC.; Pessaux P.; Baumert TF.; Lupberger J.; Impaired expression of protein tyrosine phosphatase receptor delta induces signaling alterations associated with glucose metabolism in the liver (In preparation).

Impaired expression of protein tyrosine phosphatase receptor delta induces signaling alterations associated with glucose metabolism in the liver

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Word count: 3025.

Number of figures: Three figures.

List of abbreviations:

Akt	Akt serine/threonine kinase
AURKA	Aurora kinase A
CD-HFD	Choline-deficient high-fat diet
DEN	Diethylnitrosamine
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
GSEA	Gene set enrichment analysis
HbA1c	Hemoglobin A1c
HCC	Hepatocellular carcinoma

HOMA2	Homeostasis model assessment 2
InsR	Insulin receptor
IRS1	Insulin receptor substrate 1
NASH	Non-alcoholic steatohepatitis
NAFLD	Non-alcoholic fatty liver disease
PHH	Primary human hepatocytes
PTPRD	Protein tyrosine phosphatase receptor type delta
SNP	Single nucleotide polymorphism
SOCS3	Suppressor of cytokine signaling 3
STAT3	Signal transducer and activator of transcription 3

Key words: PTPRD, Akt, diabetes, signaling.

Conflicts of interest: The authors disclose no conflicts.

Financial support: This work was supported by the *Agence Nationale de Recherche sur le Sida et les Hépatites Virales* (ECTZ4236 to J.L. and ECTZ4446 to A.A.R.S.), *Fondation pour la Recherche Médicale en France* (FRM FDT201805005763 to A.A.R.S.), by the European Union (ERC-AdG-2014 HEPCIR to T.F.B and EU H2020 HEPCAR 667273 to T.F.B and J.L.), and the French Cancer Agency (ARC IHU201301187 to T.F.B.) and has been published under the framework of the LABEX ANR-10-LAB-28 (HEPSYS).

Author's contribution: J.L. initiated and supervised the study. A.A.R.S., A.M., N.B., L.M., S.C.D., C.O. conducted experiments; S.C.D. and P.P. provided and extracted primary human hepatocytes; A.A.R.S. and F.J. performed bioinformatic analysis; J.L. and A.A.R.S. wrote the manuscript, T.F.B. and A.M. critically reviewed the manuscript.

ABSTRACT

Background and aims: The function of the phosphatase PTPRD in the liver is currently only partially understood, therefore we analyzed transcriptomic and clinical data from patients and a PTPRD-heterozygous (*Ptprd*+/-) mouse model in order to identify signaling pathways associated to its regulatory activity. **Methods:** Based on liver transcriptomic data, healthy patients were classified according to *PTPRD*

expression and GSEA was performed in order to identify signaling pathways associated to low *PTPRD* expression. We validated our findings using siRNAs targeting PTPRD in PHH and the quantification of selected targets by western blotting. RNA-seq followed by GSEA was performed on liver samples from *Ptprd*+/- mice. Fasting blood glucose levels were measured in WT and *Ptprd*+/- animals following 8 weeks of CD-HFD. Obese patients were ranked according to hepatic *PTPRD* expression and its association with diabetes markers was studied by statistical methods. **Results:** We observed signaling pathway alterations associated with glucose metabolism in the liver of healthy patients presenting low *PTPRD* expression. Silencing of PTPRD in PHH induced a decreased Akt activation following insulin stimulation. *Ptprd*+/- mice present hepatic transcriptional changes similar to the ones observed in healthy patients, leading to increased blood glucose levels when the animals were subjected to a CD-HFD. Obese patients presenting low *PTPRD* expression show increased levels of fasting blood glucose and HbA1c. Moreover, this was also associated with a higher HOMA2 score suggesting insulin resistance as a potential mechanism. **Conclusion:** Our data suggests an important regulatory role hepatic PTPRD in maintaining glucose homeostasis and how its impaired expression is associated with clinical manifestations of metabolic disease.

INTRODUCTION

Chronic liver disease represents a significant public health burden, with approximately 2 million deaths per year worldwide [1]. This stems from the presence of numerous risk factors such as viral hepatitis and the western lifestyle that favor the progression from chronic inflammation, steatosis, fibrosis, cirrhosis and ultimately to the development of hepatocellular carcinoma (HCC) [2]. Although the disease etiologies may vary, the similarities in the course of liver disease progression suggest the involvement of common deregulated signaling pathways driving this pathological process [3]. Therefore, a more detailed understanding of the common molecular alterations associated with chronic liver disease is needed to set the basis for the novel preventive or therapeutic interventions that are urgently needed.

Phosphorylation of proteins represents one of the most crucial aspects of cellular signal transduction. Indeed, the coordination between protein kinases and phosphatases allows a balance between the amplitude, rate and duration of a given transcriptional program following its activation/inhibition [4]. Hence, is no surprise that alterations in the functioning of either kinases or phosphatases often leads to pathologies [5]. In the context of liver disease, we have previously demonstrated that the impaired

expression of protein tyrosine phosphatase receptor type delta (PTPRD) following hepatitis C virus (HCV) infection is associated with an increased activity of the signal transducer and activator of transcription 3 (STAT3) pathway, which correlates with a lower patient survival from hepatocellular carcinoma (HCC) [6]. Although the regulatory role of PTPRD over additional targets such as Aurora Kinase A (AURKA) [7] and the β-catenin pathway [8] has been shown in neuroblastoma and colon cancer respectively, other than its action over the STAT3 pathway little is known regarding its function in the liver. Therefore, the purpose of this study was to employ a PTPRD-deficient mouse model in order to identify signaling pathways which are regulated by its expression. In particular, we were interested in the possible role of PTPRD over glucoregulatory pathways, since it was previously reported that single nucleotide polymorphisms (SNPs) in the *PTPRD* gene are associated with the development of type 2 diabetes [9] [10] and the treatment response in these patients [11]. These observations are of potential clinical relevance since diabetes is a risk factor for the development of non-alcoholic fatty liver disease (NAFLD) and HCC [1].

RESULTS

Low *PTPRD* expression in the normal human liver is associated with signaling pathway alterations implicated in glucose metabolism

To investigate the potential link between PTPRD and glucose metabolism in the liver, we first dissected the effect of normal variations of PTPRD expression in liver samples from healthy patients. Therefore, we associated liver transcriptomic data from 38 normal patients [12] to *PTPRD* expression levels using gene set enrichment analysis (GSEA). Our analyses showed that in normal human liver the signaling pathways associated to low *PTPRD* expression fall mainly into three categories which are upregulation of the STAT3 inflammatory response, downregulation of gene sets associated to peroxisomal function and the perturbation of glucose metabolic pathways (**Fig. 1a**). This is highly consistent with our previous findings showing that in HCV-infected livers, low *PTPRD* expression is associated with an increased transcriptional activity of the STAT3 pathway [6], which leads to an impaired peroxisomal function [3].

One of the most important circuits regulating hepatic glucose metabolism is mediated by insulin signaling. Insulin engagement of its receptor triggers a chain of phosphorylation events including Akt serine/threonine kinase (Akt) phosphorylation, which regulates the gene expression of glucose transporters and the activity of pathways associated with gluconeogenesis [13]. We investigated a potential regulatory role of PTPRD expression on Akt activation using an RNAi approach. Stimulating

primary human hepatocytes (PHH) with insulin activates Akt by phosphorylation at serine 473 (**Fig. 1b**). In PTPRD-silenced PHH however, insulin-induced Akt phosphorylation is significantly impaired (p=0.02, U-test) (**Fig. 1c**). These results suggest the functional relevance of PTPRD as regulator of hepatic glucose homeostasis by maintaining insulin signaling.

PTPRD-deficient mice present hepatic signaling pathway alterations related to glucose homeostasis

To validate our *in vitro* findings, we utilized a PTPRD-deficient mouse model [14]. In this model, genetic deletion of the region coding for the phosphatase domain 1 of PTPRD induces a significant (p=<0.0001, U-test) downregulation of *PTPRD* expression on the mRNA (**Fig. 2a**) and protein level (**Fig. 2b**). As, PTPRD KO (*Ptprd*-/-) leads to perturbed brain development and affects feeding behavior [14], this study was restricted to the WT (*Ptprd*+/+) and PTPRD heterozygous (*Ptprd*+/-) mice. The analysis of liver transcriptomic data from the *Ptprd*+/- mice revealed a significant (FDR=<0.05) upregulation of a gene signature associated with diabetes and the downregulation of signatures related to the insulin signaling pathway (**Fig. 2c**) confirming our findings in the normal human livers. To study the phenotypical effect of these transcriptional changes in the *Ptprd*+/- mice, we employed a widely used liver disease model for the study of fibrosis and non-alcoholic steatohepatitis (NASH) [15]. When WT and *Ptprd*+/- mice were subjected to a choline-deficient high fat diet (CD-HFD) we observed a similar incremental body weight in both conditions (**Fig. 2d**). Despite of this, *Ptprd*+/- animals developed significantly increased (p=0.02, T-test) fasting blood glucose levels after 8 weeks of CD-HFD (**Fig. 2e**). This suggests that impaired hepatic PTPRD expression causes a diabetic phenotype and thus represents a potential risk factor for metabolic disease.

Impaired hepatic *PTPRD* expression is associated to clinical manifestations related to altered glucose metabolism in patients

Given the observed diabetic phenotype in *Ptprd*+/- mice fed with a high-fat diet, we validated our findings in a patient cohort with liver disease. Therefore, we analyzed liver transcriptomics, blood parameters and clinical data from 737 obese patients [16]. First, the patient samples were ranked according to hepatic *PTPRD* expression and divided into two groups with 20% lowest and 20% highest *PTPRD* expression, respectively (**Fig. 3a**). Our analysis shows that patients with low *PTPRD* expression exhibit significantly (p=<0.0001, T test) higher levels of diabetic markers including fasting blood glucose and glycated hemoglobin (Hba1c) (**Fig. 3b-c**). Additionally, we observed a significant (p=<0.0001, T test) association between low *PTPRD*-expressing patients with a higher homeostasis model assessment 2 (HOMA2) score,

suggesting the presence of insulin resistance as a potential mechanism for these metabolic alterations (**Fig. 3d**).

Taken together, our results support the role of hepatic PTPRD in the regulation of glucose homeostasis, as its perturbed expression is associated with the clinical manifestations of hepatic metabolic disease.

DISSCUSSION

Complications arising from chronic liver disease are an increasing health threat associated with viral hepatitis and western lifestyle. This group of pathologies usually involves a progression from chronic inflammation, steatosis, fibrosis, cirrhosis to HCC [2]. The similarities in the course of liver disease independent of the underlying etiology are striking and thus suggest the involvement of common deregulated signaling pathways driving this pathological process [3]. For example, HCV-induced persistent epidermal growth factor receptor (EGFR) signaling during chronic infection [17] [18] [19] [20] and epidermal growth factor (EGF) polymorphisms are associated with HCC risk in non-infected patients [21]. Deregulated EGF seems to be a pan-etiological driver for liver disease progression and inhibition of its receptor EGFR attenuates fibrosis and HCC development in animal models [22]. Another example is STAT3 signaling, which like EGF is a key factor during liver regeneration [23]. We had previously demonstrated that HCV infection impairs PTPRD expression, which is associated with an increased activity of the STAT3 signaling pathway and a lower patient survival from HCC after surgical resection [6]. STAT3 is also associated with HCCs of poor prognosis in non-infected patients [24]. Here we describe a new role of PTPRD in hepatic metabolic disease independent from etiology.

Our results demonstrate that low hepatic *PTPRD* expression is associated with the alteration of glucoregulatory signaling pathways. Moreover, we established the functional link between PTPRD expression and insulin signaling by RNAi perturbation studies. Silencing of PTPRD in PHH prevents insulin-induced phosphorylation of Akt, which is one of the main signal transducers mediating insulin function.

This clearly suggests impaired PTPRD expression as a risk factor for metabolic disease in healthy individuals, but is this still the case in a liver disease context? Indeed, *Ptprd*+/- mice develop a diabetic phenotype which is not associated with an increased weight gain after 8 weeks of CD-HFD. Moreover, obese patients with low hepatic *PTPRD* expression display significantly higher diabetes marker levels in the blood, highlighting the role of hepatic PTPRD as regulator of glucose-homeostasis in the presence of metabolic risk factors. Our findings are further supported by genome-wide association studies identifying

SNPs in the *PTPRD* gene, which are associated with the development of type 2 diabetes [9] [10] and the treatment response in these patients [11].

Activation of insulin signaling is mediated by a chain of phosphorylation events on tyrosine residues present on the insulin receptor (InsR) and the insulin receptor substrate 1 (IRS-1), leading to downstream serine phosphorylation of Akt [13]. Interestingly, PTPRD has been previously shown to interact with IRS-1, although the functional significance of this remains unknown [7]. However, our mechanistic studies suggest a role of PTPRD on an additional indirect regulator of the insulin pathway since a direct action over InsR or IRS1 by PTPRD would not match our phenotypic observations. Given that PTPRD does not possess serine or threonine phosphatase activity [25], any binding candidate should be either 1) an inducer of the insulin pathway which is itself negatively regulated by tyrosine phosphorylation or, 2) an inhibitor of this pathway which is positively regulated by tyrosine phosphorylation. Although this needs to be further studied, a potentially relevant substrate falling in this second category could be again tyrosine phosphorylated STAT3, which we find strongly upregulated in the livers of *Ptprd*+/- and *Ptprd*-/- mice (data not shown). Indeed, STAT3 signaling has been associated with the development of insulin resistance in diabetic patients [26] or patients presenting *STAT3* germline mutations which enhance its transcriptional activity [27]. In this context, it has been suggested that suppressor of cytokine signaling 3 (SOCS3), which is a feedback regulator of STAT3 signaling, impairs hepatic insulin signaling when overexpressed in the liver [28]. This would suggest a functional role of PTPRD in suppressing hepatic STAT3 activation and SOCS3 expression maintaining insulin responsiveness. In a diseased hepatic state associated with chronic IL-6/STAT3 activation, like HCV infection [6] or NASH [29], unleashed STAT3 signaling by impaired PTPRD may contribute to the development of metabolic disease.

In conclusion our data suggests an important regulatory role hepatic PTPRD in maintaining hepatic glucose homeostasis and how its impaired expression is associated with clinical manifestations of metabolic disease.

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MATERIALS AND METHODS

Animal experiments

Sperm from C57BL/6 *Ptprd*-deficient mice, originally developed by Uetani *et al* [14] was obtained from McGill University for subsequent *in vitro* fertilization and repopulation at the *Institut Clinique de la Souris* (ICS, Illkirch, France). *In vivo* experiments were performed at the animal facility of Inserm U1110 according to local laws and ethics committee approval. Eight-week old *Ptprd*+/+ (n=22) and *Ptprd*+/- (n=13) mice received a single intraperitoneal (i.p.) injection of DEN (100 mg/kg) (Sigma-Aldrich) and were subsequently fed with CD-HFD (A06071302, Research Diet, NJ, USA) for eight weeks. Blood glucose levels were determined in blood collected from the tail vein using a handheld Accu-check active glucometer (Roche) after overnight fasting.

Patient cohorts

Microarray data from liver tissues and the accompanying clinical data were obtained from the gene expression omnibus GSE61260 (n=38, untreated normal patients) [12] and from GSE130991 (n=737, untreated obese patients) [16].

Primary human hepatocytes

PHH were isolated and cultured as previously described [18]. For the silencing of PTPRD, PHH were reverse transfected using siRNA against *PTPRD* (Dharmacon) and lipofectamine RNAiMAX (Thermo Fischer) for 72 hours according to the protocol suggested by the manufacturer. Post silencing, PHH were serum starved for 6 hours and stimulated with human insulin (Sigma-Aldrich) 100 nmol/L for 10 minutes prior cell lysis.

Processing of liver tissues and cells:

Liver samples from C57BL/6 mice were lysed using TRI reagent (Sigma-Aldrich) and RNA was extracted using the Direct-zol kit (Zymo Research) according to the manufacturer's indications. Proteins samples from PHH were prepared using lysis buffer 6 (R&D Systems) supplemented with protease and phosphatase inhibitors cocktails 2 and 3 (Sigma-Aldrich).

Real time quantitative PCR

Complementary DNA was generated using the Maxima first strand cDNA synthesis kit (Thermo Fisher Scientific). Quantitative polymerase chain reaction (qPCR) was performed using a CFX96 real-time PCR system (Bio-Rad, Hercules, CA) and iTaq Universal SYBR Green Supermix (Bio-Rad) as recommended. Primers were synthesized by Sigma-Aldrich: Mouse *Gapdh* (5'-GGT CCT CAG TGT AGC CCA AG-3', 5'-AAT GTG TCC GTC GTG GAT CT-3'), mouse *Ptprd* (5'- CGT AGG TCC TGT CCT TGC AG-3', 5'-CGA CTC TGC CCT CTT CCT TT-3').

Western blot

β-Actin mAb (AC-15) was obtained from Invitrogen, PTPRD antibodies (A8559 and A15713) were obtained from ABclonal, Akt (4691) and pAkt S473 (9271) were obtained from Cell Signaling Technologies. For western blot analyses, 20 µg of protein was loaded on 8-12% SDS-PAGE gels prior transfer of proteins to PVDF membranes. Bands were quantified using Image Lab software (Bio-Rad) version 5.2.1.

RNA-seq data processing

For the analysis of RNA-seq data from C57BL/6 mice, reads were mapped onto the mm10 of the *Mus musculus* genome using STAR version 2.5.3a. Quantification was performed using HTSeq version 0.6.1p1 and read counts were normalized using the median-of-ratios method. Sequencing and data processing were performed by the GenomEast platform of the *Institut de génétique et de biologie moléculaire et cellulaire* (IGBMC), Illkirch, France.

Bioinformatic analyses

Differential expression analysis of RNA-seq data from C57BL/6 mice was performed using the DESeq2 R package. P values were computed using the Wald test and adjusted for multiple testing using the Benjamini and Hochberg method [30]. Pre-ranked GSEA was performed using the gene sets belonging to the Molecular Signatures Database (MsigDB) version 6.2. For the analysis of microarray data from

GSE61260, probes were collapsed using the CollapseDataset tool available at GenePattern and samples were ranked according to *PTPRD* expression using it as a continuous class label. Significantly enriched gene sets (FDR= <0.05) presented in the figures belong to HALLMARK, KEGG and REACTOME. For the analysis of clinical data from the GSE130991 dataset, samples were ranked according to *PTPRD* expression and the 20% of patients with the highest (n=147) and lowest (n=147) expression were used to plot fasting blood glucose, Hba1c and HOMA2 index. Insulin resistance index was calculated using the HOMA2 calculator (https://www.dtu.ox.ac.uk/homacalculator/). Statistical analyses were performed using GraphPad Prism 7.

ACKNOWLEDGEMENTS

We would like to thank Michel Tremblay and Noriko Uetani (McGill University, Montreal, Quebec, Canada) for the PTPRD knockout mouse model and for helpful discussions.

FIGURE LEGENDS

Figure 1: Low *PTPRD* expression in the normal human liver is associated with signaling pathway alterations implicated in glucose metabolism. a) Low hepatic *PTPRD* expression associates with transcriptional signatures of inflammation, impaired glucose metabolism and peroxisomal function. GSEA showing the significantly enriched (FDR=<0.05) signaling pathways associated with low hepatic *PTPRD* expression in heathy humans (n=38). **b-c**) Impaired PTPRD expression alters the insulin signaling pathway *in vitro*. Silencing of PTPRD in PHH leads to a significantly impaired (p=0.02, U-test) Akt phosphorylation (S473) following insulin stimulation as assessed by western blot (n=4).

Figure 2: PTPRD-deficient mice present transcriptomic and phenotypic alterations related to glucose homeostasis. a) PTPRD-deficient mouse model. Genomic deletion of the region coding for the first phosphatase domain of PTPRD induces a significant downregulation of its expression at the mRNA level when comparing *Ptprd*+/+ (n=6), *Ptprd*+/- (n=4) and *Ptprd*-/- (n=5) mice (p=<0.0001, U-test) as assessed by RT-qPCR. **b**) PTPRD protein levels are impaired in PTPRD-deficient mice as shown in liver samples analyzed by western blotting. **c**) Liver transcriptomics of PTPRD-deficient mice exhibit a pro-diabetic transcriptional signature. GSEA of liver expression data from *Ptprd*+/- mice (n=3) as compared to *Ptprd*+/+ animals (n=3), showing a significant upregulation of the REACTOME_DIABETES_PATHWAYS gene set and a downregulation of the KEGG_INSULIN_SIGNALING gene set (FDR=<0.05). **d**) PTPRD-deficient mice with induced liver disease show similar incremental weight gain than wild type mice. Incremental weight gain of *Ptprd*+/+ (n=22) and *Ptprd*+/- (n=13) mice following CD-HFD during an eight-week period. **e**) PTPRD-deficient mice with induced liver disease show a diabetic phenotype. *Ptprd*+/- mice (n=13) present significantly (p=0.02, T test) higher levels of fasting blood glucose as compared to *Ptprd*+/+ mice (n=22).

Figure 3: Low hepatic *PTPRD* expression associates with clinical manifestations of metabolic disease in humans. **a**) Classification of obese patients according to hepatic *PTPRD* expression into high-expressing (n=147, red) and the low-expressing (n=147, blue) groups. **b-d**) Patients with low hepatic *PTPRD* expression display higher levels of diabetic markers in the blood. Analysis of clinical data from obese patients ranked according to *PTPRD* expression shows a significant association (p=<0.0001, T test) of low *PTPRD* expression with increased fasting blood glucose, Hba1c and HOMA2 insulin resistance index.

FIGURE 1

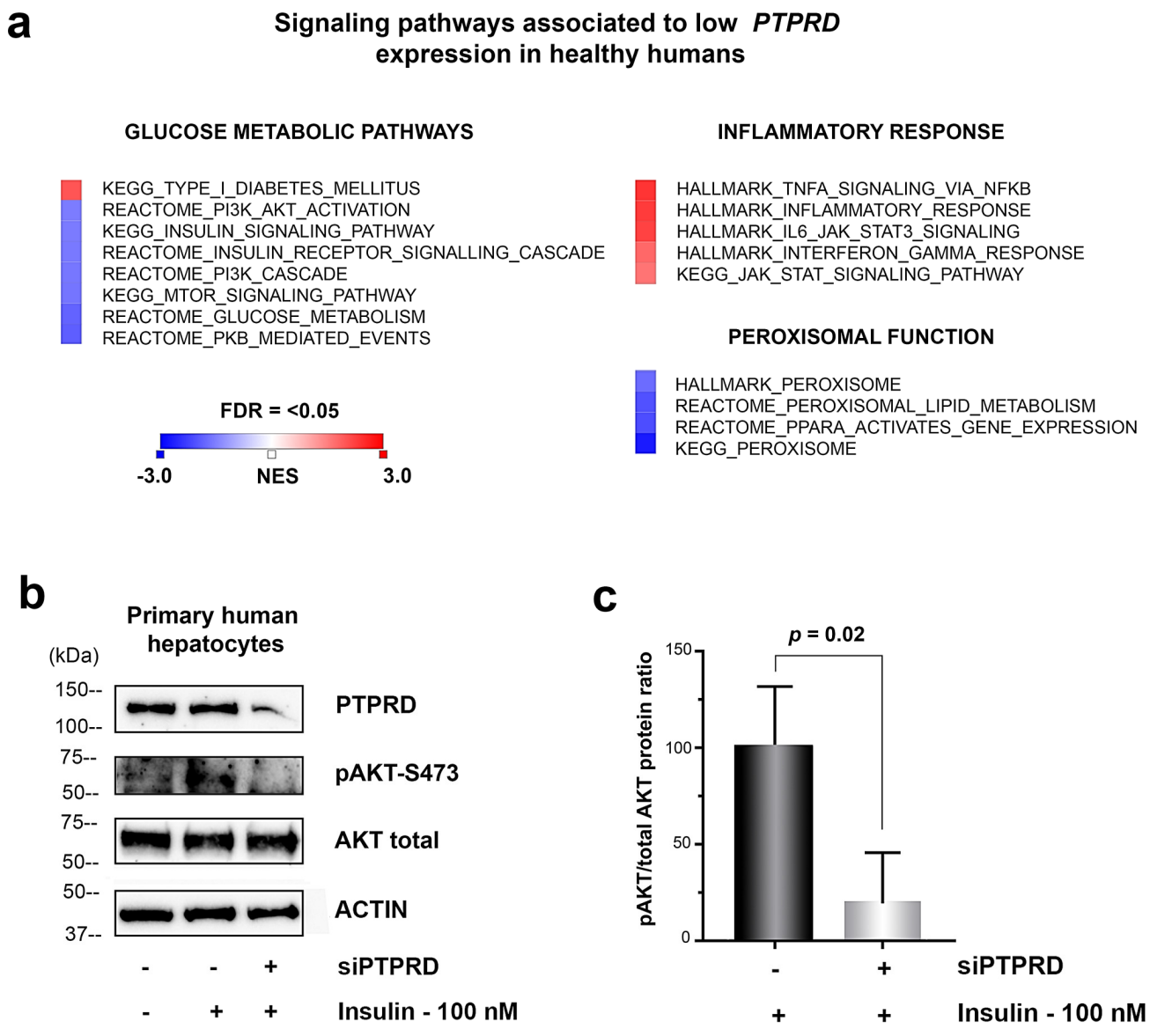

a
Signaling pathways associated to low PTPRD expression in healthy humans
GLUCOSE METABOLIC PATHWAYS
KEGG_TYPE_I_DIABETES_MELLITUS
REACTOME_PI3K_AKT_ACTIVATION
KEGG_INSULIN_SIGNALING_PATHWAY
REACTOME_INSULIN_RECEPTOR_SIGNALLING_CASCADE
REACTOME_PI3K_CASCADE
KEGG_MTOR_SIGNALING_PATHWAY
REACTOME_GLUCOSE_METABOLISM
REACTOME_PKB_MEDIATED_EVENTS
INFLAMMATORY RESPONSE
HALLMARK_TNFA_SIGNALING_VIA_NFKB
HALLMARK_INFLAMMATORY_RESPONSE
HALLMARK_IL6_JAK_STAT3_SIGNALING
HALLMARK_INTERFERON_GAMMA_RESPONSE
KEGG_JAK_STAT_SIGNALING_PATHWAY
PEROXISOMAL FUNCTION
HALLMARK_PEROXISOME
REACTOME_PEROXISOMAL_LIPID_METABOLISM
REACTOME_PPARA_ACTIVATES_GENE_EXPRESSION
KEGG_PEROXISOME
FDR = <0.05
-3.0
NES
3.0
b
Primary human hepatocytes
(kDa)
150--
100--
75--
50--
75--
50--
50--
37--
PTPRD
pAKT-S473
AKT total
ACTIN
- - +
siPTPRD
- + +
Insulin - 100 nM
c
p = 0.02
pAKT/total AKT protein ratio
150
100
50
0
- +
siPTPRD
+ +
Insulin - 100 nM

