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Carla ELLER

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Un criblage gain-de-fonction identifie *CDKN2C* comme facteur d'hôte impliqué dans le cycle viral du virus de l'hépatite B

THÈSE dirigée par :

M. BAUMERT Thomas	HDR, PU-PH, université de Strasbourg
Mme. SCHUSTER Catherine	HDR, DR Inserm, université de Strasbourg

RAPPORTEURS :

Mme. NEUVEUT Christine	HDR, DR Inserm, université de Montpellier
M. LEVRERO Massimo	HDR, PU-PH, université de Lyon

AUTRES MEMBRES DU JURY :

M. GEORGEL Philippe	HDR, PU, université de Strasbourg
M. NASSAL Michael	HDR, Prof, université de Freiburg

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ABBREVIATIONS

AASLD	American Association for the Study of Liver Diseases
AGL	Antigenic loop
AIDS	Acquired immune deficiency syndrome
ALT	Alanine aminotransferase
Anti-HBs	Antibody targeting HBsAg
Anti-HBc	Antibody targeting HBcAg
Anti-HBe	Antibody targeting HBeAg
C	Core
C-ter	Carboxy-terminal
CAM	Capsid assembly modulators
cccDNA	Covalently closed circular DNA
CDK	Cyclin-dependent kinase
CDKN2C	Cyclin dependent kinase inhibitor 2C
cDNA	Complementary DNA
CHB	Chronic hepatitis B
Cip/Kip	CDK-interacting protein/kinase-inhibitory protein
CKI	CDK inhibitor
CLIA	Chemiluminescence immunoassay
CRISPR	Clustered regularly interspaced short palindromic repeats
CsA	Cyclosporin A
CTD	C terminal domain
ctrl	Control lentivirus
DAA	Direct acting antiviral
DHBV	Duck hepatitis B virus
dHepaRG	Differentiated HepaRG
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dpi	Days post infection
DR	Direct repeat
dslDNA	Double stranded linear DNA
EASL	European Association for the Study of the Liver
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked immunosorbent assay
EMA	European medicines agency
En	Enhancer

ESRP1	Epithelial splicing regulatory protein 1
et al.	*Et alii* (and others)
ETV	Entecavir
FACS	Fluorescence activated cell sorting
FDA	Food and drug administration
G0	Gap 0
G1	Gap 1
G2	Gap 2
GFP	Green fluorescent protein
GPC5	Glypican 5
HBc	HBV core protein
HBcAg	HBV core antigen
HBe	HBV e protein
HBeAg	HBV e antigen
HBs	HBV surface protein
HBsAg	HBV surface antigen
HBV	Hepatitis B virus
HBx	HBV x protein
HBxAg	HBV x antigen
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HDV	Hepatitis D virus
HIV	Human immunodeficiency virus
HL	Hepatocyte-like
HLF	Hepatic leukemia factor
HNF4	Hepatocyte nuclear factor
HNF4α	Hepatocyte nuclear factor 4 α
hNTCP	Human sodium taurocholate co-transporting polypeptide
HPV	Human papillomavirus
HR+	Hormone receptor positive
HSPG	Heparan sulfate proteoglycan
HTA	Host targeting agent
HTLV-1	Human T-cell leukemia virus type 1
IF	Immunofluorescence
IFN-α	Interferon-α
INK4	Inhibitor of CDK4
iPS	Induced pluripotent stem

KO	Knockout
L	Large
LEE	Ribociclib (LEE011)
Log2FC	Log2 fold change
M	Middle
MOI	Multiplicity of infection
mRNA	Messenger RNA
MVB	Multivesicular body
MyrB	Myrcludex B
N-ter	Amino-terminal
NA	Nucleoside/Nucleotide analogue
NHEJ	Non-homologous end-joining
nt	Nucleotide
NTCP	Sodium taurocholate co-transporting polypeptide
ORF	Open reading frame
P	Polymerase
Palbo	Palbociclib (PD-0332991)
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PegIFN-α	PEGylated interferon-α
pgRNA	Pregenomic RNA
PHH	Primary human hepatocyte
PI	Propidium iodide
Pol	Polymerase
PTH	Primary tupaia hepatocyte
qRT-PCR	Real-time reverse transcription-PCR
Rb	Retinoblastoma
rcDNA	Relaxed circular DNA
RNA	Ribonucleic acid
RNAi	RNA interference
RT	Reverse transcriptase
S	Small
S	Surface
sgRNA	Single guide RNA
siRNA	Small interfering RNA
Smc5/6	Structural maintenance of chromosomes 5/6
SVP	Subviral particle

TAF	Tenofovir alafenamide
TDF	Tenofovir disoproxil fumarate
Tet	Tetracycline
TP	Terminal protein
WHBV	Woodchuck hepatitis B virus
WHO	World health organization
(-)	Negative
(+)	Positive

INTRODUCTION

Viral hepatitis

Viral hepatitis is an inflammation of the liver caused by viral infection. The five major hepatitis viruses A-E can cause acute hepatitis. Additionally, hepatitis B, C and D viruses often lead to chronic hepatitis. Every year, viral hepatitis causes around 1.3 million deaths worldwide, mainly through progressive liver disease including liver cirrhosis and hepatocellular carcinoma (HCC) (WHO, 2017). This number is comparable to deaths caused by tuberculosis or HIV/AIDS and higher than those caused by malaria (MacLachlan and Cowie, 2015). Around 95% of these deaths are caused by chronic infection with hepatitis viruses B and C. The goal of the World Health Organization (WHO) to eliminate viral hepatitis as a major public health threat until 2030 is ambitious and requires major advances in the fields of HBV and HCV research (WHO, 2016). Hepatitis C virus (HCV) is a RNA virus that chronically infects around 71 million people worldwide (WHO, 2017). Thanks to the recent approval of new direct acting antivirals (DAAs) targeting viral proteins, HCV cure is now possible (Chung and Baumert, 2014). Therefore, the focus of HCV research is currently shifting towards the remaining risk for HCC even after HCV cure (Kanwal *et al.*, 2017; Hamdane *et al.*, 2019; Ioannou *et al.*, 2019; Singal *et al.*, 2019). However, viral cure is only very rarely achieved with currently available antivirals for the treatment of HBV infection, leaving HBV as a threat to global health.

HBV epidemiology

About 30% of the world population shows serological evidence of past or current HBV infection (Trépo, Chan and Lok, 2014). Worldwide, there are approximately 250 million people living with chronic HBV infection (see Figure 1) (Schweitzer *et al.*, 2015). According to the Global Burden of Disease Study 2010, HBV infection caused 786 thousand deaths in 2010 and was ranked the 15th leading cause of worldwide mortality (Lozano *et al.*, 2012). Importantly, HBV infection is the leading cause of HCC worldwide, accounting for more than 50% of primary liver cancers, whose associated mortality increased by 62% from 1990 to 2010 (Lozano *et al.*, 2012; Trépo, Chan and Lok, 2014). Approximately 5% of HBV patients are co-infected with hepatitis D virus (HDV), a satellite virus depending on HBV surface antigen (HBsAg) for the production of infectious virions (Wedemeyer and Manns, 2010). Wrapped into HBV envelope proteins, HDV is expected to behave like HBV in very early steps of the life cycle as attachment and entry. In nature, HDV co- or super-infection occurs with HBV infection and worsens its outcome. Treatment of HBV-HDV patients in less effective than treatment of HBV patients (Sultanik and Pol, 2016; WHO, 2017). Although HDV is naturally packaged in HBsAg, it can take advantage of surface proteins from enveloped viruses other than HBV for viral spread (Perez-Vargas *et al.*, 2019).