FIGURE 2

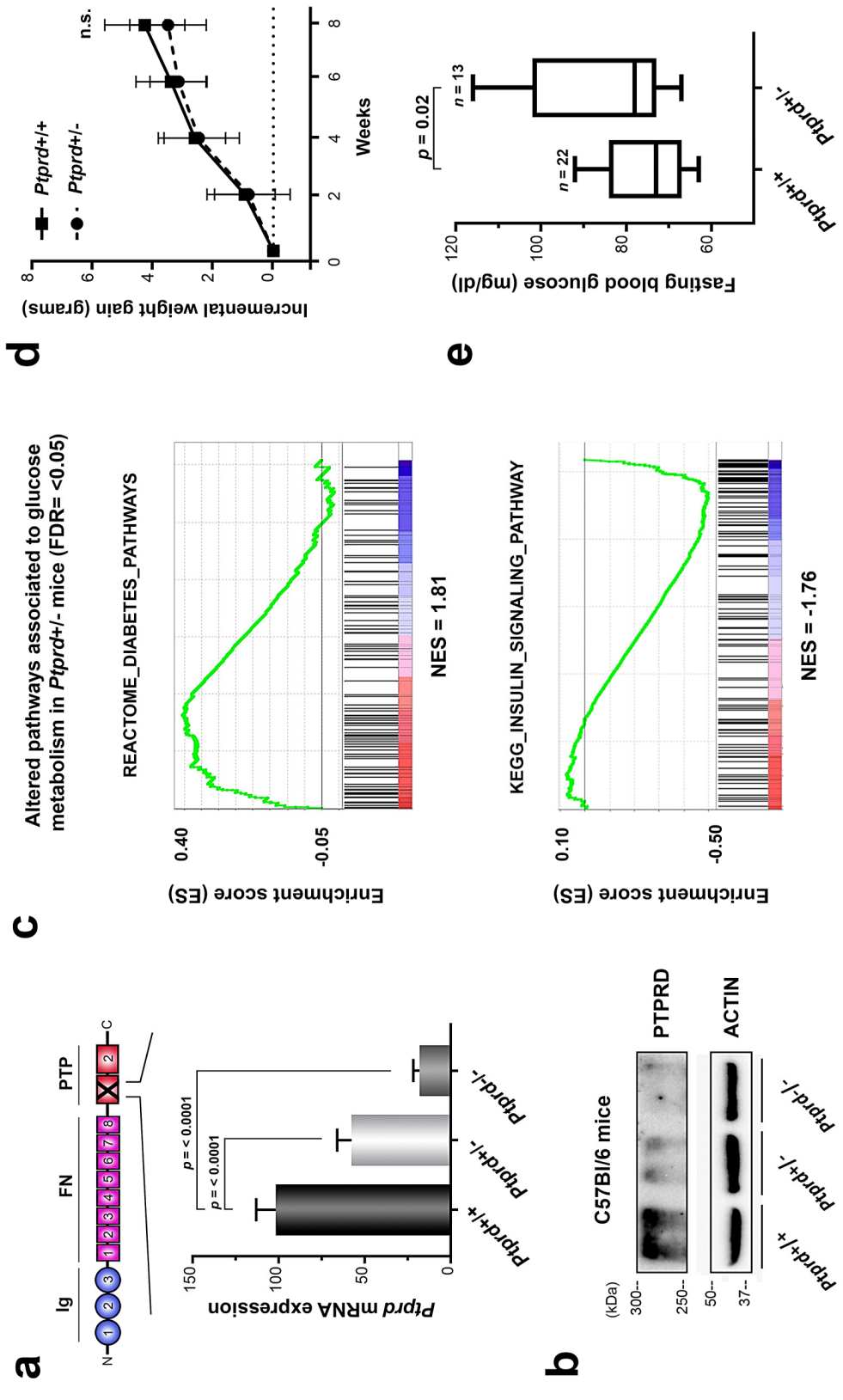
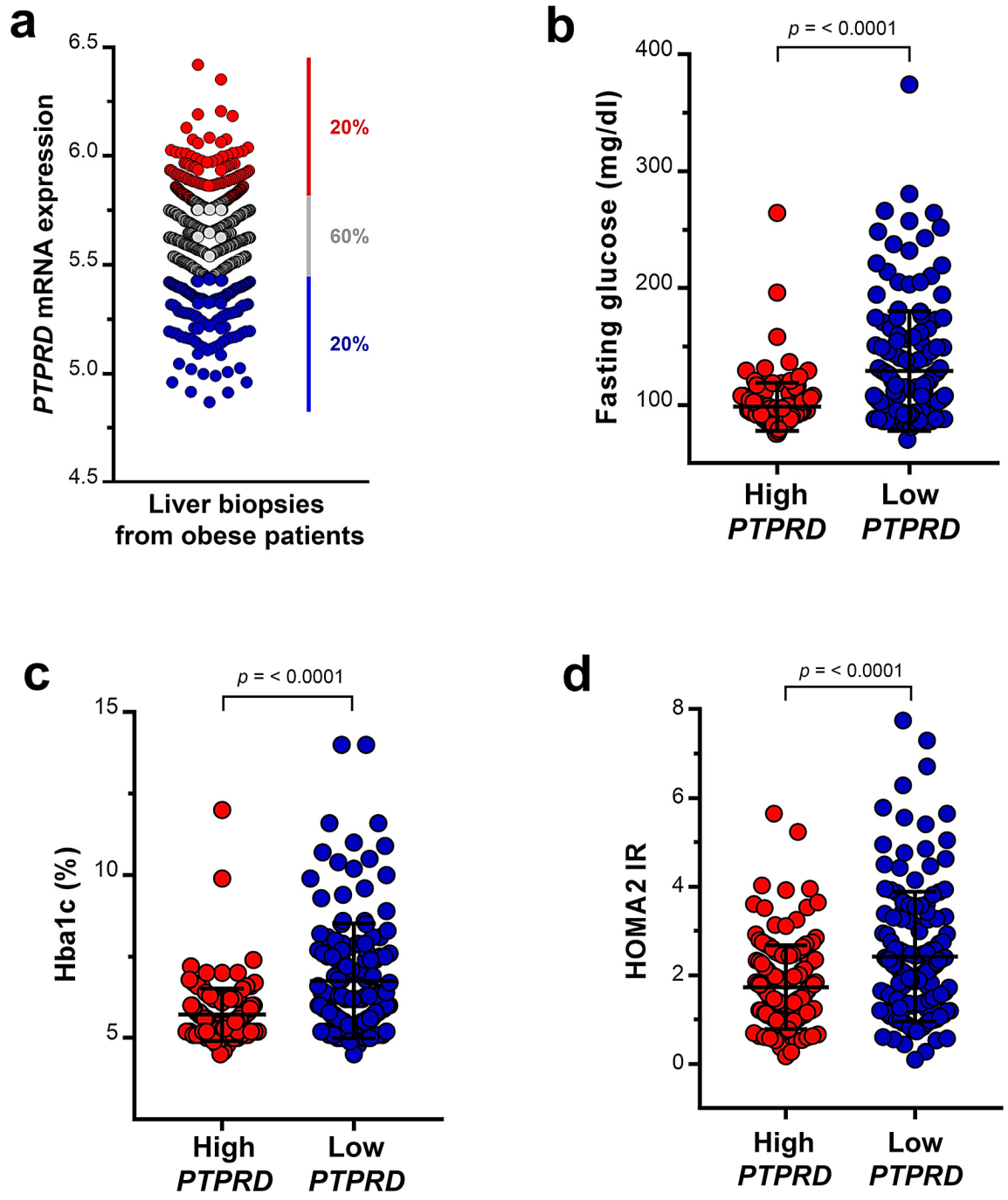

a
Ig
FN
PTP
N
C
p = < 0.0001
p = < 0.0001
Ptprd mRNA expression
Ptprd+/+
Ptprd+/-
Ptprd-/-
b
C57Bl/6 mice
(kDa)
300--
250--
50--
37--
PTPRD
ACTIN
Ptprd+/+
Ptprd+/-
Ptprd-/-
c
Altered pathways associated to glucose metabolism in Ptprd+/- mice (FDR= <0.05)
REACTOME_DIABETES_PATHWAYS
Enrichment score (ES)
NES = 1.81
KEGG_INSULIN_SIGNALING_PATHWAY
Enrichment score (ES)
NES = -1.76
d
Ptprd+/+
Ptprd+/-
n.s.
Incremental weight gain (grams)
Weeks
e
p = 0.02
n = 13
n = 22
Fasting blood glucose (mg/dl)
Ptprd+/+
Ptprd+/-

FIGURE 3


a
6.5
6.0
5.5
5.0
4.5
PTPRD mRNA expression
20%
60%
20%
Liver biopsies
from obese patients
b
p = < 0.0001
400
300
200
100
Fasting glucose (mg/dl)
High
PTPRD
Low
PTPRD
c
p = < 0.0001
15
10
5
Hba1c (%)
High
PTPRD
Low
PTPRD
d
p = < 0.0001
8
6
4
2
0
HOMA2 IR
High
PTPRD
Low
PTPRD

3.- Discussion and perspectives

In the framework of my thesis, I aimed to characterize in detail the signaling pathway alterations associated with the development and progression of liver diseases, with a special emphasis on the ones arising in the context of chronic HCV infection. Although the recent development and clinical success of DAAs may generate the impression that further fundamental research on HCV is of little practical relevance, the large number of remaining medical challenges and unanswered questions related to the pathogenesis of HCV-associated complications strongly support the notion that thorough investigations in this field must be sustained (Bartenschlager, Baumert et al. 2018). Moreover, similar signaling alterations have been observed at different stages of liver disease development independently of its etiology (Llovet, Zucman-Rossi et al. 2016). Therefore, the study of HCV/host interactions is potentially useful to understand the general molecular mechanisms behind the pathogenesis of chronic liver disease associated to risk factors other than HCV.

An example which illustrates this previous point, was our initial identification of the impaired PTPRD expression in the context of chronic HCV infection (Van Renne, Roca Suarez et al. 2018). The results that I obtained from this study showed that HCV infection induces the expression of miR-135a-5p which in turn targets the *PTPRD* mRNA and leads to its degradation. However, the existence of a population of samples in our patient cohort where both miR-135a-5p and *PTPRD* were minimally expressed suggested the existence of additional regulatory mechanisms of PTPRD expression. The answer to this question came from a subsequent study by our group, which aimed to identify HCV-induced epigenetic changes that persist in the human liver even after viral cure (Hamdane, Juhling et al. 2019). In this work, we identified significantly decreased H3K27 acetylation levels (transcriptional activation marker) in the promoter-enhancer region of the *PTPRD* gene from HCV-infected and DAA-treated patients. This finding not only showed the presence of multiple regulatory mechanisms associated with the impairment of PTPRD expression but also highlighted the relevance of this phosphatase as a potential modulator of liver disease progression. This is further supported by our results showing an association between a low *PTPRD* expression in the liver of HCV-infected patients, an increased transcriptional activity of the oncogenic STAT3 signaling pathway and a poor survival from HCC. This suggests that PTPRD could act as a potential tumor suppressor in the liver, similarly to what has been observed in other types of cancers (Julien, Dube et al. 2011).

Further evidence supporting the relevance of this persistent STAT3 activation as a driver of liver disease came from our multi-omics analysis of HCV-infected cells, mice and patients (Lupberger, Croonenborghs et al. 2019). In this study we found that HCV-induced STAT3 signaling deregulates transcriptional programs related to peroxisomal function. This finding is of clinical importance as an impaired peroxisomal gene expression showed a significant association with the progression of liver cirrhosis, HCC development and patient survival.

Although my discussion of this project has been centered around the STAT3 pathway, our multi-omics characterization of HCV infection has a broad range of potential applications in general. Indeed, it represents a useful resource data set for researchers aiming 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 the livers of HCV patients emphasizes the potential of this model for the identification of additional liver disease drivers and therapeutic targets.

Regarding PTPRD's role in regulating signaling pathways associated with glucose metabolism, the results that I have obtained until now in combination with previous independent reports (Tsai, Yang et al. 2010) (Chen, Xu et al. 2016), suggests a regulatory link. However, there are still several questions that will require further investigations and methodological improvements in order to be thoroughly answered.

One of these points concerns the identification of the molecular mechanism linking PTPRD with glucoregulatory pathways. Our observations in PHH showed that silencing of PTPRD induces a decreased phosphorylation of Akt, which is one of the main signal transducers mediating insulin function (Haeusler, McGraw et al. 2018). Given that PTPRD does not possess serine or threonine phosphatase activity (Uhl and Martinez 2019), any binding partner should be either **1**) an inducer of the insulin pathway which is itself negatively regulated by tyrosine phosphorylation, or **2**) an inhibitor of this pathway which is positively regulated by tyrosine phosphorylation. A potentially relevant substrate falling in this second category could be again tyrosine phosphorylated STAT3, which we find strongly upregulated in the livers of *Ptprd*+/- and *Ptprd*-/- mice (data not shown). Indeed, STAT3 signaling has been associated to the development of insulin resistance in diabetic patients (Mashili, Chibalin et al. 2013) or in patients presenting *STAT3* germline mutations which enhance its transcriptional activity (Flanagan, Haapaniemi et al. 2014). This hypothesis could be tested for example by the use of STAT3-inhibitors and insulin stimulation following silencing of PTPRD. If indeed STAT3 mediates the observed inhibition of the insulin

pathway, its inhibition would induce an increase of Akt phosphorylation to a level comparable to the control condition.

Our *in vivo* experiments employing the *Ptprd*+/- mice, showed that these animals present increased levels of fasting blood glucose following eight weeks of CD-HFD. After this time point, we did not observe any significant differences in blood glucose levels as compared to the *Ptprd*+/+ mice. Moreover, insulin and glucose tolerance tests were also negative following our observation of similar glucose levels between the two conditions (data not shown). These inconclusive results could be related to technical problems in our protocol. Indeed, this experiment was designed to test at the same time the potential regulatory role of PTPRD over glucose metabolic pathways and its previously described action as a tumor suppressor, hence the use of DEN and a CD-HFD. Although a choline-deficient diet is an important component of HCC animals models, it has been described to attenuate insulin resistance and glucose tolerance in mice (Raubenheimer, Nyirenda et al. 2006). This point could be addressed in the future by conducting a similar *in vivo* experiment but using a normal high-fat or western diet.

As described throughout this thesis, there is strong scientific rationale to pursue the development and clinical application of STAT3 inhibitors, but the question still remains as to why this is not the case in current medical practice? One reason why we have not observed a breakthrough in STAT3-targeting drugs so far may be that transcription factors are notoriously difficult to target and that many of the STAT3 inhibitors evaluated to date have shown to be problematic regarding their potency, bioavailability and specificity (Wong, Hirpara et al. 2017). Therefore, continued efforts need to be made by the scientific community and the pharmaceutical industry in order to surmount these challenges. Niclosamide for example, is an FDA-approved anthelmintic drug that has been described to present a poor bioavailability (Chen, Mook et al. 2018). More recently, niclosamide ethanolamine which is a niclosamide-derived salt, is characterized by a higher water solubility and has shown encouraging results in preclinical models by improving diabetic symptoms (Tao, Zhang et al. 2014), and slowing the growth of HCC tumors (Chen, Wei et al. 2017). Therefore, our data in the context of HCV infection represents a proof of principle highlighting the potential of STAT3 inhibition for the management of chronic liver disease. The evaluation of similar compounds could potentially result in a wide range of clinical applications, given the prevalence of hyperactivated STAT3 signaling in different cancer types (Huynh, Chand et al. 2019).

As a final remark, I would like to end this manuscript in a more personal note. It is evident that the cell signaling alterations involved in the development and progression of liver disease are numerous and complex. Although their characterization is a painstaking process, this task is a fundamental step in order to translate the biological insights gained into the clinic. No matter how big or small, I believe that the contributions that I have made during my doctoral studies and the work of other young researchers like myself, are valuable and will help to surmount the current challenges associated to these pathologies. This will lay the foundations for future chemo-preventive and therapeutic strategies that are urgently needed.

4.- Annexes

4.1.- Supplementary article I

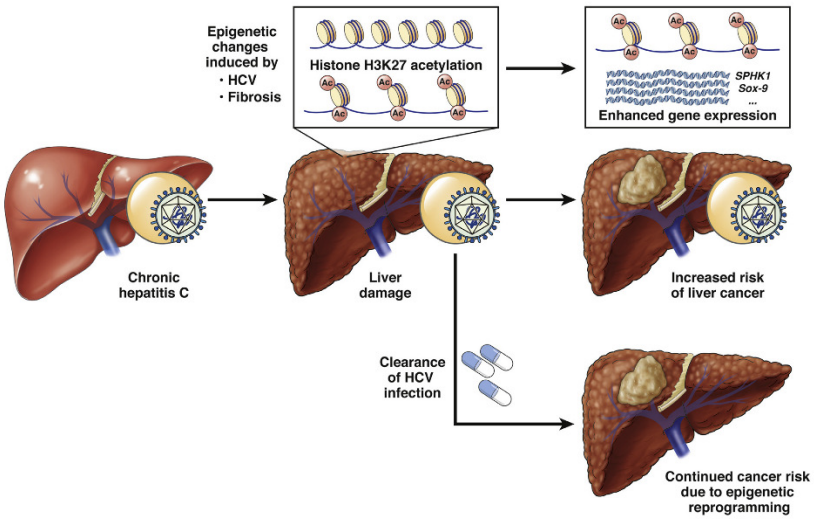


*Image from Lohmann and Bartenschlager 2019

Hamdane, N.; Juhling, F.; Crouchet, E.; El Saghire, H.; Thumann, C.; Oudot, M. A.; Bandiera, S.; Saviano, A.; Ponsolles, C.; **Roca Suarez, A. A.**; Li, S.; Fujiwara, N.; Ono, A.; Davidson, I.; Bardeesy, N.; Schmidl, C.; Bock, C.; Schuster, C.; Lupberger, J.; Habersetzer, F.; Doffoel, M.; Piardi, T.; Sommacale, D.; Imamura, M.; Uchida, T.; Ohdan, H.; Aikata, H.; Chayama, K.; Boldanova, T.; Pessaux, P.; Fuchs, B. C.; Hoshida, Y.; Zeisel, M. B.; Duong, F. H. T.; Baumert, T. F., HCV-induced Epigenetic Changes Associated With Liver Cancer Risk Persist After Sustained Virologic Response. *Gastroenterology* **2019**. doi: 10.1053/j.gastro.2019.02.038.

Gastroenterology 2019;156:2313–2329

HCV-Induced Epigenetic Changes Associated With Liver Cancer Risk Persist After Sustained Virologic Response

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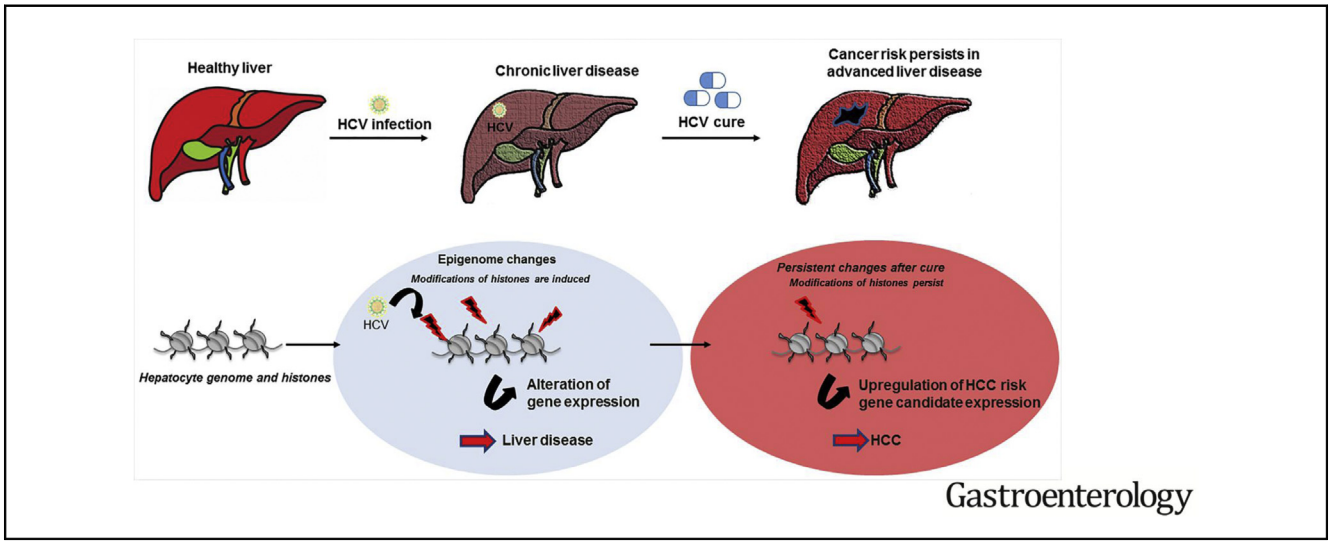


See editorial on page 2130. See Covering the Cover synopsis on 2118.

BACKGROUND & AIMS: Chronic hepatitis C virus (HCV) infection is an important risk factor for hepatocellular carcinoma (HCC). Despite effective antiviral therapies, the risk for HCC is decreased but not eliminated after a sustained virologic response (SVR) to direct-acting antiviral (DAA) agents, and the risk is higher in patients with advanced fibrosis. We investigated HCV-induced epigenetic alterations that might affect risk for HCC after DAA treatment in patients and mice

with humanized livers. **METHODS:** We performed genome-wide ChIPmentation-based ChIP-Seq and RNA-seq analyses of liver tissues from 6 patients without HCV infection (controls), 18 patients with chronic HCV infection, 8 patients with chronic HCV infection cured by DAA treatment, 13 patients with chronic HCV infection cured by interferon therapy, 4 patients with chronic hepatitis B virus infection, and 7 patients with nonalcoholic steatohepatitis in Europe and Japan. HCV-induced epigenetic modifications were mapped by comparative analyses with modifications associated with other liver disease etiologies. uPA/SCID mice were engrafted with human hepatocytes to create mice with humanized livers and given injections of HCV-infected serum samples from patients; mice were given DAAs to eradicate the virus. Pathways associated with HCC risk were identified by integrative pathway analyses and validated in analyses of paired HCC tissues from 8 patients with an SVR to DAA treatment of HCV infection. **RESULTS:** We found chronic HCV infection to induce specific genome-wide changes in H3K27ac, which correlated with changes in expression of mRNAs and proteins. These changes persisted after an SVR to DAAs or interferon-based therapies. Integrative pathway analyses of liver tissues from patients and mice with humanized livers demonstrated that HCV-induced epigenetic alterations were associated with liver cancer risk. Computational analyses associated increased expression of SPHK1 with HCC risk. We validated these findings in an independent cohort of patients with HCV-related cirrhosis (n = 216), a subset of which (n = 21) achieved viral clearance. **CONCLUSIONS:** In an analysis of liver tissues from patients with and without an SVR to DAA therapy, we identified epigenetic and gene expression alterations associated with risk for HCC. These alterations might be targeted to prevent liver cancer in patients treated for HCV infection.

Keywords: Biomarker; Biopsy; Chemoprevention; Sox9.

WHAT YOU NEED TO KNOW

BACKGROUND AND CONTEXT

Despite effective antiviral therapies, the risk for HCC is not eliminated following a sustained virologic response to direct-acting antiviral (DAA) agents, and risk is higher in patients with advanced fibrosis.

NEW FINDINGS

In an analysis of liver tissues from patients with and without a sustained virologic response to DAA therapy, and from HCV-infected mice with humanized livers, the authors identified epigenetic and gene expression alterations associated with risk for HCC.

LIMITATIONS

This was a retrospective analysis of liver tissues from patients and mice.

IMPACT

The epigenetic alterations identified in this study might be targeted to prevent liver cancer in patients treated for HCV infection.

Chronic hepatitis C virus (HCV) infection is a leading cause of hepatocellular carcinoma (HCC), the second most common and fastest rising cause of cancer-related death.[1] The development of direct-acting antivirals (DAAs) with cure rates of higher than 90% has been a major breakthrough in the management of patients with chronic HCV infection. However, although viral cure decreases the overall HCC risk in HCV-infected patients, it does not eliminate virus-induced HCC risk, especially in patients with advanced fibrosis.[2,3] Furthermore, convenient biomarkers to robustly predict HCC risk after viral cure and strategies for HCC prevention are absent.[2] These unexpected findings pose new challenges for patient management.[4–6]

Despite more than 2 decades of intensive research efforts, the pathogenesis of HCV-induced HCC and the HCC risk after DAA cure are still incompletely understood.[6,7] Although HCV is an RNA virus with little potential for integrating its genetic material into the host genome, HCV contributes to hepatocarcinogenesis through a direct and an indirect way. HCV-mediated liver disease and carcinogenesis are considered multistep processes that include chronic infection-driven hepatic inflammation and progressive liver fibrogenesis with formation of neoplastic clones that arise and progress in the carcinogenic tissue microenvironment.[4,6,8] A 186-gene expression signature in liver tissue of HCV-infected patients has been associated with HCC risk and mortality, suggesting that virus-induced transcriptional reprogramming in the liver could play a functional role in hepatocarcinogenesis.[9,10]

Epigenetic modifications of histones can lead to chromatin opening and compacting and play a major role in gene regulation in health and disease.[11] Although epigenetic changes have been identified in established HCC,[12] their role in viral hepatocarcinogenesis remains largely unknown.

Methods

Human Subjects

Liver tissues from patients undergoing surgical resection or biopsy examination were collected at the Gastroenterology and Hepatology Clinic of the Hiroshima University Hospital (Hiroshima, Japan), the Basel University Hospital (Basel, Switzerland), the Centre Hospitalier Universitaire de Reims (Reims, France), and the Hôpitaux Universitaires de Strasbourg (Strasbourg, France). Protocols for patient tissue collection

***Authors share co-first authorship.**

Abbreviations used in this paper: **DAA, direct-acting antiviral; FC, fold change; GSEA, gene set enrichment analysis; HCC, hepatocellular carcinoma; HBV, hepatitis B virus; HCV, hepatitis C virus; IFN, interferon; mTOR, mammalian target of rapamycin; NASH, nonalcoholic steatohepatitis; PCA, principal component analysis; PLS, prognostic liver signature; SVR, sustained virologic response; TNFα, tumor necrosis factor α; TSG, tumor suppressor gene.**

Most current article

0016-5085
https://doi.org/10.1053/j.gastro.2019.02.038

were reviewed and approved by the hospital ethics committees. Written and informed consent was obtained from all patients. Eligible patients were identified by a systematic review of patient charts. Histopathologic grading and staging of HCV liver biopsy specimens, according to the METAVIR classification system, were performed at the pathology institutes of the respective university hospitals. Overall, we analyzed liver tissue from 6 noninfected control patients, 18 patients with chronic HCV infection, 8 patients with DAA-cured chronic HCV, 13 patients with interferon (IFN)-cured chronic HCV, 4 patients with hepatitis B virus (HBV) infection, and 7 patients with nonalcoholic steatohepatitis (NASH). Furthermore, we studied 8 paired HCC samples with HCV-induced liver disease (Table 1).

HCV Infection of Human Hepatocyte Chimeric Mice and DAA Treatment

cDNA-uPA$^{+/+}$/SCID$^{+/+}$ (uPA/SCID) mice were engrafted with human hepatocytes and intravenously inoculated with serum samples containing approximately 10^5 HCV particles. HCV-infected mice were treated with a combination of MK-7009 and BMS-788329 DAAs.[13] Elimination of HCV in treated mice was confirmed by the absence of HCV viremia 12 weeks after cessation of therapy. See the Supplementary Materials for further details.

ChIPmentation-Based ChIP-Seq

ChIPmentation-based ChIP-Seq on liver tissue using H3K27ac antibody (number 39134, Activ Motif , La Hulpe, Belgium) was performed as described previously[14] and adapted as follows. To perform ChIP-Seq on human and mouse livers, tissues were cut in small pieces of 2–3 mm, crosslinked with 0.4% formaldehyde for 10 minutes at room temperature, and quenched with glycine 125 mmol/L for 5 minutes at room temperature. Then, tissue was homogenized using a glass potter and ChIPmentation was performed as described previously.[14]

Processing of Raw ChIPmentation Data

Reads were aligned to the human genome (hg19) and peaks were called in uniquely mapped reads using MACS2.[8] Peaks within all samples were intersected and used for counting reads if they overlapped in at least 2 samples. Read counts of genes were defined as the sum of all reads in peak regions overlapping the gene body or the promoter region, that is, the region up to 1500 bp ahead of the transcription start site. See the Supplementary Materials for further details.

RNA Extraction and Next-Generation Sequencing

Liver tissues were lysed in TRI-reagent (Molecular Research Center; Cincinnati, OH) and RNA was purified using Direct-zol RNA MiniPrep (Zymo Research, Irvine, CA) or RNeasy kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. RNA quantity and quality were assessed using NanoDrop (Thermo Scientific, Waltham, MA) and Bioanalyzer 2100 (Illumina, San Diego, CA). Libraries of extracted RNA were prepared and sequenced as described previously.[3,9]

Processing of RNA-Seq Data

Reads were counted with htseq-count, and a differentially expression analysis was performed with DESeq2 applying GENCODE 19.[15] Reads were taken from our RNA-Seq experiments as described earlier and from external sources: RNA-Seq from infected (low ISG) vs control patients was retrieved from the GEO dataset GSE84346 (low ISG samples). See the Supplementary Materials for further details.

Pathway Enrichment and Correlation Analyses

Pathway enrichment analyses were performed using gene set enrichment analysis (GSEA) with all gene sets included in MSigDB 6.0.[16] We used the pre-ranked version of GSEA and genes were ranked for *P* values of differential expression and modification analyses. Figures showing enriched pathways and gene sets, Spearman correlations, and oncogene $\log_2$ fold change (FC) were drawn using ggplot2 and the R environment (R Foundation, Vienna, Austria). Gene network analysis was performed based on 3 MSigDB subsets: Hallmark gene sets, curated gene sets, and gene ontology gene sets. See the Supplementary Materials for further details.

Western Blot

Expression of SPHK1 and SOX9 proteins was assessed by western blot and quantified using ImageJ software (National Institutes of Health, Bethesda, MD). See the Supplementary Materials for further details.

Association of Hepatic Gene Expression With Prognostic Cox Score for Overall Death

Prognostic association of hepatic gene expression was determined using the Cox score for time to overall death in HCV-infected patients with advanced liver disease and HCC as previously described.[17]

Gene Expression and Assessment of HCC Risk in HCV Cohorts

Patients with early-stage HCV cirrhosis (n = 216[10]; GSE15654) and a subgroup of patients who had achieved a sustained virologic response (SVR) before the biopsy (n = 21) were classified into SPHK1-high and -low expression groups based on the cutoff value of 1 sample standard deviation above the mean. Cumulative probabilities of HCC development were calculated using the Kaplan-Meier procedure and compared by log-rank test.

Data Availability

The Sequence Read Archive accession number for the data reported in this study is SRP170244.

Results

Virus-Induced Modifications of Histone Mark H3K27ac Persist in Human Liver After DAA Cure in HCV-Infected Patients

To investigate whether chronic HCV infection triggers persistent epigenetic modifications after cure, we performed a genome-wide analysis using ChIPmentation-based

Table 1. Characteristics of Studied Patients

	Biopsy ID	Sex	Age	Diagnosis	Viral genotype	Viral load (*IU/mL*)	METAVIR grade	METAVIR stage	Antiviral treatment
Controls	C1	F	55	Minimal hepatitis	N/A	N/A	N/A	F0	N/A
	C2	M	46	Minimal hepatitis	N/A	N/A	N/A	F0	N/A
	C3	F	40	Lobular hepatitis	N/A	N/A	N/A	F0	N/A
	C4	F	53	Minimal hepatitis	N/A	N/A	N/A	F0	N/A
	C5	M	56	Lobular hepatitis	N/A	N/A	N/A	F0	N/A
	C6	F	58	Minimal hepatitis	N/A	N/A	N/A	F0	N/A
	C7	F	51	Chronic indeterminate hepatitis	N/A	N/A	N/A	F3	N/A
	C8	F	37	Acute partially cholestatic hepatitis	N/A	N/A	N/A	F0	N/A
	C9	F	44	Cholestatic hepatitis	N/A	N/A	N/A	F1	N/A
	C10	M	78	Adjacent liver from CCM resection	N/A	N/A	N/A	N/A	N/A
	C11	F	58	Adjacent liver from CCM resection	N/A	N/A	N/A	N/A	N/A
	C12	F	70	Adjacent liver from CCM resection	N/A	N/A	N/A	N/A	N/A
	C13	M	63	Adjacent liver from CCM resection	N/A	N/A	N/A	N/A	N/A
	C14	M	70	Adjacent liver from CCM resection	N/A	N/A	N/A	N/A	N/A
	C15	F	69	Adjacent liver from CCM resection	N/A	N/A	N/A	N/A	N/A
	C16	M	53	Adjacent liver from CCM resection	N/A	N/A	N/A	N/A	N/A
	C17	M	71	Adjacent liver from CCM resection	N/A	N/A	N/A	N/A	N/A
HBV	B1	F	46	HBV	N/A	N/A	N/A	F4	NUC
	B2	M	65	HBV and HCC	N/A	N/A	N/A	F4	NUC
	B3	M	57	HBV and HCC	N/A	N/A	N/A	F4	NUC
	B4	M	58	HBV and HCC	N/A	N/A	N/A	F4	NUC
NASH	N1	M	27	NASH and HCC	N/A	N/A	N/A	F4	N/A
	N2	M	63	NASH and HCC	N/A	N/A	N/A	F4	N/A
	N3	M	73	NASH and HCC	N/A	N/A	N/A	F4	N/A
	N4	M	76	NASH and HCC	N/A	N/A	N/A	F4	N/A
	N5	F	65	NASH and HCC	N/A	N/A	N/A	F4	N/A
	N6	F	47	NASH and HCC	N/A	N/A	N/A	F4	N/A
	N7	F	68	NASH and HCC	N/A	N/A	N/A	F4	N/A
HCV infected	H1	F	62	Chronic HCV	1a	5140000	A1	F1	Naïve
	H2	M	44	Chronic HCV	1a	7.41E + 06	A1	F2	Naïve
	H3	F	23	Chronic HCV	3a	2.46E + 02	A2	F2	Naïve
	H4	F	60	Chronic HCV	2	2.70E + 06	A2	F2	Naïve
	H5	M	23	Chronic HCV	1a	1.76E + 06	A1	F1	Intolerant to Peg-IFN/RBV
	H6	M	48	Chronic HCV	1a	5.93E + 06	A1	F2	Naïve
	H7	F	38	Chronic HCV	1b	7.95E + 05	A1	F2	Naïve
	H8	M	58	Chronic HCV	4	4.08E + 06	A3	F2	Nonresponder to Peg-IFN/RBV
	H9	M	52	Chronic HCV	1a	6.60E + 05	A3	F3	Naïve
	H10	M	54	Chronic HCV and HCC	1b	4.40E + 04	A1	F4	Relapse to SOF/DCV/RBV
	H11	M	68	Chronic HCV and HCC	2a	2.51E + 05	A3	F3	Naïve

Table 1. Continued

	Biopsy ID	Sex	Age	Diagnosis	Viral genotype	Viral load (*IU/mL*)	METAVIR grade	METAVIR stage	Antiviral treatment
	H12	M	51	Chronic HCV	3a	3.30E + 06	A2	F1	Naïve
	H13	M	54	Chronic HCV	4	3.31E + 06	A2	F1	Naïve
	H14	F	48	Chronic HCV	3a	1.15E + 06	A3	F4	Naïve
	H15	M	65	Chronic HCV	1b	2.25E + 06	A2	F4	Naïve
	H16	M	81	Chronic HCV and HCC	1b	1.85E + 06	A1	F1	Nonresponder to Peg-IFN/RBV
	H17	M	51	Chronic HCV and HCC	3a	3.79E + 06	A2	F4	Relapse to SOF/RBV
	H18	F	71	Chronic HCV and HCC	1b	3.93E + 06	A1	F1	Naïve
	H19	F	49	Chronic HCV	3a	3.50E + 06	A3	F4	Naïve
	H20	M	34	Chronic HCV	N/A	2.21E + 06	A3	F4	Naïve
	H21	M	53	Chronic HCV	1	1.35E + 06	A3	F4	Naïve
	H22	F	62	Chronic HCV	N/A	6.10E + 06	A3	F4	Naïve
	H23	F	59	Chronic HCV	4	2.68E + 06	A3	F4	Naïve
	H24[a]	M	79	Chronic HCV and HCC	1b	2.00E + 06	A2	F2	N/A
	H25[a]	M	56	Chronic HCV and HCC	1b	2.00E + 06	A3	F4	N/A
	H26[a]	F	79	Chronic HCV and HCC	1b	5.01E + 05	A2	F4	N/A
	H27[a]	M	85	Chronic HCV and HCC	1b	3.16E + 05	A3	F3	N/A
	H28[a]	M	64	Chronic HCV and HCC	2b	1.00E + 07	A2	F4	N/A
	H29[a]	F	76	Chronic HCV and HCC	1b	6.31E + 06	A2	F4	N/A
	H30[a]	F	84	Chronic HCV and HCC	1b	5.01E + 04	A2	F3	N/A
	H31[a]	M	61	Chronic HCV and HCC	1b	3.98E + 04	A2	F2	N/A
HCV cured	D1[a]	M	65	Cured HCV and HCC	1b	Undetectable	A0	F2	SOF/DCV
	D2[a]	M	58	Cured HCV and HCC	1a	Undetectable	A0	F4	SOF/LDV
	D3[a]	F	79	Cured HCV and HCC	1b	Undetectable	A2	F4	DCV/ASV
	D4[a]	M	63	Cured HCV and HCC	2a	Undetectable	A2	F4	SOF/RBV
	D5[a]	M	69	Cured HCV and HCC	1b	Undetectable	A2	F3	DCV/ASV
	D6[a]	M	73	Cured HCV and HCC	1b	Undetectable	A2	F3	DCV/ASV
	D7[a]	M	75	Cured HCV and HCC	1b	Undetectable	A2	F3	SOF/LDV
	D8[a]	F	75	Cured HCV and HCC	1b	Undetectable	A2	F3	SOF/LDV
	D9[a]	M	71	Cured HCV and HCC	1B	Undetectable	A3	F2	DCV/ASV
	D10[a]	M	73	Cured HCV and HCC	1B	Undetectable	A2	F3	DCV/ASV
	D11[a]	F	76	Cured HCV and HCC	1B	Undetectable	A2	F2	DCV/ASV
	D12[a]	M	61	Cured HCV and HCC	2A	Undetectable	A2	F3	SOF/RBV
	D13[a]	F	71	Cured HCV and HCC	1B	Undetectable	A2	F4	DCV/ASV
	D14[a]	M	79	Cured HCV and HCC	1B	Undetectable	N/A	N/A	DCV/ASV
	D15[a]	M	64	Cured HCV and HCC	1B	Undetectable	A2	F3	SOF/LDV
	D16[a]	M	78	Cured HCV and HCC	1B	Undetectable	A1	F1	SOF/LDV
	I1	M	68	Cured HCV and HCC	2A	Undetectable	N/A	F3	Peg-IFN/RBV
	I2	M	61	Cured HCV and HCC	2A	Undetectable	A2	F4	Peg-IFN/RBV
	I3	F	74	Cured HCV and HCC	2B	Undetectable	A2	F3	IFN/RBV
	I4	M	69	Cured HCV and HCC	1B	Undetectable	A1	F2	Peg-IFN/RBV
	I5	M	66	Cured HCV and HCC	2B	Undetectable	A2	F4	IFN

Table 1. Continued

Biopsy ID	Sex	Age	Diagnosis	Viral genotype	Viral load (IU/mL)	METAVIR grade	METAVIR stage	Antiviral treatment
I6	F	68	Cured HCV and HCC	1B	Undetectable	A2	F2	IFN
I7	M	54	Cured HCV and HCC	1B	Undetectable	A2	F4	Peg-IFN/RBV
I8	M	66	Cured HCV and HCC	1B	Undetectable	A1	F3	IFN
I9	M	74	Cured HCV and HCC	2A	Undetectable	A2	F1	Peg-IFN
I10	M	80	Cured HCV and HCC	1B	Undetectable	A1	F2	Peg-IFN/RBV
I11	F	77	Cured HCV and HCC	1B	Undetectable	A1	F4	Peg-IFN/RBV
I12	M	70	Cured HCV and HCC	1B	Undetectable	A1	F1	Peg-IFN
I13	M	65	Cured HCV and HCC	1B	Undetectable	A2	F2	Peg-IFN/RBV

NOTE. Biopsy identification number, sex, age, pathologic diagnosis, HCV genotype and load, antiviral treatment (for HCV-infected and HCV-cured patients), and METAVIR grade (when applicable) and score are presented.
ASV, asunaprevir; CCM, colon cancer metastasis; DCV, daclatasvir; F, female; IU, international unit; LDV, ledipasvir; M, male; N/A, not applicable; NUC, nucleos(t)ide analogues; Peg, pegylated; RBV, ribavirin; SOF, sofosbuvir.
[a]Paired analysis of HCC and nontumor tissue.

ChIP-Seq[14] profiling the well-characterized histone modification H3K27ac in liver tissues from 18 patients with chronic HCV infection, 21 patients with DAA- or IFN-based curative therapy, and 6 noninfected controls (Figure 1*A* and Table 1). The H3K27ac modification is associated with active promoters and enhancers and with activation of transcription.[18] We observed significant changes in specific H3K27ac modifications in HCV-infected patients compared with noninfected controls (Figures 1*B* and Supplementary Figure 1). To study whether these were etiology specific, we performed comparative analyses of liver tissues with chronic HCV infection (n = 18), chronic HBV infection (n = 4), and NASH (n = 7). Using principal component analysis (PCA), we found that the distribution of H3K27ac changes in the epigenome of livers of noninfected, HCV-infected, HBV-infected, and NASH samples formed distinct clusters on the PCA plot, suggesting that an important part of the changes are etiology specific (Figure 2*A*). Next, we performed a correlation analysis of H3K27ac changes among HCV-infected, HBV-infected, and NASH samples. Our data showed a positive correlation of H3K27ac changes (Figure 2*B*) among patients with NASH ($r = 0.83$; $P < 10^{-10}$), or patients with HBV infection ($r = 0.79$; $P < 10^{-10}$), or HCV infection, suggesting that some epigenetic modifications are shared among etiologies. To analyze the impact of epigenetic changes in genes related to immune responses, we extracted immune-related genes from MSigDB and performed a restricted correlation study that showed lower correlation coefficients (NASH vs HCV, $r = 0.75$, $P < 10^{-10}$; HBV vs HCV, $r = 0.62$, $P < 10^{-10}$) compared with analyses composed of all genes (Supplementary Figure 2). These findings suggest that epigenetic modifications in immune genes associated with inflammatory responses are only partly responsible for the similarities between etiologies.

Recent studies have reported a correlation between fibrosis and an increased incidence of HCC.[6] However, the molecular mechanism of fibrosis-induced HCC is not well understood. Our comparative analysis showed that H3K27ac modifications, separated based on fibrosis score along the primary component (dimension 1), accounted for 42% of the variation between samples. This suggests that a substantial fraction of the observed H3K27ac alterations is related to liver fibrosis. Interestingly, we did not observe any significant correlation between these epigenetic changes and the activity score (ie, reflecting liver inflammation), suggesting that aberrant H3K27 acetylation is less dependent of necro-inflammatory activity but rather dependent on the fibrosis stage (Figure 1*B*).

By comparing H3K27ac modifications in liver tissue with chronic HCV infection before DAA treatment and in liver tissue with successful DAA cure, we studied whether epigenetic changes persisted in cured patients. Interestingly, we found a significant and positive correlation of H3K27ac modifications after comparing HCV-infected and DAA-cured samples ($r = 0.87$; $P < 10^{-10}$; Figure 2*C*). A comparative analysis showed a strong positive correlation between epigenetic changes in liver samples of DAA-cured and IFN-cured patients ($r = 0.91$; $P < 10^{-10}$; Supplementary Figure 1*B*), suggesting that HCV-

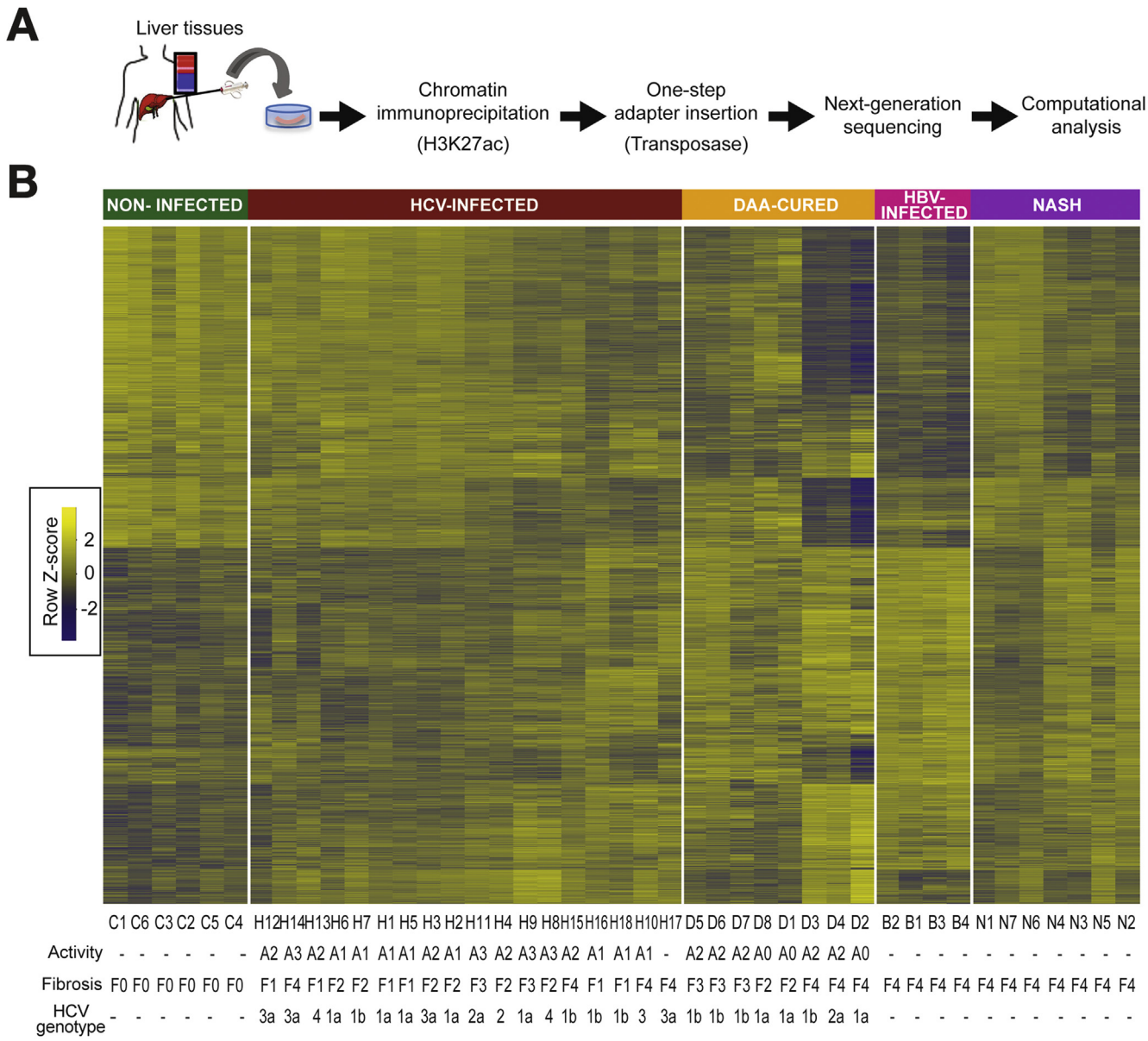
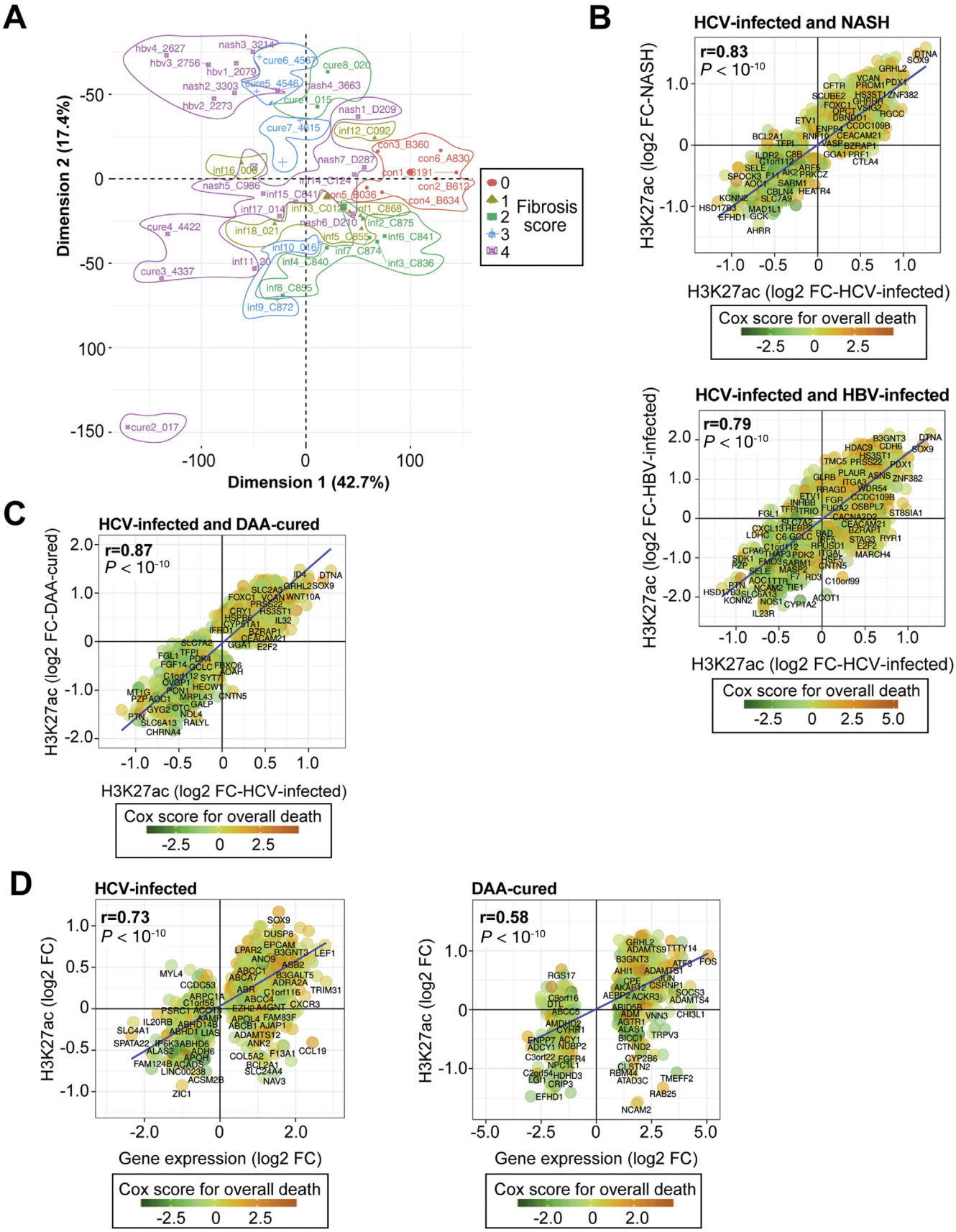


Figure 1. HCV-induced epigenetic changes persist after HCV clearance in patient-derived liver tissue. (*A*) Approach: HCV-induced H3K27ac histone modifications were measured genome-wide using a ChIPmentation-based ChIP-Seq protocol optimized for low input material such as patient-derived liver biopsy samples and resections. (*B*) Unsupervised clustering of normalized read counts in ChIP-Seq peaks of 12,700 genes linked with significant ($P < .05$) H3K27ac modifications in HCV-infected (n = 18), DAA-cured (n = 8), HBV-infected (n = 4), or NASH (n = 7) vs noninfected control (n = 6) patients.

induced epigenetic changes persist after DAA- and IFN-based therapies.

To address the potential clinical relevance, we next analyzed genes that were epigenetically modulated by HCV infection by integrating ChIP-Seq data and by assigning a gene expression–based Cox score for overall death based on the clinical outcome of a cohort of 216 HCV-induced cirrhotic patients who later developed HCC.[10] We chose this score because it is has been shown to robustly predict clinical outcome of patients with advanced HCV liver disease.[10] Importantly, we found that persistent H3K27ac modifications were linked with genes associated with a high Cox score for overall death in HCV-infected patients and advanced liver disease[17] (Figure 2*C*), confirming the clinical impact of these findings. Next, we compared H3K27ac enrichment and transcriptomic changes in HCV-infected and in DAA-cured patients. We found a positive correlation between H3K27ac and gene expression changes in HCV-infected and DAA-cured patients ($r = 0.73$; $P < 10^{-10}$ and $r = 0.58$; $P < 10^{-10}$, respectively; Figure 2*D*), supporting the functional relevance of these epigenetic changes for the deregulation of gene transcription that persists after cure.


A
Dimension 2 (17.4%)
Dimension 1 (42.7%)
Fibrosis score
0
1
2
3
4
B
HCV-infected and NASH
r=0.83
P < 10-10
H3K27ac (log2 FC-NASH)
H3K27ac (log2 FC-HCV-infected)
Cox score for overall death
HCV-infected and HBV-infected
r=0.79
P < 10-10
H3K27ac (log2 FC-HBV-infected)
H3K27ac (log2 FC-HCV-infected)
Cox score for overall death
C
HCV-infected and DAA-cured
r=0.87
P < 10-10
H3K27ac (log2 FC-DAA-cured)
H3K27ac (log2 FC-HCV-infected)
Cox score for overall death
D
HCV-infected
r=0.73
P < 10-10
H3K27ac (log2 FC)
Gene expression (log2 FC)
Cox score for overall death
DAA-cured
r=0.58
P < 10-10
H3K27ac (log2 FC)
Gene expression (log2 FC)
Cox score for overall death

Persistent Epigenetic Changes Are Associated With Liver Carcinogenesis After Cure

Epigenetic regulation is an indispensable process for normal development and preservation of tissue-specific gene expression profiles. Thus, any perturbation in the epigenetic landscape can lead to shifted gene function and malignant cellular transformation. We addressed the potential functional role of the observed alterations for virus-induced liver disease and hepatocarcinogenesis by performing a pathway enrichment analysis of genes associated with H3K27ac changes in liver tissues from HCV-infected and cured patients. We found that chronic HCV infection induces significant epigenetic H3K27ac changes on genes that belong to pathways related to tumor necrosis factor α (TNFα), inflammatory response, and interleukin 2 and signal transducer and activator of transcription 5 signaling (Figure 3*A*). Furthermore, we observed lower levels of H3K27ac within genes related to pathways associated with coagulation and metabolism, such as oxidative phosphorylation, fatty acid metabolism, or adipogenesis (Figure 3*A*). Remarkably, several altered pathways persisted after cure (eg, TNFα signaling, inflammatory response, G2M checkpoint, epithelial–mesenchymal transition, and phosphoinositide 3-kinase, Akt, and mammalian target of rapamycin [mTOR]; Figure 3*A*). We also observed lower levels of H3K27ac mapping to genes related to oxidative phosphorylation pathways (Figure 3*A*). Overall, our data provide evidence supporting a functional role for H3K27ac changes in establishing gene expression patterns that persist after cure and contribute to carcinogenesis.

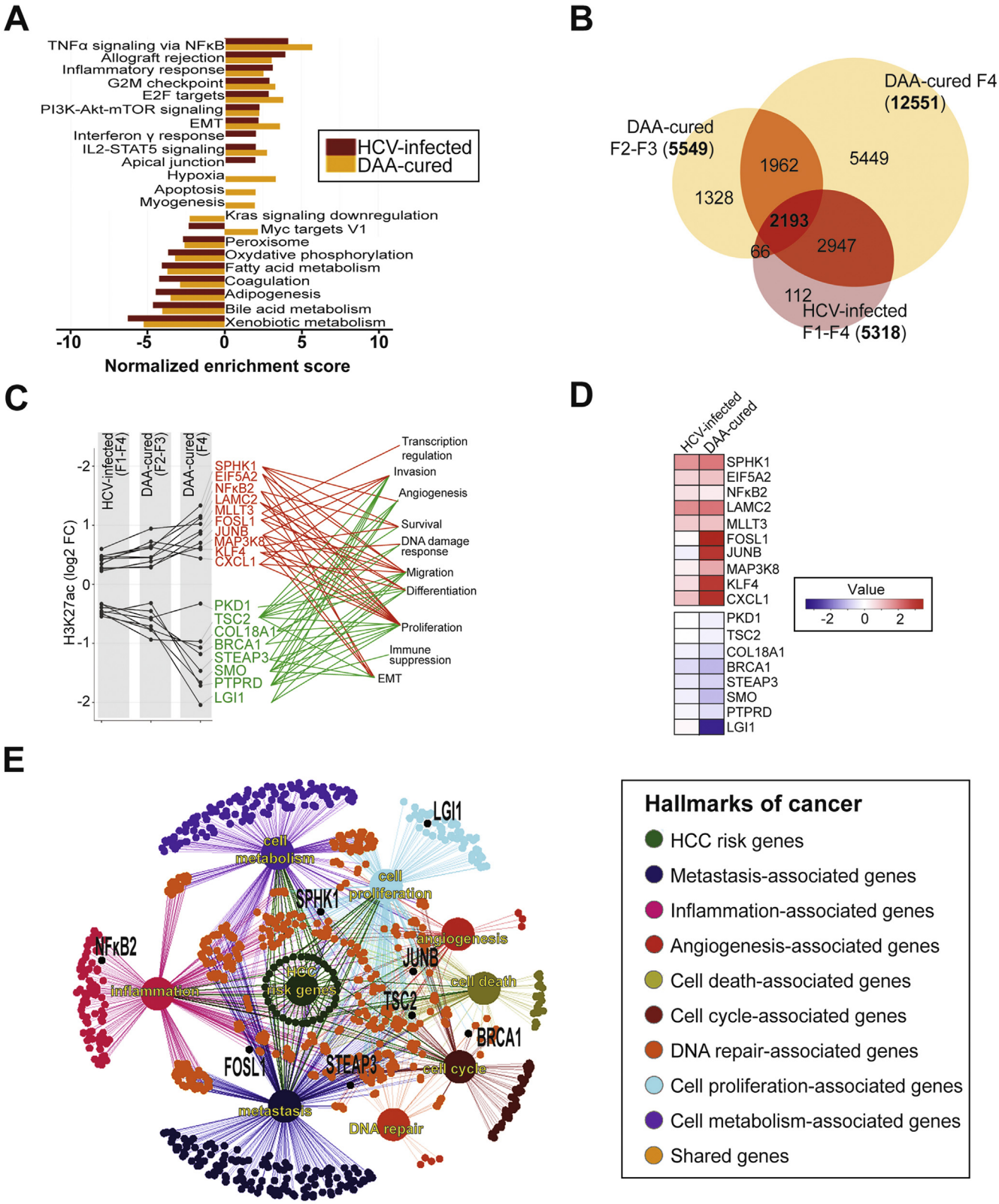
We proceeded to study the impact of fibrosis on persistence of epigenetic modifications. Our analysis showed that H3K27ac changes observed in HCV-infected patients were partly reversed in cured patients with stage F2–3 fibrosis. This group shared 2259 of the 5318 (42.5%) modified genes in the HCV-infected group (Figure 3*B*). In contrast, in DAA-cured patients with advanced liver disease (F4), the HCV-induced H3K27ac changes largely persisted. The HCV-infected group shared nearly all modified genes (96.6%, 5140 of 5318 genes) with F4 cured patients (Figure 3*B*). Collectively, we identified significant changes of H3K27ac levels on 2193 genes persisting in the 2 DAA-cured patient groups (Figure 3*B* and Supplementary Table 1). Among these candidates, we identified oncogenes and tumor suppressor genes (TSGs) that are associated with, respectively, increased or decreased levels of H3K27ac (Figure 3*C*). These alterations were even more pronounced in patients with advanced fibrosis (Figure 3*C*), correlating with an enhanced risk for developing HCC in F4 vs F2–F3.[2,3] Importantly, we found a clear correlation between transcriptomic and epigenomic changes of the identified oncogenes and TSGs, supporting the biological relevance of the findings (Figure 3*D*). Among these oncogenes was *SPHK1*, a lipid kinase mediating the phosphorylation of sphingosine to form SP1, which is a major regulator of cell apoptosis inhibition and proliferation promotion. SPHK1 and SP1 play key roles in the TNFα and nuclear factor κB signaling pathways.[19] SPHK1 expression is increased and associated with tumor size and progression in patients with HCC.[20] Among the TSGs with significantly decreased H3K27ac level in HCV-infected patient livers were *PTPRD*, *TSC2*, and the major regulator of DNA repair, *BRCA1*. PTPRD has been identified as a candidate tumor suppressor in the liver impaired by HCV infection.[21] TSC2 has been reported to be a negative regulator of the mTOR signaling pathway. Its down-regulation is associated with metabolic defects, liver disease progression, and carcinogenesis.[7] Collectively, the overexpressed oncogenes and down-regulated TSGs that are enriched or decreased for the H3K27ac mark in chronic HCV infection, respectively, are involved in processes that favor carcinogenesis.

To further confirm that the persistent H3K27ac changes are linked to HCC risk, we referred to the genes of the recently reported 186-gene prognostic liver signature (PLS) and a 32-gene subset thereof for predicting liver disease progression, HCC development, and death for all HCC etiologies.[9,17,22] We analyzed functional links, that is, commonly shared pathways in MsigDB, among the 32-gene set, the 2193 genes with persistent epigenetic and transcriptional modifications, and the hallmarks of cancer.[23] We found that 1411 of the identified genes are closely connected to the PLS through shared pathways. Then, we assigned categories related to the hallmarks of cancer to the deregulated genes to understand the pathophysiologic impact of chronic HCV infection. Our analyses showed that approximately 900 genes of the genes with epigenetic modifications are directly linked with carcinogenesis. A network of these genes associated with at least 1 hallmark of cancer is shown in Figure 3*E*.

Next, we investigated whether H3K27ac alterations persist in cancer tissues after cure. We performed pairwise comparison of HCC and adjacent nontumorous tissue from the individual DAA-treated patient. We found a genome-wide H3K27ac enrichment in adjacent nontumorous and in tumorous tissues compared with noninfected samples (Figure 4). Deeper analysis showed that 52% of H3K27ac enriched genes are specific to tumorous tissues, 31% are

Figure 2. HCV-infection induces specific epigenetic changes in the liver of HCV-infected patients. (*A*) PCA for control, noninfected, HCV-infected, DAA-cured, IFN-cured, HBV-infected, and NASH patient samples. Comparative analysis of epigenetic modifications separated based on fibrosis score along the primary component (dimension 1). (*B*) H3K27ac modifications among HCV-infected patients correlate (Spearman rank correlation coefficients and *P* values) with H3K27ac modifications among NASH or HBV-infected patients. Common H3K27ac modifications were analyzed. Prognostic association of hepatic gene expression was determined by using Cox score for time to overall death in a cohort of patients as previously described.[17] (*C*) HCV-induced and persistent epigenetic changes after DAA cure in patient-derived liver tissue are associated with a decreased survival and death. H3K27ac modifications among HCV-infected correlate with persistent H3K27ac modifications among DAA-cured patients. (*D*) H3K27ac modifications correlate with significantly differentially expressed genes in HCV-infected and DAA-cured patients.

specific to adjacent nontumorous tissues, and 17% are common to the paired tissue. These data suggest that epigenetic alterations persist from advanced fibrosis to HCC and therefore could play a pathogenic role in hepatocarcinogenesis before and after cure. Furthermore, the presence of epigenetic modifications in adjacent tumor tissue suggests that the epigenetic modifications might precede hepatocarcinogenesis.



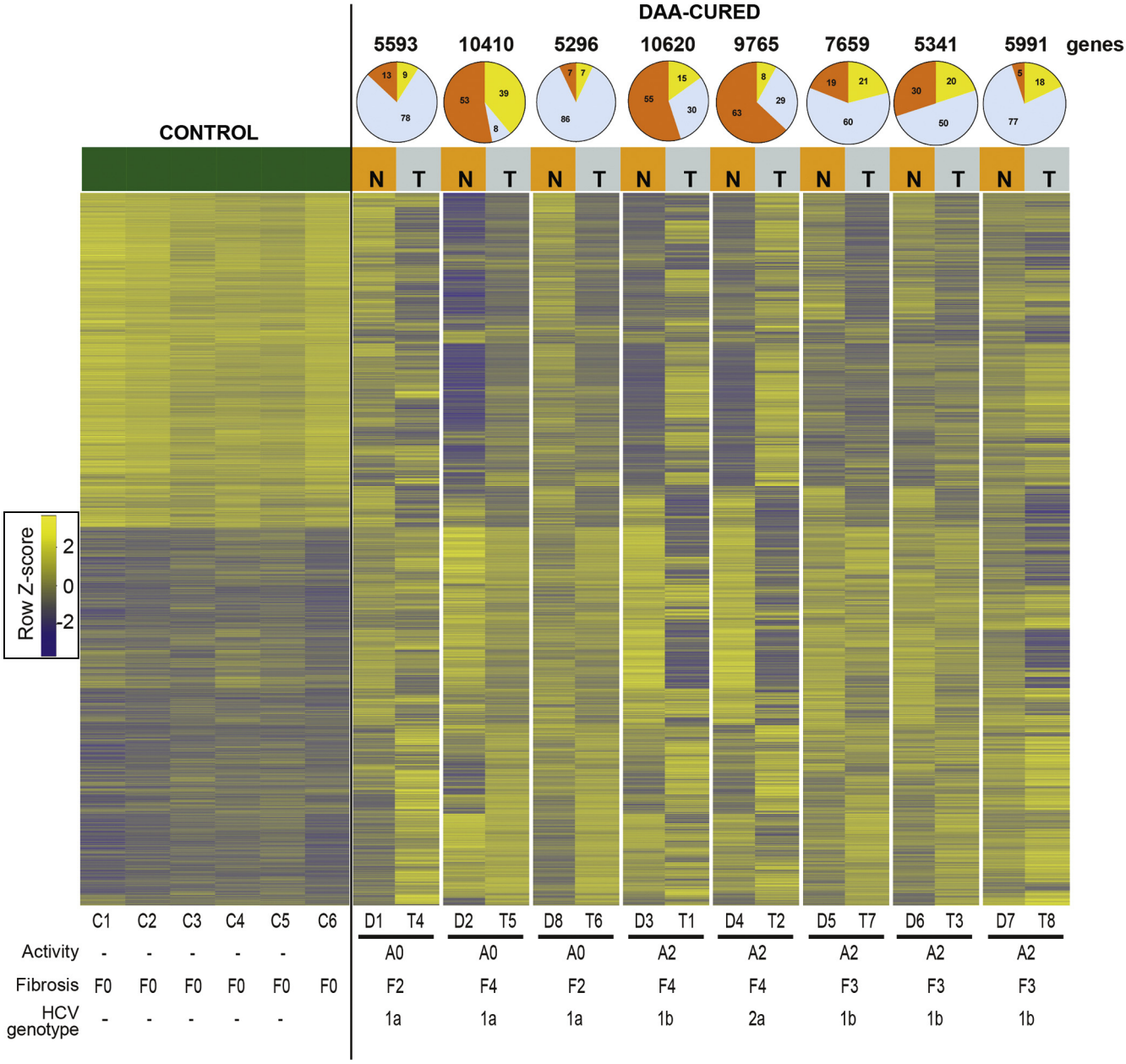
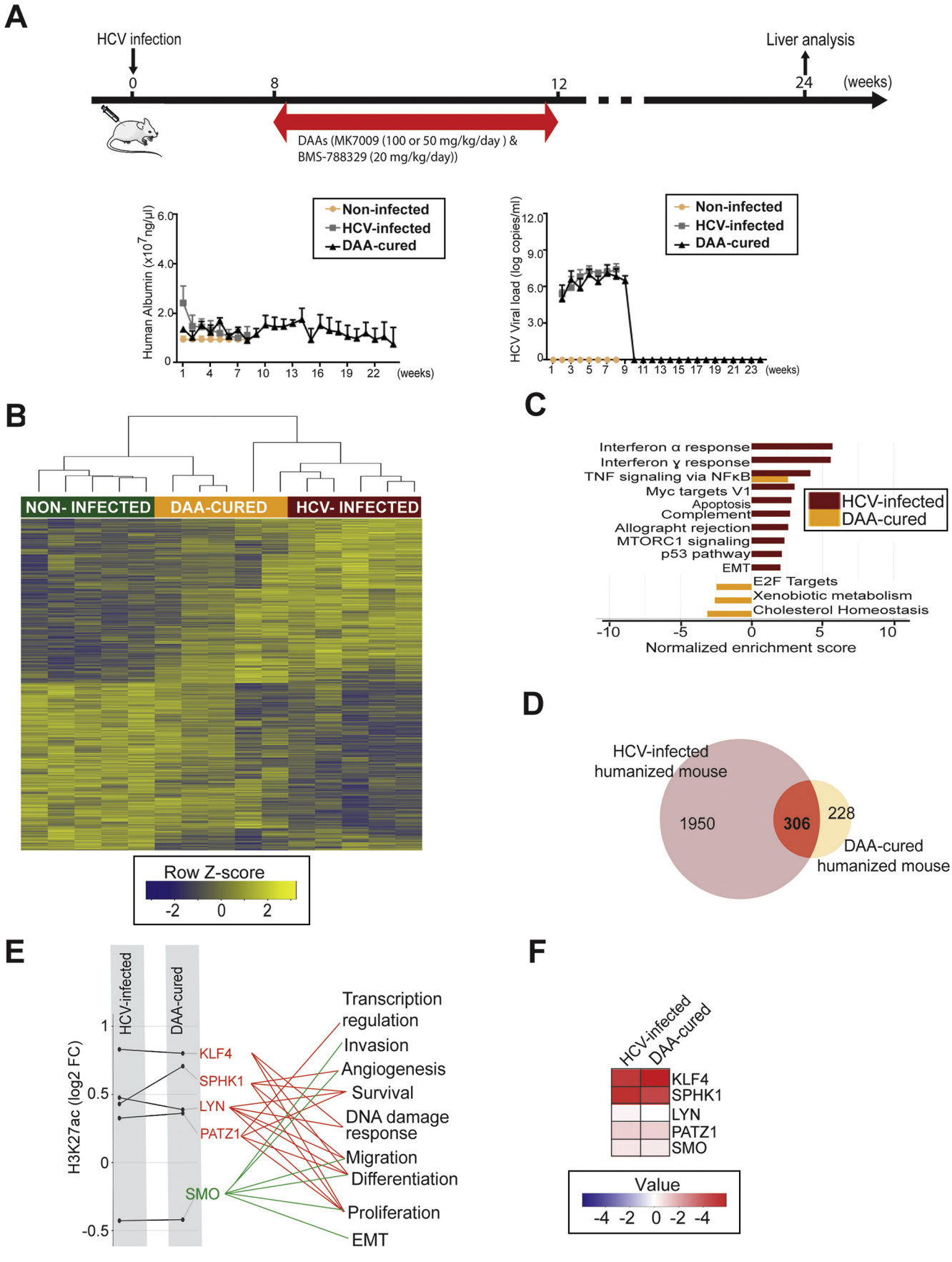


Figure 4. HCV-induced epigenetic changes persisting after DAA-based cure are present in the tumor tissue of patients with DAA-cured HCC. H3K27ac modifications from patient-derived resections of tumor and nontumor adjacent paired tissue samples. Similar to the analysis shown in Figure 1*B*, we performed an unsupervised clustering of normalized read counts in ChIP-Seq peaks of 7609 genes linked with significant ($q < 0.05$) H3K27ac modifications in DAA-cured adjacent ($n = 8$) or paired-tumor ($n = 8$) tissues vs noninfected control patients ($n = 6$). The proportions (percentages) of common (*yellow*) or distinct genes associated with changes in H3K27ac levels in tumor (*blue*) or nontumor paired-adjacent tissues (*orange*) are represented as a pie chart. N, nontumor; T, tumor.

Figure 3. Pathway analysis of epigenetic and transcriptional reprogramming in HCV-infected patients unravels candidate genes driving carcinogenesis after DAA cure. (*A*) Hallmark pathways significantly enriched for H3K27ac modifications in infected ($n = 18$) or/and DAA-cured ($n = 8$) compared with control ($n = 6$) patient samples. A large overlap of enriched pathways persists in DAA-cured patients. (*B*) Venn diagram showing HCC risk gene candidates as the overlap of significantly modified genes in HCV-infected (F1–F4) and DAA-cured (F2–F3 and F4) patients derived from the ChIP-Seq experiment shown in Figure 1*B*. (*C*) Oncogenes (*red*) and TSGs (*green*) from the 2193 potential HCC risk gene candidates, with their biological functions indicated. (*D*) Heat map depicting transcriptional changes of the oncogenes and TSGs described in *C* in HCV-infected and DAA-cured patients. (*E*) Genes with persistent HCV-induced H3K27ac modifications after DAA cure, linked with the 32-gene prognostic liver signature predicting HCC in HCV-infected patients,[9,17] and overlapped with the hallmarks of cancer. Oncogenes shown in *D* are highlighted in *black*. This network includes 910 potential HCC risk gene candidates, highlighting a strong enrichment for modifications linked to carcinogenesis. EMT, epithelial–mesenchymal transition; IL2, interleukin 2; PI3K, phosphoinositide 3-kinase; STAT5, signal transducer and activator of transcription 5.


A
HCV infection
0
8
12
24
(weeks)
Liver analysis
DAAs (MK7009 (100 or 50 mg/kg/day) &
BMS-788329 (20 mg/kg/day))
Human Albumin (x10^7 ng/µl)
Non-infected
HCV-infected
DAA-cured
(weeks)
HCV Viral load (log copies/ml)
B
NON- INFECTED
DAA-CURED
HCV- INFECTED
Row Z-score
C
Interferon α response
Interferon γ response
TNF signaling via NFκB
Myc targets V1
Apoptosis
Complement
Allographt rejection
MTORC1 signaling
p53 pathway
EMT
E2F Targets
Xenobiotic metabolism
Cholesterol Homeostasis
Normalized enrichment score
D
HCV-infected
humanized mouse
1950
306
228
DAA-cured
humanized mouse
E
H3K27ac (log2 FC)
KLF4
SPHK1
LYN
PATZ1
SMO
Transcription
regulation
Invasion
Angiogenesis
Survival
DNA damage
response
Migration
Differentiation
Proliferation
EMT
F
Value

Identification of HCV-Specific Epigenetic and Transcriptional Modifications That Are Independent of Inflammation and Fibrosis Using a Human Liver Chimeric Mouse Model

In the HCV-infected patient livers, epigenetic and transcriptional changes are most likely due to direct HCV-hepatocyte interactions and indirect mechanisms caused by chronic inflammation and fibrosis. Furthermore, our analysis is based on bulk tissue in which hepatocyte-related changes are difficult to distinguish from those in non-parenchymal cells. To clarify which fraction of the observed changes is dependent on HCV–hepatocyte interactions, we applied an HCV-permissive human liver chimeric mouse model.[13] In this model HCV efficiently infects the engrafted human hepatocytes without detectable liver fibrosis and inflammation. Moreover, human-specific sequencing reads in the ChIP-Seq pipeline are hepatocyte related because in liver bulk tissue only engrafted hepatocytes are of human origin. HCV-infected animals were cured using a combination of DAAs. Measurements of human albumin and HCV viral load in animals confirmed the viability of the engrafted hepatocytes and viral cure, respectively (Figure 5*A*). Similar to the findings in patients, we observed significant changes in H3K27ac levels in HCV-infected mice persisting after DAA cure (Figure 5*B*). Kyoto Encyclopedia of Genes and Genomes network analysis showed that pathways of genes showing epigenetic alterations included TNF signaling by nuclear factor κB, IFNα/γ responses, complement, apoptosis, and mTOR signaling (Figure 5*C*). We found a persistence of TNF signaling through the nuclear factor κB pathway, whereas the other HCV-induced pathways (ie, apoptosis, mTORC1 signaling, and IFNα/γ response) were restored to basal level after DAA-mediated cure (Figure 5*C*).

By intersecting genes associated with significant H3K27ac modifications from infected and cured mice, we identified 306 genes with persistent H3K27ac modifications after cure (Figure 5*D* and Supplementary Table 2). We found *SPHK1* and *KLF4* oncogenes and *SMO* TSGs, previously identified in patient samples (Figure 3*C*), to be associated with increased or decreased level of H3K27ac, respectively, in DAA-cured mice (Figure 5*E*), supporting the biological relevance of the findings in humanized mice. Similar to the results obtained in patients, we found a strong correlation between transcriptomic and epigenomic changes (Figure 5*F*).

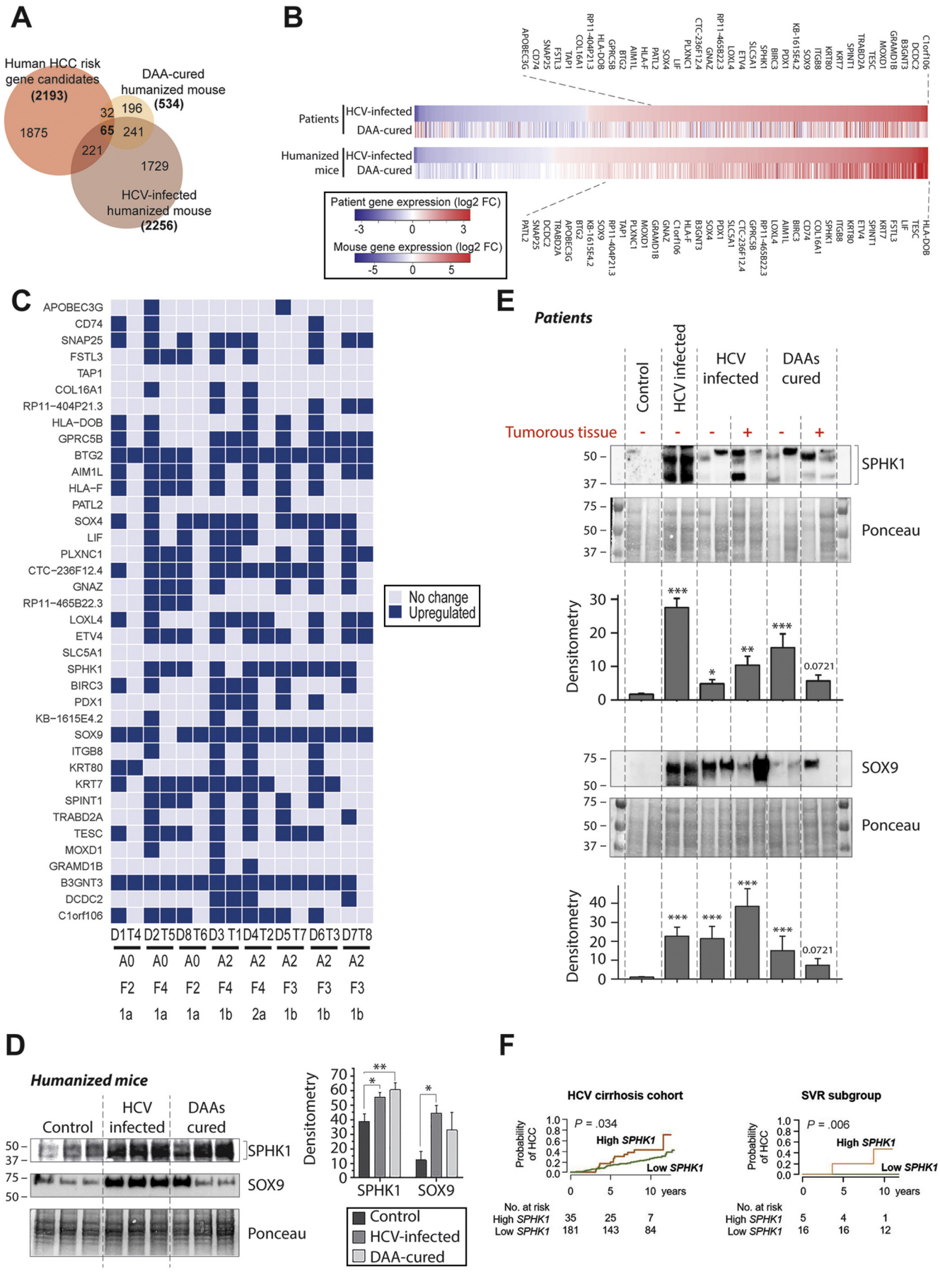
Next, we identified HCV-specific epigenetic modifications in hepatocytes that are associated with HCC development by integrative analysis of epigenomic and transcriptomic data from patient and mouse liver samples. A comparative analysis of genes with persistent H3K27ac modifications in patients and mice showed a set of 65 commonly modified genes ($P = 2.94 \times 10^{-9}$; Figure 6*A*). Further analysis identified that some of these 65 genes have their transcripts significantly correlated to epigenetic changes after DAA cure in patients and humanized mice. We ranked their transcript expression based on the FC relative to the noninfected samples. This approach identified 38 genes that were enriched for H3K27ac and that are associated with a significant positive FC of their transcripts after HCV infection and DAA cure compared with noninfected samples (Figure 6*B*). We further studied the biological function of these 38 genes by performing gene set analysis and found that they are associated to KRas, TNFα, and interleukin 2 and signal transducer and activator of transcription 5 signaling or to p53, epithelial–mesenchymal transition, apoptosis, glycolysis, and inflammation pathways (Supplementary Figure 3). Because they were identified by integrative analysis of data from patients and immunodeficient humanized mice, we hypothesize that inflammation-related genes derive from the innate response of infected hepatocytes.

To obtain further evidence that these alterations play a role in hepatocarcinogenesis after cure, we compared their H3K27ac levels in paired liver tissues of nontumorous adjacent and HCC. We found that most of them already harbored changes in the nontumorous sample that remained in HCC tissue (Figure 6*C*). For instance, changes were observed for *SPHK1* in nontumorous tissue in 7 of 8 patients and persisted in HCC tissue in 4 patients. H3K27ac modifications in *SOX9*, a gene that is associated to ductular reaction, was found in nontumorous tissue in all DAA-cured patients and remained in HCC tissue in 7 of 8 patients.

HCV and Hepatocyte-Specific Epigenetic Modifications Translate Into Liver Protein Expression Changes and Are Associated With HCC Development in HCV Cirrhosis and SVR Cohorts

To further validate the biological relevance of HCV-induced epigenetic and transcriptional changes, we studied

Figure 5. Analysis of H3K27ac changes in livers of HCV-infected humanized mice identifies virus-specific modifications in human hepatocytes. (*A*) Our experimental setup: uPA-SCID mice were infected with HCV for 8 weeks and cured with a combination of DAAs MK7009 (50 or 100 mg/kg/d) and BMS-788329 (20 mg/kg/d) for 16 weeks. Livers were analyzed at week 24 by ChIP-Seq and RNA-Seq. Human albumin level (*left*) and HCV viral load (*right*) were measured to monitor functional engrafted human hepatocytes and HCV clearance after DAA treatment, respectively. (*B*) Unsupervised clustering of normalized read counts in ChIP-Seq peaks of 2483 genes linked with significant ($q < 0.05$) H3K27ac modifications in HCV-infected ($n = 5$) or DAA-cured ($n = 5$) vs noninfected control ($n = 5$) mice. (*C*) Hallmark pathways significantly enriched for H3K27ac modifications in infected ($n = 5$) or/and DAA-cured ($n = 5$) compared with noninfected ($n = 5$) mice samples. A significant overlap of enriched pathways persists in DAA-cured mice. (*D*) Venn diagram showing the HCV-induced and persistent genes with H3K27ac changes as the overlap of significantly modified genes in HCV-infected and DAA-cured mice derived from the ChIP-Seq experiment shown in *B*. (*E*) Oncogenes (*red*) and TSGs (*green*) with persistent HCV-induced H3K27ac modifications identified in the 306 HCV-induced and persistent genes with H3K27ac changes, with their biological functions indicated. (*F*) Heat map depicting transcriptional changes of the oncogenes and TSGs described in *E* in HCV-infected humanized and DAA-cured mice. EMT, epithelial–mesenchymal transition; NFκB, nuclear factor κB.


A
Human HCC risk gene candidates (2193)
DAA-cured humanized mouse (534)
HCV-infected humanized mouse (2256)
1875
32
196
65
241
221
1729
B
Patients
HCV-infected
DAA-cured
Humanized mice
Patient gene expression (log2 FC)
Mouse gene expression (log2 FC)
C
No change
Upregulated
D1T4 D2T5D8T6 D3 T1 D4T2D5T7 D6T3 D7T8
A0 A0 A0 A2 A2 A2 A2 A2
F2 F4 F2 F4 F4 F3 F3 F3
1a 1a 1a 1b 2a 1b 1b 1b
D
Humanized mice
Control
HCV infected
DAAs cured
SPHK1
SOX9
Ponceau
Densitometry
Control
HCV-infected
DAA-cured
E
Patients
Control
HCV infected
HCV infected
DAAs cured
Tumorous tissue
SPHK1
Ponceau
Densitometry
SOX9
Ponceau
Densitometry
F
HCV cirrhosis cohort
P = .034
High SPHK1
Low SPHK1
Probability of HCC
10 years
No. at risk
High SPHK1 35 25 7
Low SPHK1 181 143 84
SVR subgroup
P = .006
High SPHK1
Low SPHK1
No. at risk
High SPHK1 5 4 1
Low SPHK1 16 16 12

whether the expression of the identified genes correlates with corresponding protein abundance. We quantified the protein expression of *SPHK1* and *SOX9* genes by immunoblotting in patient and mouse liver samples (Figure 6*D* and *E* and Supplementary Figures 4–6). We found increased SPHK1 and SOX9 protein levels at HCV infection that remained increased after DAA cure. Importantly, by comparing pairwise liver tissue from adjacent nontumorous areas and HCC, we found that the expression of SPHK1 and SOX9 were already increased in adjacent nontumorous tissue (Figure 6*D* and *E*), suggesting that the up-regulation of these proteins preceded tumor development.

To assess the potential of the expression of these genes as biomarkers to predict HCC risk, we assessed the association of *SPHK1* expression with the long-term probability to develop HCC over a decade in a cohort of patients with HCV cirrhosis (n = 216), among which a subset of patients achieved SVR (n = 21). We found that high expression of *SPHK1* is significantly associated with HCC risk in the 2 cohorts ($P < .034$ for HCV cirrhosis and $P < .006$ for SVR; Figure 6*F*), identifying a potential predictor of HCC risk post SVR.

Discussion

Our study exposes a previously undiscovered paradigm showing that chronic HCV infection induces H3K27ac modifications that are associated with HCC risk and that persist after HCV cure. Thus far, only limited data have shown that HCV infection can induce epigenetic changes.[24] Previous attempts to connect specific histone marks to HCC development were inconclusive because of semi-quantitative approaches.[25,26] For the first time, our study provides an integrative genome-wide approach that combines analyses in patient liver tissue and a humanized animal model.

Long-term epigenetic alterations also were observed after Epstein-Barr virus infection[27] or after transient hyperglycemia.[28] Indeed, latent Epstein-Barr infection triggered persistent epigenetic reprogramming, possibly resulting in the establishment of immortal growth and cancer, whereas transient hyperglycemia resulted in persistent enrichment of H3K4me1 on the *p65* gene promoter and subsequently in oxidative stress and increased cancer risk. Importantly, these data suggest that persistent epigenetic changes also can occur through environmental changes, independently from direct viral infection.

Epigenetic changes in patient liver tissue can result from infected hepatocytes and from virus-induced inflammatory or fibrotic responses in the liver microenvironment. Interestingly, PCA showed a clear correlation of epigenetic changes with fibrosis stage (Figure 2*A*), suggesting that HCV-induced histone modifications and fibrogenesis are interdependent from the progression of liver disease. Indeed, epigenetic changes are considered as orchestrating fibrogenesis,[29] including the activation of hepatic stellate cells. In contrast, the induction of fibrosis triggers a liver response to injury, implicating the epigenetic machinery to mediate the activation of dedicated genes,[30] and thereby enhancing HCV-established epigenetic changes. Because distinct epigenetic changes were found in patient liver tissue and humanized mouse liver tissue (Figures 3 and 5), where no necro-inflammatory response or fibrosis is present, it is likely that a fraction of the observed changes is caused by direct HCV–hepatocyte interactions. Collectively, our results suggest that direct virus–hepatocyte interactions and indirect mechanisms, such as disease-induced fibrosis mediated by the liver non-parenchymal cells, contribute to the observed epigenetic changes in the livers of HCV-infected patients. Importantly, our data provide a previously undiscovered mechanism for persistent HCC risk after DAA cure in advanced fibrosis and could explain why a small number of patients develop HCC even in the absence of fibrosis.[2] However, we point out that this mechanism is not exclusive, and many other factors most likely contribute to hepatocarcinogenesis after cure.

Although we did not perform extensive functional studies, our data provide evidence that HCV-induced H3K27ac modifications on specific genes are causal factors for HCC risk after DAA cure. Our hypothesis is strongly supported by (1) altered expression of genes known to promote and drive carcinogenesis, (2) the correlation of epigenetic changes with a clinical Cox score for overall death and a HCC risk score,[17] (3) the positive correlation between

Figure 6. Intersection of ChIP-Seq and RNA-Seq analyses from livers of patients and humanized mice uncovers HCV-induced persistent epigenetic changes associated with HCC risk after SVR. (*A*) Venn diagram showing the overlap of H3K27ac modifications between the human HCC risk gene candidates and significantly modified genes in HCV-infected and DAA-cured mice derived from the ChIP-Seq experiments shown in Figures 1*B* and 5*B*, respectively. (*B*) Expression data of genes with significant H3K27ac changes from livers of HCV-infected and DAA-cured patients (n = 32) and mice (n = 15) were intersected to uncover common genes with HCV-induced and persistent epigenetic and transcriptional changes after DAA. (*C*) Presence of epigenetic modifications on the 38 identified genes in pairwise liver tissues from DAA-cured patients. H3K27ac modifications (vs control liver samples) were assessed on the corresponding genes in nontumorous adjacent and HCC liver tissues from DAA-cured patients. *Dark blue squares* represent increased H3K27ac changes and *light blue squares* represent unchanged status. (*D*) Analysis of protein level of SPHK1 and SOX9 protein in control, HCV-infected, and DAA-cured mice by western blot. (*E*) Analysis of SPHK1 and SOX9 protein levels in control (n = 7), HCV-infected (non-HCC and HCC; n = 8) and DAA-cured (non-HCC and HCC; n = 8) patients by western blot. One representative gel of 4 is shown. Graphs show quantification of western blot intensities in arbitrary units normalized to total protein level (Ponceau staining). Results show mean ± standard error of the mean of integrated blot densities. (*F*) Probability of HCC development according to the gene expression level of SPHK1 among 216 patients with HCV-induced cirrhosis or 21 patients with HCC occurrence after HCV cure.

the magnitude of epigenetic changes and fibrosis stage, which is the strongest clinical risk factor for HCC,[6] and (4) the presence of H3K27ac modifications in HCC tumors of the same patients. Collectively, these findings suggest that epigenetic modifications precede hepatocarcinogenesis. Among the identified genes, functional knockout of *SOX9* has been reported to decrease liver cancer cell growth,[31] and *SPHK1* deletion decreased diethyl-nitrosamine–induced liver cancer in mice,[32] whereas ETS translocation variant 4 (*ETV4*) is up-regulated and is associated to HCC progression.[33] Importantly, extended analysis in additional cohorts showed that those genes that were epigenetically changed by HCV infection and that persisted after DAA cure predicted HCC risk in cohorts of patients with HCV cirrhosis and SVR (Figure 6*C*). Although we do not have experimental evidence that HCV-mediated modulation of SPHK1 or SOX9 gene expression is sufficient to promote cancer, our data combined with published knowledge on the role of these proteins in cancer biology[31,32] nevertheless suggest that SPHK1 and SOX9, among additional tumor-associated proteins, participate in HCV-induced HCC. This strongly supports the hypothesis that H3K27ac alterations of the identified genes precede HCC onset.

Other well-known causes for HCC development are chronic HBV infection and NASH.[2] Interestingly, we found that H3K27ac modifications also are present in these etiologies (Figures 1*B* and 2*B*). In-depth analyses including PCA (Figures 2*A* and Supplementary Figure 2) showed etiology-independent and etiology-specific epigenetic profiles in liver disease.

Because of the difficulty of obtaining liver tissue after HCV cure, which was available only for patients with concomitant HCC, the number of patient tissues is limited. Because it impossible to obtain healthy liver tissue for ethical reasons, the control samples from patients with nonviral minimal liver disease or adjacent tissue from patients undergoing surgery for metastasis for colorectal cancer exhibited heterogeneity. Furthermore, the H3K27ac mark constitutes only a part of the epigenetic gene regulation program. Nevertheless, the robust results obtained by clustering and statistical analyses combined with consistent results from patients of different cohorts and clinical centers and confirmation of the key concept in humanized mouse engrafted with hepatocytes from the same donor and infected with the same viral inoculum allowed arresting conclusions.

HCC is often asymptomatic and thus remains undiagnosed until the late stage. Therefore, there is an urgent medical need for biomarkers to predict HCC risk. A large body of literature has shown the association between the human epigenome and cancer development.[34] In this study, showing that HCV induces persistent epigenetic alterations after DAA cure provides a unique opportunity to uncover novel biomarkers for HCC risk, that is, from plasma through the detection of epigenetic changes of histones bound to circulating DNA complexes. Furthermore, by uncovering virus-induced epigenetic changes as therapeutic targets, our findings offer novel perspectives for HCC prevention—a key unmet medical need.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at https://doi.org/10.1053/j.gastro.2019.02.038.

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Received December 10, 2018. Accepted February 27, 2019.

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Acknowledgments
We acknowledge the Centre de Ressources Biologiques (Biological Resource Centre; Strasbourg, France) for the management of patient-derived liver tissues. We acknowledge the work of the IGBMC high-throughput sequencing facility and the Quantitative Genomics Facility at the Department of Biosystems Science and Engineering at the scientific central facilities of ETH Zurich. The IGBMC high-throughput sequencing facility is a member of the France Génomique consortium (ANR10-INBS-09-08). The laboratory of Irwin Davidson is an *équipe labellisée* of the Ligue Nationale contre le Cancer.

Author contributions: Thomas F. Baumert initiated and coordinated the study. Thomas F. Baumert, Nourdine Hamdane, Frank Jühling, Mirjam B. Zeisel, Yujin Hoshida, and Bryan C. Fuchs designed experiments and analyzed data. Nourdine Hamdane, Emilie Crouchet, Christine Thumann, Marine A. Oudot, Clara Ponsolles, Armando Andres Roca Suarez, and Shen Li performed experiments. Frank Jühling performed computational analyses. Nabeel Bardeesy contributed to the concept and approach of the study. Christian Schmidl and Christoph Bock performed sequencing of ChIPmentation experiments. Irwin Davidson, Houssein El Saghire, Antonio Saviano, Naoto Fujiwara, Catherine Schuster, Atsushi Ono, and Yujin Hoshida analyzed data. Patrick Pessaux, Michio Imamura, Takuro Uchida, Hideki Ohdan, Hiroshi Aikata, Kazuaki Chayama, Tullio Piardi, Daniele Sommacale, François Habersetzer, Michel Doffoël, and Tujana Boldanova provided clinical liver tissue samples. Nourdine Hamdane, Frank Jühling, François H.T. Duong, Bryan C. Fuchs, Joachim Lupberger, Mirjam B. Zeisel, and Thomas F. Baumert wrote the manuscript.

Conflicts of interest
Authors declare no conflict of interest.

Funding
This work was supported by the ARC (Paris) and the Institut Hospitalo-Universitaire (Strasbourg; TheraHCC IHUARC IHU201301187 to Thomas F. Baumert), the Foundation of the University of Strasbourg and Roche Institute (HEPKIN to Thomas F. Baumert and Yujin Hoshida), the Agence Nationale de Recherches sur le Sida et les Hépatites Virales (2017/1633 to Thomas F. Baumert), the U.S. Department of Defense (W81XWH-16-1-0363 to Yujin Hoshida and Thomas F. Baumert), the Cancéropôle du Grand-Est (AAP Emergence 2017 to Joachim Lupberger), the National Institutes of Health (DK099558 to Yujin Hoshida), and the Research Program on Hepatitis from the Japan Agency for Medical Research and Development, AMED (17fk0210104h0001 to Kazuaki Chayama). This project has received funding from the European Union's Horizon 2020 research and innovation program under grant agreement 667273 (HEPCAR to Thomas F. Baumert and Joachim Lupberger). This project has received funding from the European Research Council under the European Union's Horizon 2020 Research and Innovation Program under grant 671231 (HEPCIR to Thomas F. Baumert and Yujin Hoshida). This work has been published under the framework of the LABEX ANR-10-LABX-0028_HEPSYS, and PLAN CANCER 2014–2019 and benefits from a funding from the state managed by the French National Research Agency as part of the Investments for the Future Program, the French National Cancer Institute, and INSERM.

Supplementary Methods

HCV Infection of Human Hepatocyte Chimeric Mouse and DAA Treatment

cDNA-uPA$^{+/+}$/SCID$^{+/+}$ (uPA/SCID) mice were created and human hepatocytes were transplanted as described previously.[1] Mice were intravenously inoculated with serum samples containing 10^5 HCV particles. The viremic serum was obtained from an HCV-infected (genotype 1b) DAA-naïve patient who provided written informed consent to participate in the study, according to the process approved by the ethical committee of the hospital and in accordance with the ethical guidelines of the 1975 Declaration of Helsinki. Blood sampling was done weekly, and serum samples were divided into small aliquots and stored in liquid nitrogen before measurement of HCV RNA. All animal protocols were performed in accordance with the Guide for the Care and Use of Laboratory Animals (https://grants.nih.gov/grants/olaw/guide-for-the-care-anduse-of-laboratory-animals.pdf). The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of the Graduate School of Biomedical Sciences at Hiroshima University (A14-195). Sixteen mice were divided into 3 groups: 6 mice were infected with HCV and treated with DAAs, 5 mice were infected with HCV but were not treated with DAAs, and 5 uninfected and untreated mice were used as controls. After the establishment of stable viremia, HCV-infected mice were treated with a combination of MK-7009 (vaniprevir; Merck Sharp & Dohme Corp, Kenilworth, NJ) and BMS-788329 (NS5A inhibitor; Bristol-Meyers Squibb, New York, NY) as described previously.[2] Elimination of HCV in 6 treated mice was confirmed by the absence of HCV viremia 12 weeks after cessation of therapy and by undetectable HCV RNA by reverse transcription-nested polymerase chain reaction from extracted liver tissue. Five viremic mice and 5 control mice were sacrificed at week 8. All liver samples were snap frozen and stored at −80°C before analysis.

Processing of Raw ChIPmentation Data

Reads were aligned to the human genome (hg19) using HISAT2[3] reporting up to 100 alignments per read. Data from humanized mice were mapped similarly, but to an artificial genome consisting of all human (hg19) and mouse (mm10) chromosomes, and only reads mapping to human chromosomes were kept for further analysis. Sorting, indexing, and other basic operations on alignments were performed with samtools[4] and intersections of annotations and peaks with alignments were performed using bedtools intersect.[5] Peaks were called in uniquely mapped reads filtered for duplicates using MACS2[6] in standard mode and with corresponding input sequence data. Only samples with at least 10,000 peaks were used for further analyses. Peaks within all samples were intersected and used for counting reads if they overlapped in at least 2 samples. Close peak regions with a maximal distance of 500 bp were merged. Read counts of genes were defined as the sum of all reads in peak regions overlapping the gene body or the promoter region, that is, the region up to 1500 bp ahead of the transcription start site.

Processing of RNA-Seq Data

Raw reads of patient's samples had to be trimmed for primer and quality using cutadapt.[7] Reads were mapped using HISAT2[3] to the human genome hg19 (patients) or to hg19 and mm10 (humanized mice) as described earlier for raw ChIPmentation data. Reads were counted with htseq-count, and a differentially expression analysis was performed with DESeq2 applying GENCODE 19.[8] Reads were taken from our RNA-Seq experiments as described earlier and from external sources: RNA-Seq from infected vs control patients was taken from the GEO dataset GSE84346 (low ISG samples).

Pathway Enrichment and Correlation Analyses

The full downstream ChIP-Seq analysis was based on read counts in ChIP-Seq peaks called as described earlier. Differentially modified genes (GENCODE 19 annotation) and $\log_2$ FCs were identified using these peak read counts as input for edger.[9] Pathway enrichment analyses were performed using local javaGSEA with all gene sets included in MSigDB 6.0.[10] We used the pre-ranked version of javaGSEA and genes were ranked for *P* values of differential expression and modification analyses. Figures showing enriched pathways and gene sets, Spearman correlations, and oncogene $\log_2$ FCs were drawn using ggplot2 and the R environment. Immune-related genes used for calculating correlations were selected from MSigDB by including only genes from pathways with the term "IMMUNE" in their title. Heat maps of gene expression and histone modifications were generated by applying the heatmap.2 function in combination with clustering through Spearman correlation included in the R package gplots. Gene network analysis was performed based on 3 MSigDB subsets: Hallmark gene sets, curated gene sets, and gene ontology gene sets. Genes were assigned with the hallmarks of cancer in case they were found in gene sets whose designation matches a corresponding term. Network figures were generated manually using Cytoscape.[11] Genes were defined as to be "connected to the PLS" in the case they shared at least 1 common pathway listed in MsigB 6.0 with at least 1 of the 32 PLS genes.

Western Blot and Antibodies

The expression of SPHK1 and SOX9 proteins was assessed by western blot using polyclonal rabbit antibodies anti-SPHK1 (D1H1L; number 12071) and anti-SOX9 (D8G8H; number 82630) from Cell Signaling (Danvers, MA). Protein expression was quantified using ImageJ software. Because anti-SPHK1 antibody detects all 3 isoforms[12] of SPHK1 and it is only partially understood which isoform or which post-translational modification on the oncogene SPHK1 predominantly triggers carcinogenesis, all apparent bands were included in the densitometry analysis.

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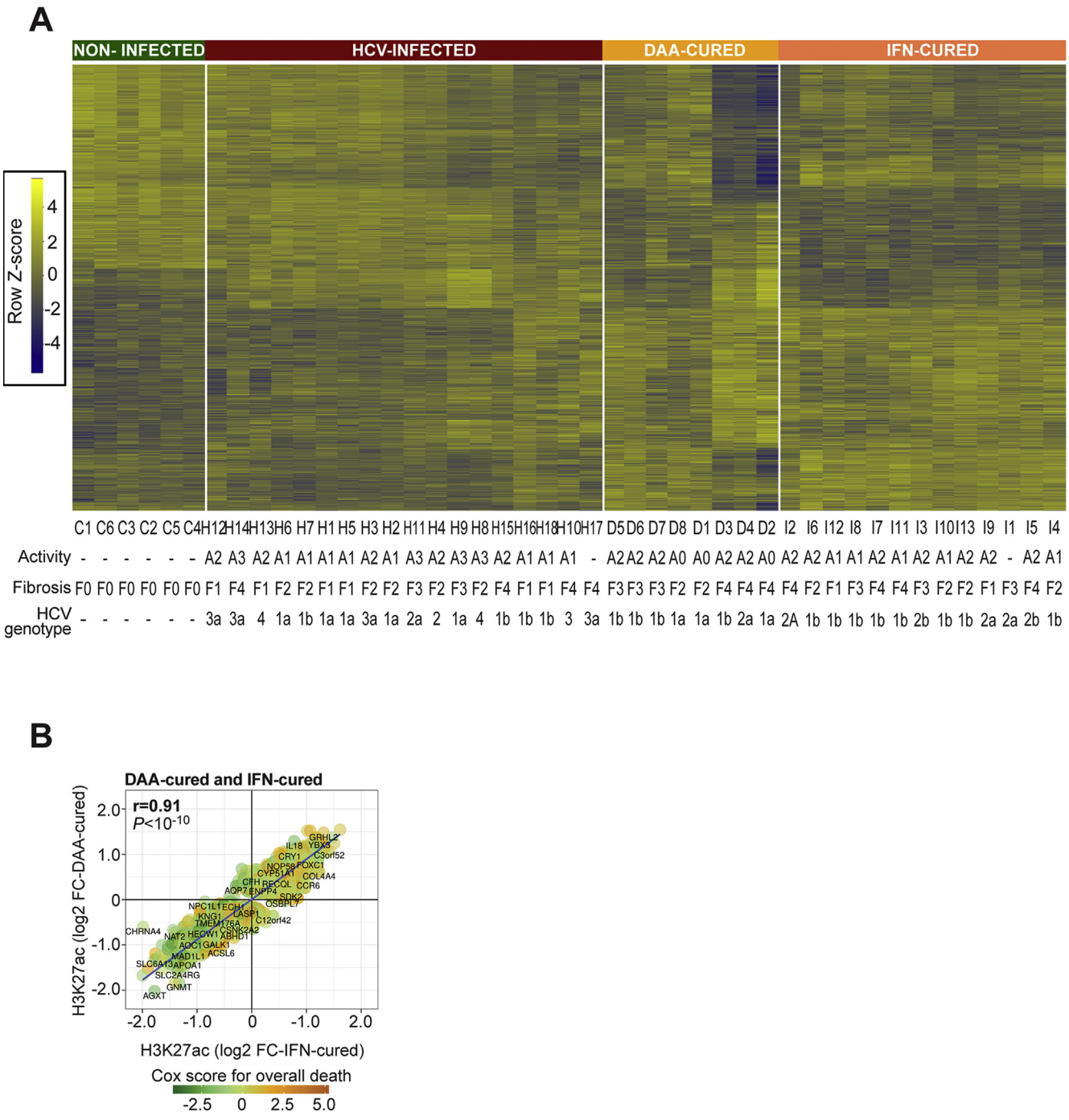
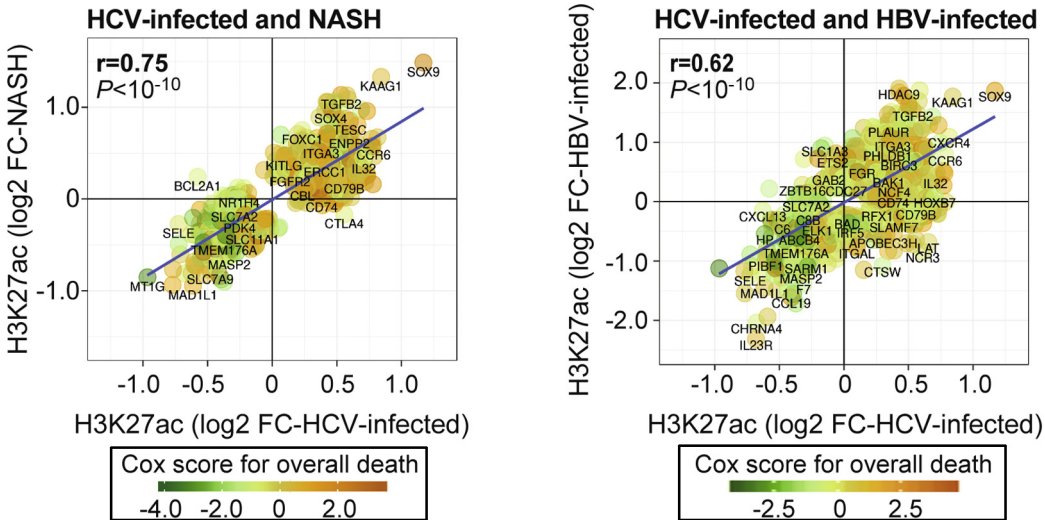
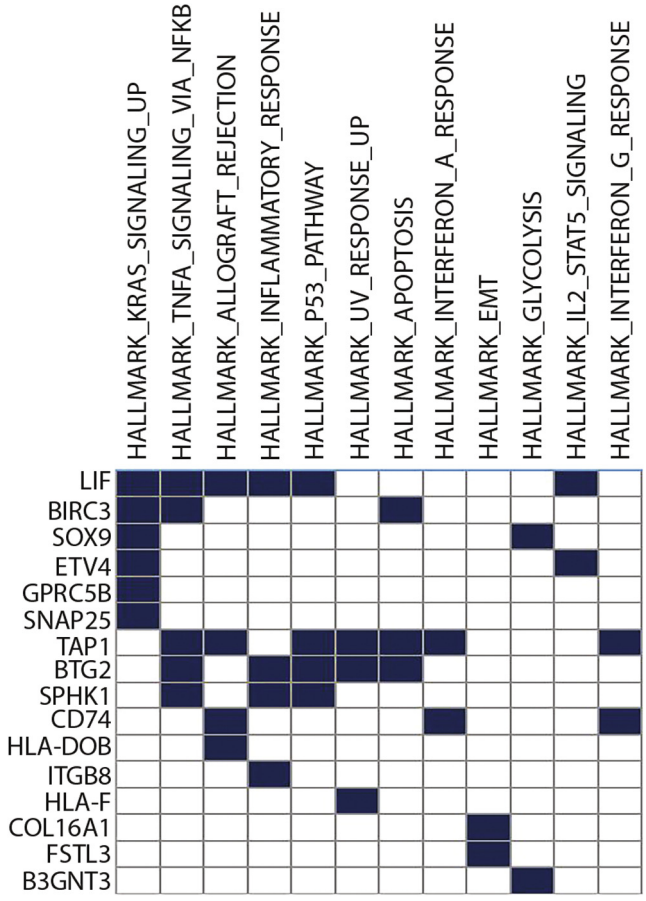
Author names in bold designate shared co-first authorship.



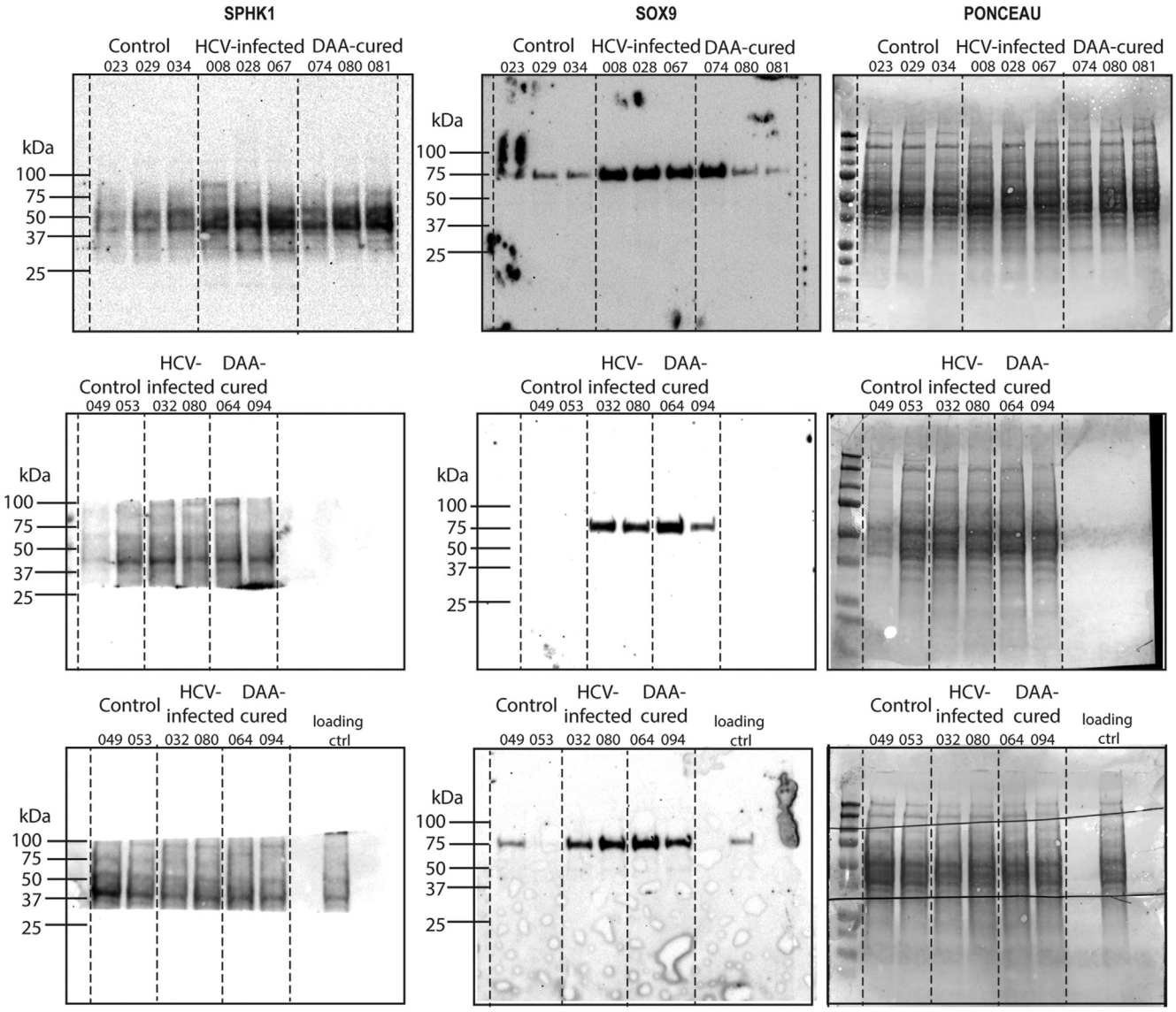
Supplementary Figure 1. Persistent H3K27ac modifications in the livers of DAA- and IFN-cured HCV-infected patients. (*A*) Unsupervised clustering of normalized read counts in ChIP-Seq peaks of genes linked with significant ($P < .05$) H3K27ac modifications in HCV-infected (n = 18), DAA-cured (n = 8), and IFN-cured (n = 13) vs noninfected control (n = 6) patients. (*B*) Persistent H3K27ac modifications among DAA-cured and IFN-cured patients correlate (see Spearman rank correlation coefficients r and *P* values) with H3K27ac modifications among IFN-cured patients.



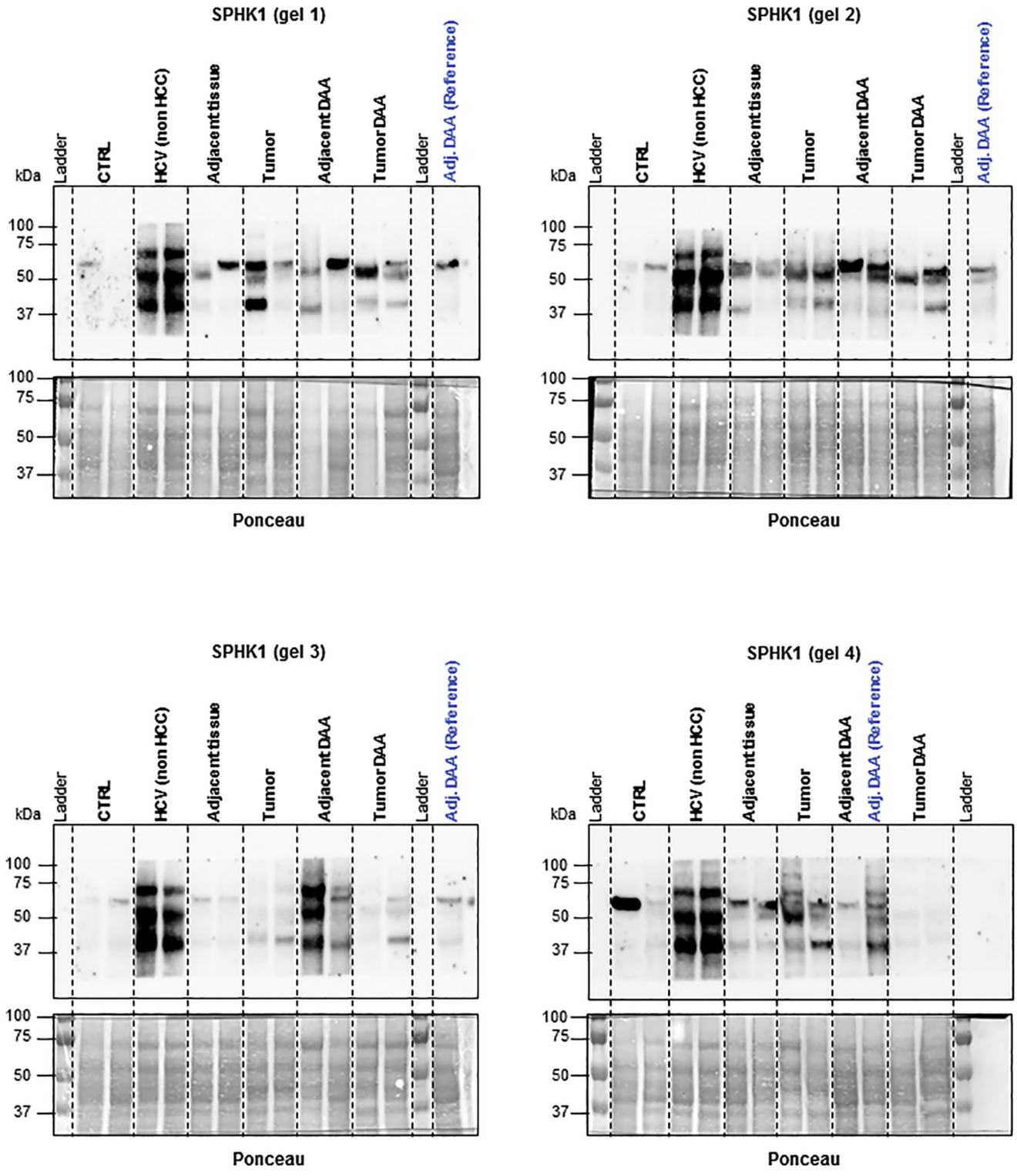
Supplementary Figure 2. Differential epigenetic modifications on immune-related gene signature among HCV-infected, NASH, and HBV-infected liver samples. To analyze the role of epigenetic changes in the disease immune responses, we extracted immune-related genes from MSigDB and performed a restricted correlation study of genes with H3K27ac modifications among NASH, HBV-infected, and HCV-infected patients. Common H3K27ac modifications were analyzed and Spearman rank correlation coefficients and *P* values are shown.



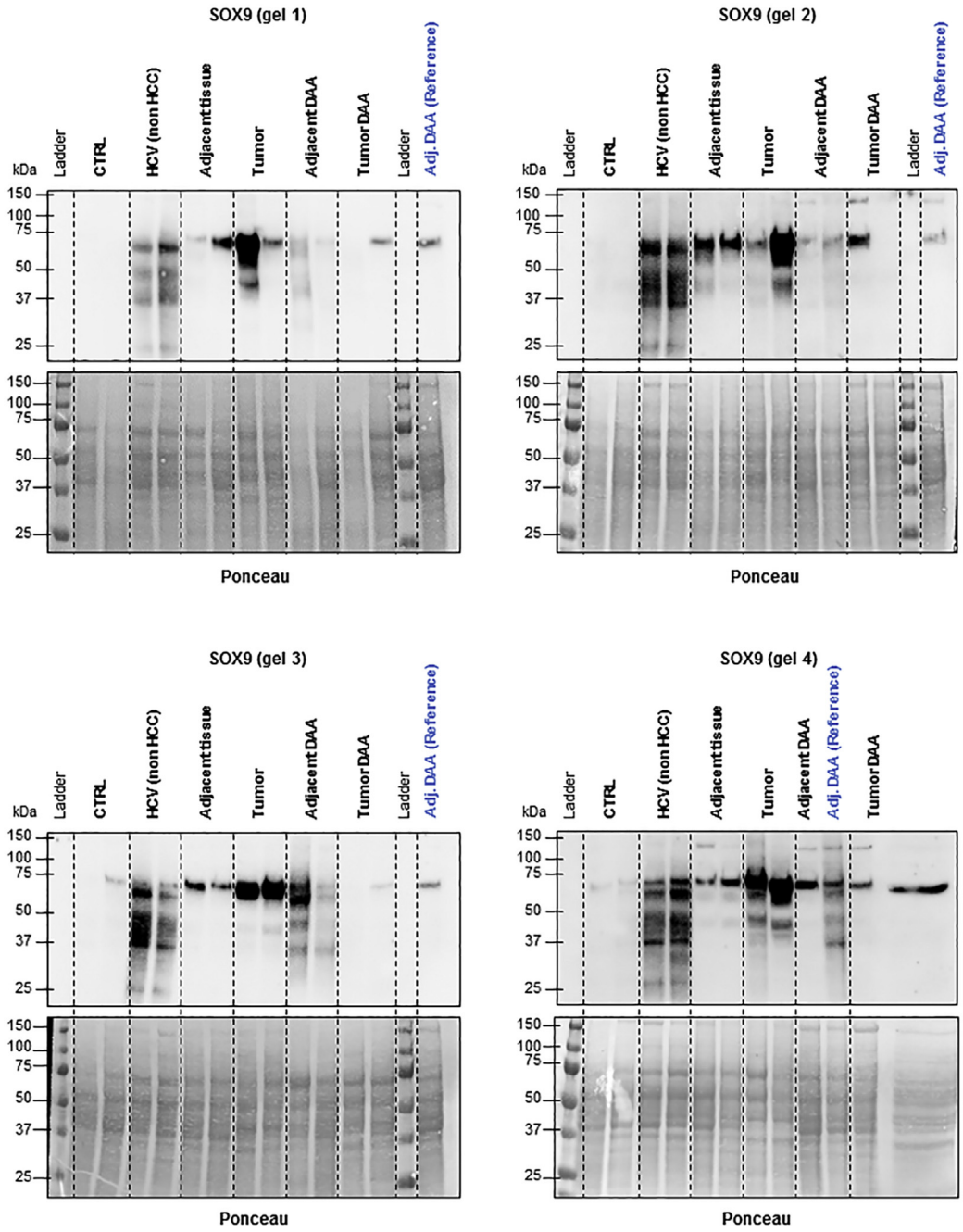
Supplementary Figure 3. Hallmark pathway analysis of the 35 genes enriched for H3K27ac modifications and overexpressed in infected and cured human (n = 32) and mice (n = 15) samples. The 38 genes harboring significant H3K27ac changes from the livers of HCV-infected and DAA-cured patients and mice were subjected to GSEA using hallmark gene sets from the MSigDB Molecular Signatures Database.



Supplementary Figure 4. Full-length immunoblots of SPHK1 and SOX9 protein levels in the livers of control, HCV-infected, and DAA-cured humanized mice. Full-length blots corresponding to representative blots shown in Figure 6*D* are shown. Reducing 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis of mouse liver lysates was performed as described in the Methods section.

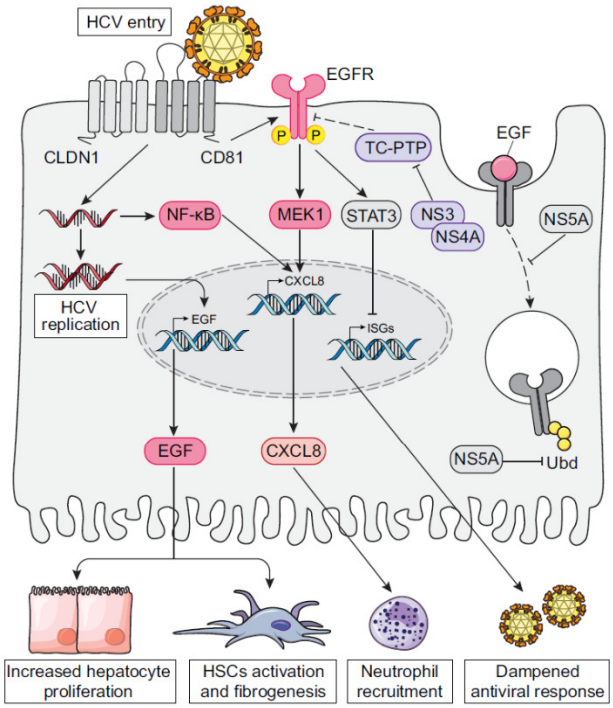


Supplementary Figure 5. Full-length immunoblots of SPHK1 protein level in the livers of control, HCV-infected, and DAA-cured patients (HCC and adjacent tissue). For patients with HCC, SPHK1 was detected in tumor and surrounding tissues (adjacent tissue). A reference sample was loaded on each gel for data normalization. Full-length blots of SPHK1 corresponding to representative blots shown in Figure 6*E*. Reducing 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis of liver biopsy lysates was performed as described in the Methods section. Multiple weight products visible on the blot could be a result of post-translational protein modifications including glycosylation, phosphorylation, and/or ubiquitination. CTRL, control.



Supplementary Figure 6. Full-length immunoblots of SOX9 protein levels in the livers of control, HCV-infected and DAA-cured patients (HCC or adjacent tissue). For patients with HCC, SOX9 was detected in tumor and surrounding tissues (adjacent tissue). A reference sample was loaded on each gel for data normalization. Full-length blots of SOX9 corresponding to representative blots shown in Figure 6*E*. Reducing 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis of liver biopsy lysates was performed as described in the Methods section. Multiple weight products visible on the blot could be a result of post-translational protein modifications including glycosylation, phosphorylation, and/or ubiquitination. CTRL, control.

4.2.- Supplementary article II



Roca Suarez, A. A.; Baumert, T. F.; Lupberger, J., Beyond viral dependence: The pathological consequences of HCV-induced EGF signaling. *J Hepatol* **2018,** *69* (3), 564-566.

Editorial

JOURNAL OF HEPATOLOGY

Beyond viral dependence: The pathological consequences of HCV-induced EGF signaling

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See Article, pages 594–602

Chronic hepatitis C virus (HCV) infection affects approximately 71 million individuals worldwide,[1] being a major etiological factor for the development of liver cirrhosis and hepatocellular carcinoma (HCC). Acute HCV infection often progresses to chronicity and is characterized by a non-resolving liver inflammation leading to a broad range of alterations in the tissue microenvironment. About ninety percent of HCC cases arise in the context of chronic liver inflammation, highlighting the central role of this persistent immune response in disease pathogenesis.[2] Despite efficient antiviral therapy by direct acting antivirals (DAA), the risk of HCC development cannot be fully eliminated in patients with advanced liver disease.[3] In this regard, accumulating evidence suggests a potentially persisting proto-oncogenic environment created by virus-induced changes in cell signaling.[4–7] Therefore, even in the DAA era, the understanding of virus-host interactions during chronic HCV-associated inflammation is key to identify and treat patients at high risk of developing HCC.

In this context, a recent article in *Journal of Hepatology* by Johannes G. Bode's laboratory at the Heinrich-Heine University in Germany provides a novel mechanism by which HCV infection contributes to this pathologic inflammatory response.[8] Aiming to identify chemokines regulated by HCV, the authors performed a functional screen using an HCV subgenomic replicon system and identified an HCV-induced upregulation of C-X-C motif chemokine receptor 2 (CXCR2) ligands (CXCLs) 1, 2, 3 and 8. Consistently, similar results were obtained upon HCV infection using the cell culture-derived strain Jc1. Having previously shown that HCV infection enhances epidermal growth factor (EGF) signaling, the authors next explored the possible involvement of this pathway on CXCR2 ligand expression. EGF receptor (EGFR) perturbation studies combining RNAi knockdown of EGF and the use of MAPK inhibitors, confirmed an HCV-induced upregulation of *CXCL8* via EGFR and the MAP kinase kinase, MEK1 (MAP2K1). Additionally, knockdown of the p65 subunit of the NF-κB complex was sufficient to abrogate basal and EGF-induced *CXCL8* expression in replicon-expressing cells, while in HCV-infected cells this mainly affected basal *CXCL8* levels. This suggests that the observed enhancement of chemokine expression during HCV infection not only depends on the EGFR pathway but also on the activation of additional transcription factors such as NF-κB. The *in vivo* relevance of the data is emphasized by an association of HCV viral load with CXCL8 serum levels in chronically infected patients. Similarly, serum levels of EGF and CXCL8 tend to positively correlate, although this did not reach statistical significance in their study cohort.

In a previous study, the authors demonstrated that HCV enhances EGFR signaling via NS3/4A-mediated proteolytic cleavage of T-cell protein tyrosine phosphatase (TC-PTP [PTPN2]), one of the major negative regulators of EGFR tyrosine-kinase activity.[9] Indeed, here they demonstrate that NS3/4A expression alone enhances EGF-inducible *CXCL8* expression, an effect that can be mimicked by knocking down TC-PTP. As the major role of chemokines is the recruitment of immune cells to the site of inflammation, the authors next evaluated if in the context of HCV replication EGF-induced release of chemokines influences leukocyte migration. Remarkably, the authors demonstrate that media from EGF-treated cell lines expressing the HCV subgenomic replicon enhances the migration of neutrophils, an effect that was not observed with EGF-conditioned media alone. This suggests that HCV infection modulates chemoattraction of immune cells to the liver via EGF-regulated chemokine secretion.

The findings of Christina Groepper and co-workers are not just relevant for our understanding of HCV-EGFR interaction but most importantly provide insight into the pathologic consequences of derailed EGF signaling for liver inflammation and HCC development (Fig. 1). EGFR is a host factor for HCV by facilitating the assembly of the host entry complex, viral glycoprotein-dependent membrane fusion and cell-to-cell transmission of the virus.[7] HCV requires EGFR signaling to maintain its life cycle but also induces these signals itself during binding to the receptor complex.[6,10] Moreover, during HCV infection the non-structural protein NS5A prolongs EGFR signaling by perturbing its internalization and subsequent degradation.[11,12] This leads to a persistent EGFR activation during chronic HCV infection that potentially contributes to an impaired antiviral response by modulating interferon alpha signaling via STAT3.[13]

Keywords: EGF; HCV; TC-PTP; Chemokines; Virus-host interaction.
Received 23 May 2018; accepted 27 May 2018
☆ DOI of original article: http://dx.doi.org/10.1016/j.jhep.2018.04.005.
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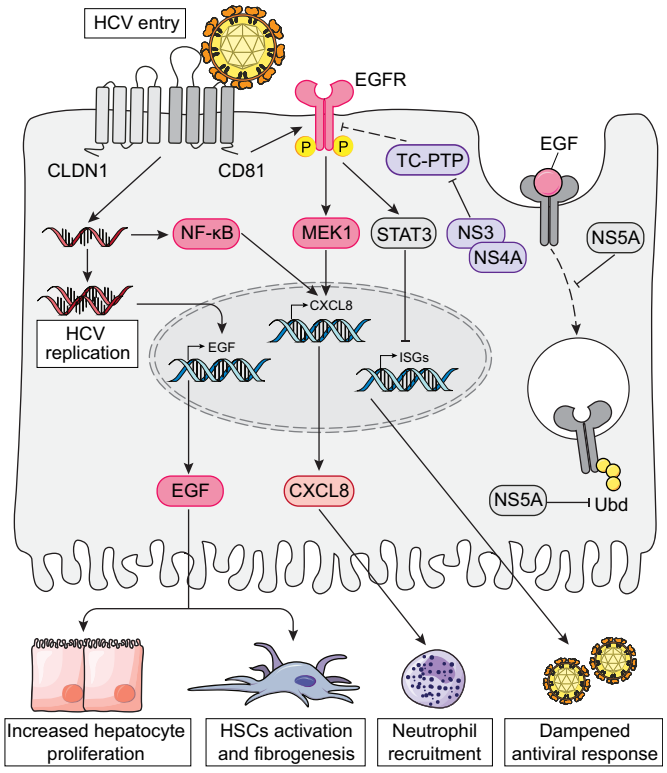

Fig. 1. Refined model of HCV-EGFR modulation and its impact on liver disease development. HCV binding to the HCV entry receptor complex (*i.e.* CD81, CLDN1) at the cell surface induces EGFR phosphorylation and downstream signaling. EGFR activity is prolonged by the NS5A-mediated perturbation of EGFR internalization and degradation. As a consequence, prolonged EGFR activity is associated with an increased hepatocyte proliferation, HSC activation, fibrogenesis and a dampened antiviral response via modulation of STAT3. Groepper *et al.*, demonstrated that HCV replication enhances the expression of CXCR2 ligands (e.g. *CXCL8*) by an EGF-dependent mechanism and activation of the NF-κB signaling pathway. This is further favored via the proteolytic cleavage of TC-PTP by NS3/4A, resulting in increased EGFR activation. Upon EGF stimulation, the production of CXCL8 during HCV replication promotes the recruitment of neutrophils.

Their finding that HCV replication promotes EGF expression is highly relevant in the study of HCV-induced chronic liver disease, as the EGF pathway is a key driver associated with progression towards cirrhosis[14] and HCC development.[15] Equally interesting is the observation that HCV-induced EGF expression is a regulator of CXCR2 ligands. For example, HCV infection has previously been described to promote CXCL8 expression, which inhibits interferon antiviral activity and facilitates viral infection.[16] Hepatic CXCL8 is detected at low maintenance levels during acute HCV infection, although marked increases in serum and hepatic levels have been observed in HCV-infected patients with progressive inflammation and cirrhosis.[17] Indeed, CXCL8, which is associated with poor outcome in patients with HCC, has been suggested as an HCC biomarker.[18] Here, Groepper and co-workers validated a mechanistic concept between EGFR signaling and CXCL8 during HCV infection, that has been previously proposed for hepatomas.[19] Moreover, they provide a previously undescribed mechanism linking EGFR signaling to chemoattraction of immune cells. In macrophages EGFR knockout attenuates HCC development in mice.[20] EGF-mediated recruitment of neutrophils during HCV infection is potentially relevant for liver pathobiology, since it has detrimental effects on the host by contributing to the necro-inflammatory process.[21]

Although further studies in larger patient cohorts are needed to consolidate the model proposed by Groepper and co-workers, the impact of their findings for liver disease and its association to EGF signaling is evident.[22] In future studies, it would be very interesting and potentially relevant to follow-up HCV-induced EGF expression pattern in liver tissue and blood samples before and after sustained viral response and to compare them to liver fibrosis scores. Furthermore, does HCV genotype influence EGF and chemokine expression profiles since genotype 3 is associated with more severe liver disease manifestations? Taken together, this paper represents a further corroboration for the clinical potential of HCC chemo-preventive strategies based on regulators of signal transduction. Indeed, EGFR which is phosphorylated in hepatic stellate cells has been successfully targeted by the clinical EGFR inhibitor erlotinib in animal models, demonstrating proof of concept that EGF-based therapies attenuates chemically induced liver fibrosis and HCC nodules.[14] Therefore, EGFR or MAPK modulators could be part of a personalized immuno-therapeutic strategy modulating chemokine profiles and inflammatory responses associated with liver disease progression.

Financial support

TFB acknowledges support by the European Union Horizon 2020 research and innovation program (ERC AdG HEPCIR – No. 667273; H2020 HEP-CAR – No. 667273; ERC POC PRELICAN – No. 755460), the NCI of the National Institutes of Health (1R21CA209940–01A1), the French National Research Agency (LABEX ANR-10-LABX-0028 HEPSYS) and the Office of the Assistant Secretary of Defense for Health Affairs (No. W81XWH-16–1-0363). JL and AARS acknowledge the French *Agence Nationale de Recherche sur le Sida et les Hépatites Virales* (ANRS) (ECTZ4236; ECTZ4446).

Conflict of interest

The authors declare no conflicts of interest that pertain to this work.

Please refer to the accompanying ICMJE disclosure forms for further details.

Authors' contributions

All authors conceived, wrote and reviewed the manuscript.

Acknowledgements

All authors thank Atish Mukherji (Inserm U1110, Strasbourg, France) for critical reading of the manuscript.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.jhep.2018.05.033.

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Author names in bold designate shared co-first authorship

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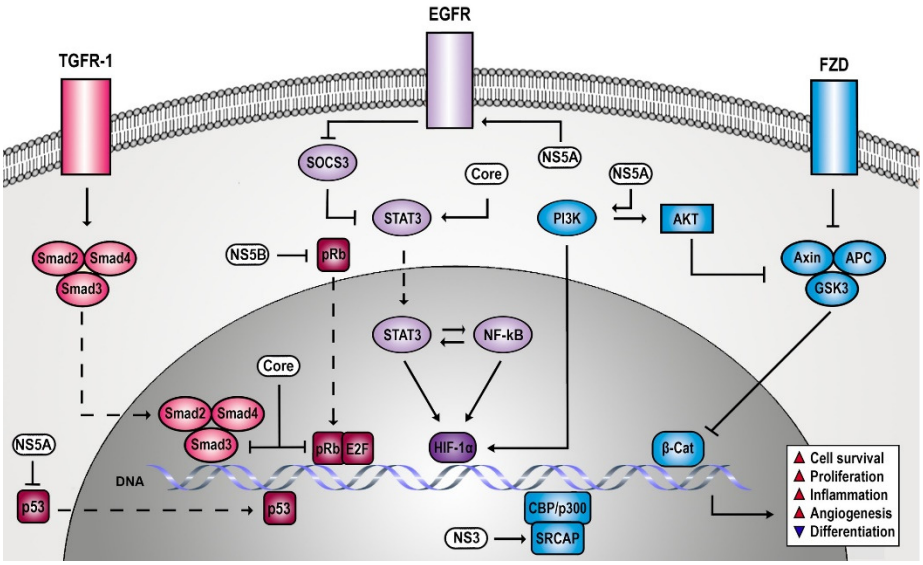
4.3.- Supplementary article III



Virzi, A.; **Roca Suarez, A. A.**; Baumert, T. F.; Lupberger, J. Oncogenic Signaling Induced by HCV Infection. *Viruses* **2018,** *10* (538).

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Review

Oncogenic Signaling Induced by HCV Infection

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Received: 1 September 2018; Accepted: 30 September 2018; Published: 2 October 2018

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.

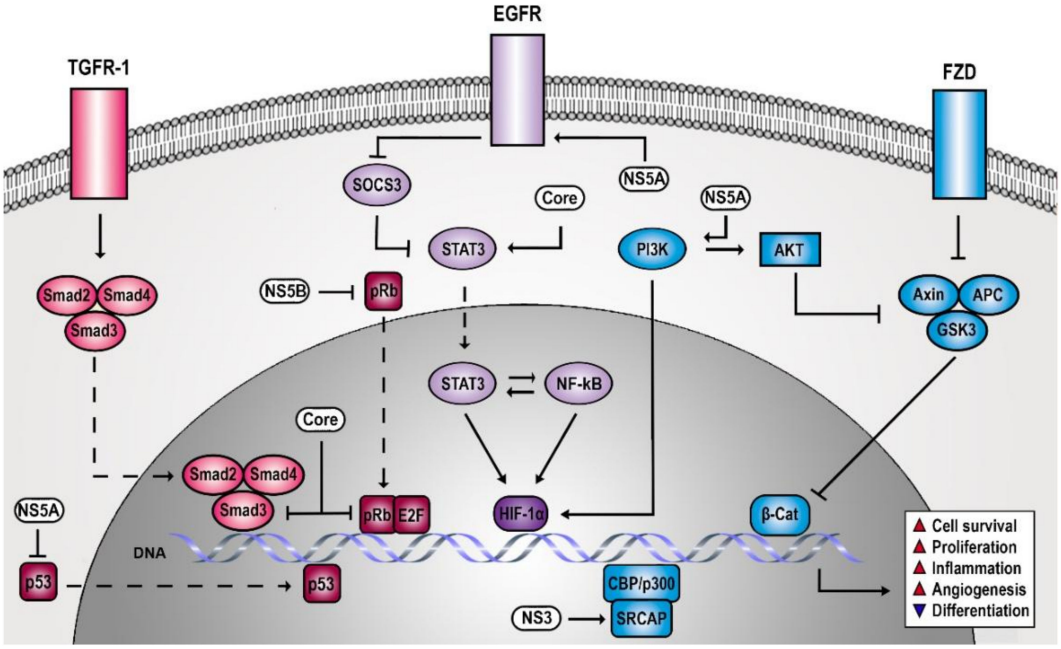

Figure 1. Hepatitis C Virus (HCV)-induced oncogenic signaling. HCV infection creates a procarcinogenic effect through the simultaneous dysregulation of cell survival, proliferation, inflammatory, angiogenic, and differentiation signaling pathways. The tight control of target genes involved in transcriptional 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.

Funding: This research was funded by the European Union (ERC-AdG-2014 HEPCIR to T.F.B., EU H2020 HEPCAR 667273 to T.F.B. and J.L.), the French Cancer Agency (ARC IHU201301187 to T.F.B.), the US Department of Defense (W81XWH-16-1-0363) to T.F.B., the National Institutes (NCI 1R21CA209940-01A1, NIAID R03AI131066, NIAID 5U19AI123862-02 to T.F.B.), and the French Agence Nationale de Recherche sur le Sida et les Hépatites Virales (ANRS) (ECTZ4236; ECTZ4446 to J.L. and A.A.R.S). This work has benefitted from the support of the Initiative of excellence IDEX-Unistra (ANR-10-IDEX-0002-02 to J.L. and A.V.) and has been published under the framework of the LABEX ANR-10-LAB-28. IDEX and LABEX are initiatives from the French program "Investments for the future".

Conflicts of Interest: The authors declare no conflict of interest.

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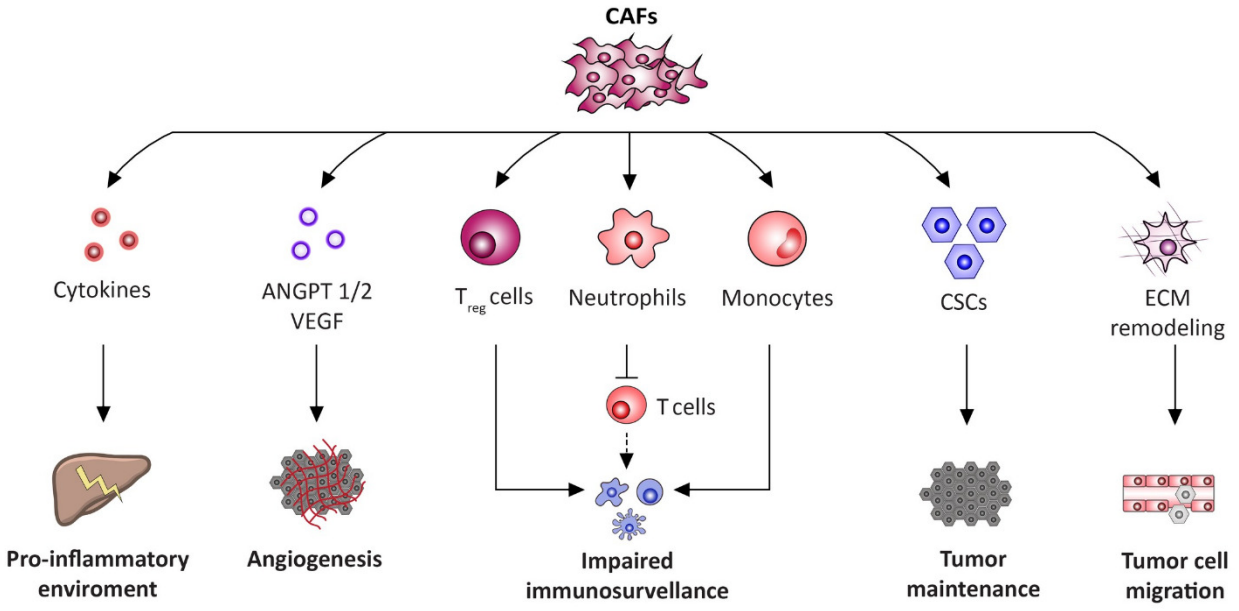
4.4.- Suppementary article IV



Saviano A, Roehlen N, Virzì A, **Roca Suarez A. A.,** Hoshida Y, Lupberger J, et al. Stromal and Immune Drivers of Hepatocarcinogenesis. In: Hoshida Y, editor. Hepatocellular Carcinoma: Translational Precision Medicine Approaches. Cham: Springer International Publishing; **2019**. p. 317-331.

Chapter 15
Stromal and Immune Drivers of Hepatocarcinogenesis

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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

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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

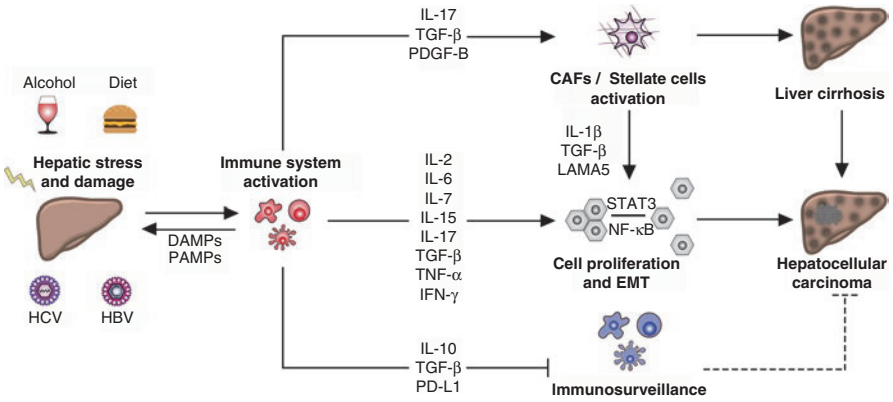

Fig. 15.1 Chronic inflammation is a pan-etiological driver of hepatocarcinogenesis. Hepatocarcinogenesis can be induced by multiple etiological and environmental conditions. Chronic HBV and HCV infections, as well as chronic alcohol abuse and metabolic syndrome trigger the activation of the innate immune 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 microenvironment, 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

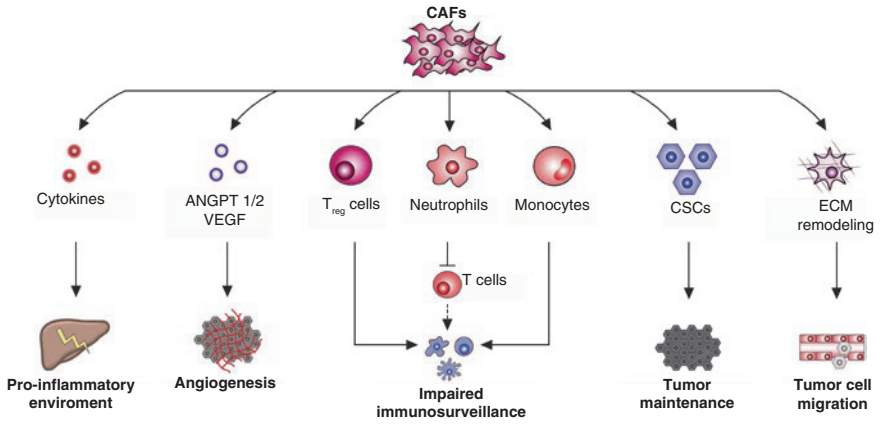

Fig. 15.2 Cancer-associated fibroblasts (CAFs) characterize the stromal tumor microenvironment and promote hepatocarcinogenesis, tumor progression and treatment resistance. Tumor microenvironment in HCC is predominantly characterized by cancer-associated fibroblasts (CAFs) that contribute actively to 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.

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5.- Résumé de la thèse de doctorat

5.1.- Introduction

5.1.1.- La charge globale du virus de l'hépatite C et ses complications

D'après de récentes estimations, environ 1 % de la population mondiale est infectée de manière chronique par le virus de l'hépatite C (VHC) (WHO., Global Hepatitis Report 2017), et l'on dénombre environ 500.000 décès par an dus aux complications secondaires de l'infection (Thrift, El-Serag et al. 2017). Malgré la disponibilité de méthodes efficaces de dépistage, seulement 20 % des patients ont connaissance de leur condition, car l'infection est asymptomatique pendant des années (WHO., Global Hepatitis Report 2017). Au cours du temps quand une cirrhose hépatique est établie, le risque de développer un carcinome hépatocellulaire (CHC) est très élevé (Villanueva 2019). La prise en charge de ces patients représente un coût de soins de santé considérable, en raison du prix des traitements et de la forte proportion de transplantations hépatiques liées au VHC (Terrault and Pageaux 2018). De plus, les traitements disponibles pour les patients atteints d'un CHC sont peu efficaces et limités en nombre (Villanueva 2019). L'ensemble de ces facteurs font du VHC et des complications hépatiques associées un problème majeur de santé publique qui nécessite une compréhension plus approfondie des altérations cellulaires associées à cette infection virale.

5.1.2.- Caractérisation des voies de signalisation liées au VHC et aux maladies hépatiques

Prenant en compte ces facteurs, la prévention du développement d'un CHC semble être la stratégie la plus efficace pour améliorer la survie des personnes infectées de manière chronique par le VHC et ses complications. L'identification de biomarqueurs et la caractérisation des voies de signalisation associées aux maladies hépatiques sont un domaine de recherche qui pourrait contribuer à la détection précoce des lésions, à un stade où les thérapies potentiellement curatives peuvent encore être utilisées (Hoshida, Fuchs et al. 2014). Les travaux réalisés par notre équipe de recherche sur les kinases cellulaires impliquées dans l'entrée du VHC en sont un exemple. En utilisant un criblage fonctionnel à ARN interférant et des pseudoparticules du VHC (VHCpp) mimant les étapes d'entrée du virus, 58 protéines tyrosine kinases (PTKs) ont été identifiées. Parmi ces PTKs, il a été montré que le récepteur à EGF (EGFR) joue un rôle clé dans l'entrée du VHC (Lupberger, Zeisel et al. 2011). Ces résultats sont pertinents non seulement pour la

compréhension du VHC, mais également pour le traitement de ses complications. Ainsi, l'efficacité de l'erlotinib, un inhibiteur du EGFR, dans la prévention du développement du CHC a été testée dans des modèles animaux de fibrose-cirrhose capables de développer un CHC. En effet, l'administration de l'erlotinib après les premiers signes de dommage hépatique dans un modèle de rat dans lequel les injections répétées du diethylnitrosamine (DEN) ont induits une fibrose progressive du foie, a permis de démontrer que l'inhibition de l'EGFR induit une régression de la fibrose hépatique au niveau histologique ainsi qu'une modulation de l'expression des gènes associés à la fibrogénèse (Fuchs, Hoshida et al. 2014).

5.1.3.- Rôle des phosphatases dans l'infection par le VHC et le développement du CHC

Contrairement aux PTKs, le rôle des protéines tyrosine phosphatases (PTPs) dans l'infection par le VHC et la progression vers le CHC est encore peu étudié. Comme dans le cas des kinases cellulaires, la compréhension de la fonction et de la régulation de ces phosphatases pourrait constituer de potentielles cibles thérapeutiques pour une meilleure maîtrise clinique des pathologies hépatiques associées à l'infection.

Dans les paragraphes suivants, je présente nos travaux concernant l'identification de la protéine tyrosine phosphatase récepteur delta (PTPRD) comme un gène candidat suppresseur de tumeur dans le foie, la caractérisation des voies de signalisation régulées par cette protéine et les possibles approches thérapeutiques qui peuvent être utilisées pour moduler les altérations associées à la diminution de son expression.

5.2.- Résultats

5.2.1.- miR-135a-5p induit une diminution de la protéine tyrosine phosphatase récepteur delta dans la carcinogenèse hépatique associée au VHC

Avec l'objectif d'étudier l'impact de l'infection par le VHC sur l'expression des protéines phosphatases humaines, nous avons analysé par qRT-PCR l'expression de 84 phosphatases humaines dans des biopsies de foie de patients infectés chroniquement par le VHC. Parmi celles-ci, 24 montrent une expression dérégulée de manière significative par rapport aux patients non infectés par le VHC (**Fig. 14a**). Quelques-unes de ces phosphatases ont été décrites comme gènes suppresseurs de tumeur comme par exemple la

phosphatase PTPRD (Veeriah, Brennan et al. 2009), qui présente une expression diminuée chez les patients infectés par le VHC.

Etant donné que la machinerie des miRNA est impliquée dans la régulation de l'expression génique cellulaire et qu'elle est détournée par le VHC pour favoriser sa réplication, nous avons utilisé des outils bio-informatiques pour examiner si des miRNA font partie du mécanisme responsable de la baisse d'expression de PTPRD. Ces analyses nous ont permis d'identifier miR-135a-5p comme un régulateur négatif de PTPRD avec une corrélation entre le taux de *PTPRD* et de miR-135a-5p chez les patients infectés (**Fig. 14b**).

PTPRD est une phosphatase qui a été précédemment impliquée comme gène suppresseur de tumeur dans le développement du glioblastome via son activité régulatrice de la voie STAT3 (Veeriah, Brennan et al. 2009). Par conséquent, une diminution de PTPRD pourrait induire l'activité de la voie STAT3 également dans le foie. En effet, nos résultats issus de la méthode du « *gene set enrichment analysis* » (GSEA) montrent une augmentation de l'activité de la voie de signalisation STAT3 chez les patients qui présentent une faible expression de *PTPRD* dans le foie (**Fig. 14c**). Ce déséquilibre entre PTPRD et STAT3 pourrait être un des mécanismes expliquant l'association d'une faible expression de *PTPRD* avec une diminution de la survie chez les patients atteints d'un CHC (**Fig. 14d**).

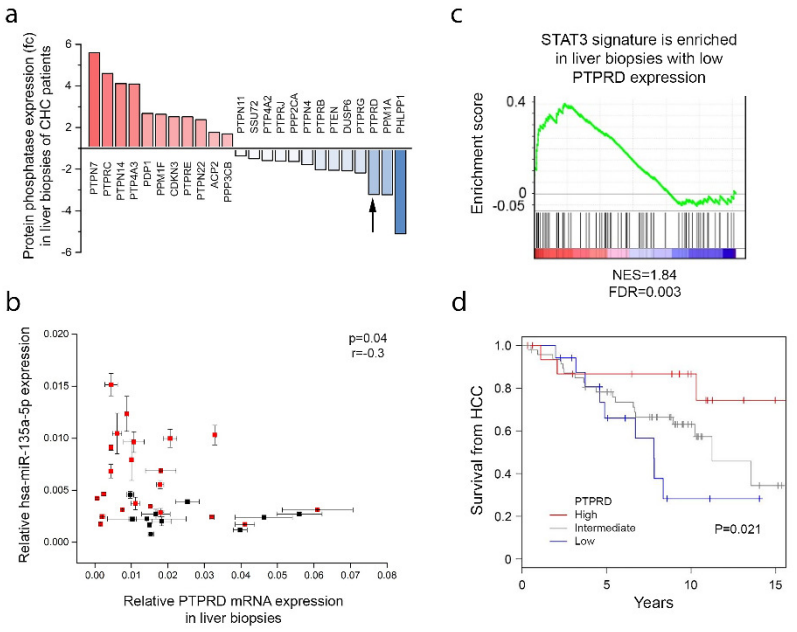

Figure 14 : miR-135a-5p induit une diminution de la protéine tyrosine phosphatase récepteur delta dans la carcinogenèse hépatique associée au VHC. **a**) Expression du mRNA de 24 phosphatases significativement dérégulées (p=<0.01, U-test) dans les biopsies du foie provenant de patients infectés par le VHC (n=6) par rapport aux patients non infectés (n=6). La phosphatase PTPRD est indiquée par une flèche. **b**) L'expression du mRNA de *PTPRD* est significativement et inversement corrélée (p=0.04, r=−0.03, Spearman's test) avec l'expression du miR-135a-5p dans les biopsies de foie humaines. **c**) Augmentation significative (NES=1.84, FDR=0.003) de la voie IL6-STAT3 dans les biopsies hépatiques humaines avec une faible expression du *PTPRD*. **d**) Les patients atteints du CHC ayant une faible expression du *PTPRD* présentent un taux de survie diminué (p=0.021, log-rank test).

5.2.2.- Analyses du métabolome, protéome et transcriptome des cellules infectées par le VHC pour l'identification des voies de signalisation associées au développement des maladies hépatiques

En parallèle de mon projet principal sur la phosphatase PTPRD et avec l'objectif d'avoir une vision globale sur les altérations cellulaires induites par l'infection du VHC, nous avons réalisé des analyses métabolomiques, transcriptomiques et protéomiques sur des modèles *in vitro*, *in vivo* et des échantillons humains. D'après nos résultats, nous avons observé une augmentation de l'activité de la voie STAT3 et une diminution des voies associées aux processus métaboliques y compris la voie du peroxysome, avec des résultats similaires dans des cellules huh7.5.1, des souris chimériques et des échantillons de foie de patients infectés par le VHC (**Fig. 15a**).

Etant donné qu'au niveau des patients des altérations de la fonction du peroxysome peuvent être pertinentes, nous avons analysé des biopsies hépatiques provenant des patients avec une cirrhose associée au VHC. Ainsi, nous avons observé que les patients avec des peroxysomes fonctionnellement altérés présentaient une probabilité de survie diminuée par rapport aux patients avec des peroxysomes intacts (**Fig. 15b**).

L'activation des voies inflammatoires régulant la fonction des peroxysomes (Taniguchi and Karin 2018), nous avons analysé l'inhibition de STAT3 afin de voir si elle pouvait moduler l'altération des peroxysomes induite par le VHC. Nous avons ainsi pu montrer que le traitement des cellules infectées par le VHC avec du niclosamide, un inhibiteur de STAT3, reverse l'inhibition des gènes associés aux fonctions des peroxysomes induite par le VHC (**Fig. 15c**).

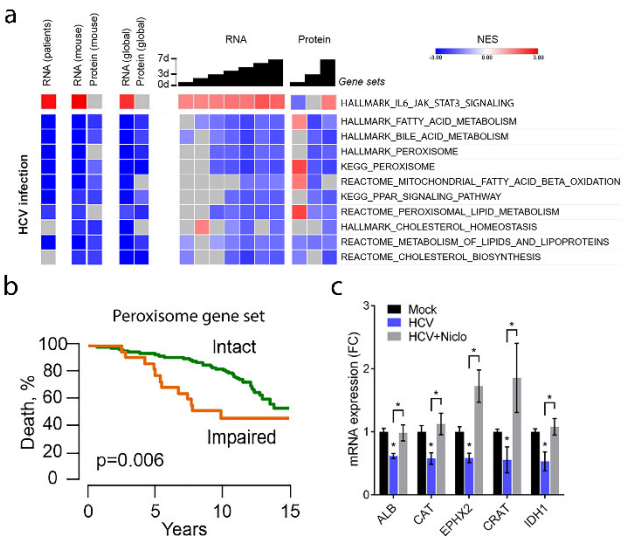

Figure 15 : Analyses du métabolome, protéome et transcriptome des cellules infectées par le VHC pour l'identification des voies de signalisation associées au développement des maladies du foie. a) GSEA sur des échantillons de patients infectés par le VHC par rapport aux patients non infectés (n=25 vs 6), des souris chimériques infectées et non infectées (n=3 vs 3) et au cours du temps de cellules huh7.5.1 infectées par le VHC jusqu'à 7 jours (n=2). **b**) Les patients atteints d'une cirrhose hépatique liée au VHC (n=216) et qui présentent une fonction altérée des peroxysomes montrent un taux de survie diminué (p=0.006). **c**) Des altérations des peroxysome induites par le VHC sont modulées par le traitement des cellules infectées avec l'inhibiteur de STAT3, le niclosamide. Quantification par qPCR de 5 *leading-edge genes* qui appartiennent au gene set HALLMARK_PEROXISOME et qui montrent une augmentation significative après traitement avec le niclosamide (p=<0.05, T test).

5.2.3.- La diminution du PTPRD induit des altérations dans les voies de signalisation associées aux processus métaboliques *in vivo*

Afin d'identifier de nouvelles voies de signalisation régulées par PTPRD dans le foie, nous avons analysé des biopsies de foie provenant de patients sains en utilisant la méthode du « GSEA » (Horvath, Erhart et al. 2014). Nos résultats montrent des altérations dans les voies de signalisation métaboliques du glucose chez les patients qui présentent une faible expression de *PTPRD* (**Fig. 16a**). Nous avons ensuite validé ces observations *in vivo* en utilisant un modèle animal hétérozygote pour *PTPRD* (*Ptprd*+/-). En réalisant du RNA-seq suivi d'une analyse de type « GSEA » sur des échantillons de foie des souris *Ptprd*+/-, nous avons obtenu des résultats similaires qui montrent par exemple une diminution de l'activité de la voie de l'insuline (**Fig. 16b**).

Les phénotypes potentiellement associés à ces altérations transcriptionnelles ont été étudié chez les souris *Ptprd*+/- recevant un régime déficient en choline et riche en gras (CD-HFD). Cette expérience a montré qu'après huit semaines du régime CD-HFD, les souris *Ptprd*+/- présentent un niveau significativement plus élevé de glucose dans le sang par rapport aux souris *wild-type* (**Fig. 16c**). Des résultats similaires ont été obtenu par l'analyse d'une cohorte de patients obèses (Margerie, Lefebvre et al. 2019), qui a montré des niveaux plus élevés de glucose dans le sang chez les patients présentant une faible expression du *PTPRD* dans le foie (**Fig. 16d**).

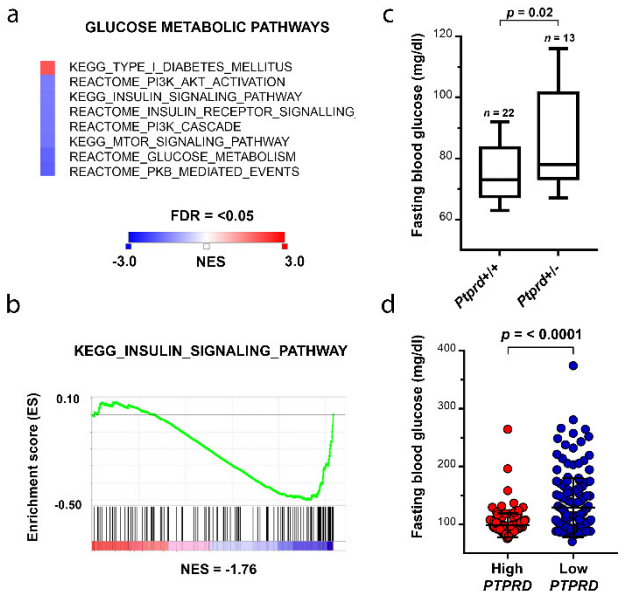

Figure 16 : La diminution du PTPRD induit des altérations dans les voies de signalisation associées aux processus métaboliques *in vivo*. **a)** Analyse de type « GSEA » montrant des altérations des voies métaboliques du glucose de manière significative (FDR=<0.05) chez les patients sains présentant une faible expression dans le foie du *PTPRD* (n=38). **b**) Diminution significative (FDR=<0.05) de l'activité de la voie de l'insuline chez les souris *Ptprd*+/- (n=3) par rapport aux souris WT (n=3). **c**) Augmentation des niveaux du glucose sanguin chez les souris *Ptprd*+/- (n=13) par rapport aux souris WT (n=22) (p=0.02, T test). **d**) Augmentation significative (p=<0.0001, T test) des niveaux de glucose sanguin chez les patients qui présentent une faible expression du *PTPRD* dans le foie (n= 147 vs 147).

5.3.- Discussion et perspectives

Les maladies chroniques du foie progressent généralement de la stéatose, vers la fibrose/cirrhose et aboutissent au développement du CHC. La similitude dans les étapes de cette progression indépendamment de l'étiologie (infection virale, alcoolisme, suralimentation), suggère l'implication de voies de signalisation communes à ces pathologies du foie. Dans ce cadre, nos travaux initiaux sur le VHC ont permis l'identification de la phosphatase PTPRD comme un facteur régulateur de l'activité de l'oncogène STAT3 dans le foie (Van Renne, Roca Suarez et al. 2018).

Ces résultats sont pertinents non seulement dans le contexte du CHC, mais aussi pour la compréhension des mécanismes impliqués dans le développement des complications métaboliques hépatiques. En effet, nos résultats ont montré que l'augmentation de l'activité de la voie STAT3 est associée à des altérations transcriptionnelles impliquées dans la fonction des peroxysomes et que l'inhibition de STAT3 permet d'améliorer l'activité de ce type de voies métaboliques (Lupberger, Croonenborghs et al. 2019).

De plus, nous avons montré qu'une faible expression de PTPRD induit des altérations transcriptionnelles et phénotypiques liées au métabolisme du glucose dans un contexte non-infectieux (Roca Suarez *et al*., en préparation). Ces résultats sont en accord avec des études précédentes qui ont montré une association entre des polymorphismes nucléotidiques dans le gène *PTPRD*, le développement du diabète (Tsai, Yang et al. 2010) et la réponse au traitement chez ces patients (Pei, Huang et al. 2013).

Comme perspectives, des expériences en cours de réalisation ont pour objectif la caractérisation du mécanisme moléculaire associé à l'action du PTPRD sur ces voies métaboliques. Une explication possible tient en l'action de STAT3, en effet les souris *Ptprd*+/- montrent une augmentation de la phosphorylation de cette protéine (données non montrées) et l'activité de STAT3 a été associée au développement de la résistance à l'insuline (Mashili, Chibalin et al. 2013).

En conclusion, l'ensemble de nos travaux ont permis la caractérisation des voies de signalisation impliquées dans les fonctions métaboliques altérées par des facteurs de risques comme le VHC, et la possible utilisation d'inhibiteurs de STAT3 comme une stratégie pour moduler la progression des maladies du foie.

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7.- Curriculum vitae

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Date of birth: 26/02/1988
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Degrees and diplomas:

2015 – 2019 **PhD in Molecular and Medical Virology:** Expression of human protein phosphatases during chronic HCV infection and HCC development. Inserm U1110 Institute of Viral and Liver Diseases, Strasbourg - France.
Supervisors: Prof. Dr. Thomas F. Baumert and Dr. Joachim Lupberger.

2013 – 2015 **Master's in Science, Technology and Health - Biology of Microorganisms (virology).** Mention: Bien. University of Strasbourg - France.

2006 – 2012 **Degree in Medicine and Surgery (MC, Médico Cirujano):** Catholic University of Bolivia (UCB), Santa Cruz - Bolivia.

Previous work experience:

2015 (January - June) **Master II internship:** Expression of human protein phosphatases during chronic HCV infection and HCC development. Inserm U1110 Institute of Viral and Liver Diseases, Strasbourg - France.
Supervisor: Dr. Joachim Lupberger.

2014 (June) **Master I internship:** Incorporation and reassortment of genomic RNA segments from the Influenza A PR8 strain. Institute of Molecular and Cellular Biology (IBMC) UPR 9002, Strasbourg - France.
Supervisor: Dr. Catherine Isel.

2013 – 2015 **Health carer:** *Abrapa aide à domicile*, Strasbourg - France.

2012 (May - June) **General physician:** Health prevention and epidemiology, campaign against Chagas disease and parasitic infections. San Jose Obrero hospital, Santa Cruz - Bolivia.

2011 – 2012 **Medicine internship:** Clinical oncology, surgical oncology, pediatric oncology, anatomic pathology, digestive surgery, clinical immunology, clinical genetics, endocrinology, reproductive medicine, cardiac surgery.
Louis Pasteur University, University Hospitals of Strasbourg - France.

Professional skills:

Scientific techniques: Cell culture, RT-qPCR, western blotting, immunofluorescence, infection models (Biosafety level 3 experience), molecular biology and oncology, Gene Set Enrichment Analysis (GSEA), CRISPR-Cas9 system, expertise in the study of cell signaling pathways.

Software: GenePattern, Graphpad Prism, Adobe Photoshop, Fiji ImageJ, MS office.

Languages: English (excellent), French (fluent), Spanish (mother tongue).

Funding awards:

- **Fondation pour la Recherche Médicale (FRM).** Expression of human protein phosphatases during chronic HCV infection and HCC development. Funding for end of PhD (8 months).

- **Cancéropôle Est**. 25th International Symposium on Hepatitis C Virus and Related Viruses. Dublin 8 – 11 October 2018. Funding for the participation in an international congress.

- **Leiden University.** Eurolife summer school Molecular Mechanisms in Cancer - Translating Discoveries into Personalized Therapies. Leiden 9 – 13 July 2018. Funding for the participation in an international congress.

- **Agence Nationale de Recherche sur le Sida et les hépatites virales (ANRS).** Protein tyrosine phosphatase delta is a candidate tumor suppressor of HCV-associated hepatocarcinogenesis. Funding for PhD (36 months).

References:

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Original publications:

- Lupberger, J.; Croonenborghs, T.; **Roca Suarez, A.A.**; Van Renne, N.; Jühling, F.; Oudot, M.A.; Virzì, A.; Bandiera, S.; Jamey, C.; Meszaros, G.; Brumaru, D.; Mukherji, A.; Durand, S.C.; Heydmann, L.; Verrier, E.R.; El Saghire, H.; Hamdane, N.; Bartenschlager, R.; Fereshetian, S.; Ramberger, E.; Sinha, R.; Nabian, M.; Everaert, C.; Jovanovic, M.; Mertins, P.; Carr, S.A.; Chayama, K.; Dali-Youcef, N.; Ricci, R.; Bardeesy, N.M.; Fujiwara, N.; Gevaert, O.; Zeisel, M.B.; Hoshida, Y.; Pochet, N.; Baumert, T.F.; Combined Analysis of Metabolomes, Proteomes, and Transcriptomes of HCV-infected Cells and Liver to Identify Pathways Associated With Disease Development, *Gastroenterology* **2019**. doi: 10.1053/j.gastro.2019.04.003. (**IF= 20.877**).

- Hamdane, N.; Jühling, F.; Crouchet, E.; El Saghire, H.; Thumann, C.; Oudot, M.A.; Bandiera, S.; Saviano, A.; Ponsolles, C.; **Roca Suarez, A.A.**; Li, S.; Fujiwara, N.; Ono, A.; Davidson, I.; Bardeesy, N.; Schmidl, C.; Bock, C.; Schuster, C.; Lupberger, J.; Habersetzer, F.; Doffoel, M.; Piardi, T.; Sommacale, D.; Imamura, M.; Uchida, T.; Ohdan, H.; Aikata, H.; Chayama, K.; Boldanova, T.; Pessaux, P.; Fuchs, B.C.; Hoshida, Y.; Zeisel, M.B.; Duong, F.H.T.; Baumert, T.F.; HCV-induced Epigenetic Changes Associated With Liver Cancer Risk Persist After Sustained Virologic Response. *Gastroenterology* **2019**. doi: 10.1053/j.gastro.2019.02.038. (**IF= 20.877**).

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- Jühling, F.; Hamdane, N.; Crouchet, E.; Li, S.; El Saghire, H.; Mukherji, A.; Thumann, C.; Oudot, M.A.; Saviano, A.; **Roca Suarez, A.A.**; Masia, R.; Soojodi, M.; Arora, G.; Ono, A.; Tabrizian, P.; Schwartz, P.; Davidson, I.; Schmidl, C.; Bock, C.; Schuster, C.; Chayama, K.; Pessaux, P.; Tanabe, K.; Hoshida, Y.; Zeisel, M.B.; Duong, F.H.T.; Fuchs, B.C.; Baumert, T.F.; Targeting clinical epigenetic reprogramming for chemoprevention of metabolic and viral hepatocellular carcinoma. (In revision, *Gut*).

- **Roca Suarez, A.A.**; Mukherji, A.; Brignon, N.; Mailly, L.; Jühling, F.; Durand, S.C.; Oudot, M.A.; Schaeffer, E.; Virzi, A.; Obringer, C.; Saviano, A.; Van Renne, N.; Habersetzer, F.; Pessaux, P.; Baumert, T.F.; Lupberger, J.; Impaired expression of protein tyrosine phosphatase receptor delta induces signaling alterations associated with glucose metabolism in the liver. (In preparation).

Reviews and comments:

- Virzi, A.; **Roca Suarez, A.A.**; Baumert, T.F.; Lupberger, J.; Rewiring Host Signaling: Hepatitis C Virus in Liver Pathogenesis. Hepatitis C viruses: The Story of a Scientific and Therapeutic Revolution. *Cold Spring Harbor Perspectives in Medicine* **2019**, 10.1101/cshperspect.a037366. (**IF= 5.564**).

- **Roca Suarez, A.A.**; Van Renne, N.; Baumert, T.F.; Lupberger, J.; Viral manipulation of STAT3: Evade, exploit, and injure. *PLoS Pathog* **2018,** *14* (3), e1006839. (**IF= 6.158**).

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- Goto, K.; **Roca Suarez, A.A.**; Wrensch, F.; Lupberger, J.; Baumert, T.F.; Hepatitis C virus and hepatocellular carcinoma: when the host loses its grip. (In preparation, *Int. J. Mol. Sci*).

Oral presentations and posters:

- **Roca Suarez, A.A.**; Jühling, F.; Baumert, T.F.; Lupberger, J.; *In vivo* characterization of PTPRD signaling pathways relevant to HCV infection and liver disease. Annual meeting AC42 – ANRS Hepatitis National Network. Paris, 7 – 8 February 2019 (Presentation).

- **Roca Suarez, A.A.**; Jühling, F.; Baumert, T.F.; Lupberger, J.; Candidate tumor suppressor PTPRD regulates signaling pathways relevant to HCV infection and hepatocellular carcinoma *in vivo*. 11eme forum du Canceropole Est. Reims 15 – 16 November 2018 (Presentation).

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- **Roca Suarez, A.A.**; Jühling, F.; Baumert, T.F.; Lupberger, J.; Expression of human protein phosphatases during chronic HCV infection and HCC development. Eurolife summer school - Molecular Mechanisms in Cancer - Translating Discoveries into Personalized Therapies. Leiden 9 – 13 July 2018 (Presentation).

- **Roca Suarez, A.A.**; Jühling, F.; Baumert, T.F.; Lupberger, J.; Impact of hepatic PTPRD expression on liver homeostasis and pathogenesis. 7^{ieme} colloque génomique fonctionnelle du foie. Lyon 14-16 mars 2018 (Presentation).

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Armando Andres ROCA SUAREZ

École Doctorale des Sciences de la Vie et de la Santé STRASBOURG

Expression des protéines phosphatases humaines pendant une infection chronique par le VHC et le développement du CHC

Résumé

L'infection chronique par le virus de l'hépatite C (VHC) est un facteur étiologique majeur menant au développement de maladies hépatiques. Ce processus est favorisé par le VHC via l'altération des voies de signalisation impliquées dans l'inflammation chronique du foie. Etant donné que les voies de signalisation cellulaires sont notamment régulées par les protéines phosphatases, tout déséquilibre de leur activité peut entraîner des conséquences désastreuses pour la cellule. Dans ce contexte, les résultats obtenus lors de mon travail de doctorat ont démontré que l'infection par le VHC induit la diminution de la protéine phosphatase récepteur type delta (PTPRD), un suppresseur de tumeur impliqué dans le développement de plusieurs cancers humains. La fonction perturbée du PTPRD favorise l'activité du facteur de transcription STAT3 dans le foie des patients, entraînant la progression de la maladie et conduisant finalement au développement du carcinome hépatocellulaire (CHC). Mes résultats suggèrent qu'une évaluation plus approfondie des inhibiteurs de STAT3 pourrait conduire à de nouvelles stratégies chimio-préventives ciblant la formation du CHC chez les patients à risque.

Mots clés : Carcinome hépatocellulaire, virus de l'hépatite C, phosphatase, PTPRD, STAT3.

Résumé en anglais

Chronic hepatitis C virus (HCV) infection is a major etiological factor leading to liver disease development. This process is favored by HCV through the alteration of signaling pathways mediating chronic liver inflammation. Since signal transduction is tightly regulated by protein phosphatases, any imbalance in their activity can elicit dire consequences for the cell. In this context, the results obtained during my PhD studies demonstrated how HCV infection induces the downregulation of protein tyrosine phosphatase receptor type delta (PTPRD), a tumor suppressor implicated in the development several human cancers. This perturbed PTPRD function promotes STAT3 transcriptional activity in the liver of patients, driving disease progression and ultimately leading to the development of hepatocellular carcinoma (HCC). My results suggest that further evaluation of STAT3-inhibitors could lead to novel chemo-preventive strategies targeting HCC formation in patients at risk.

Keywords: Hepatocellular carcinoma, hepatitis C virus, phosphatase, PTPRD, STAT3.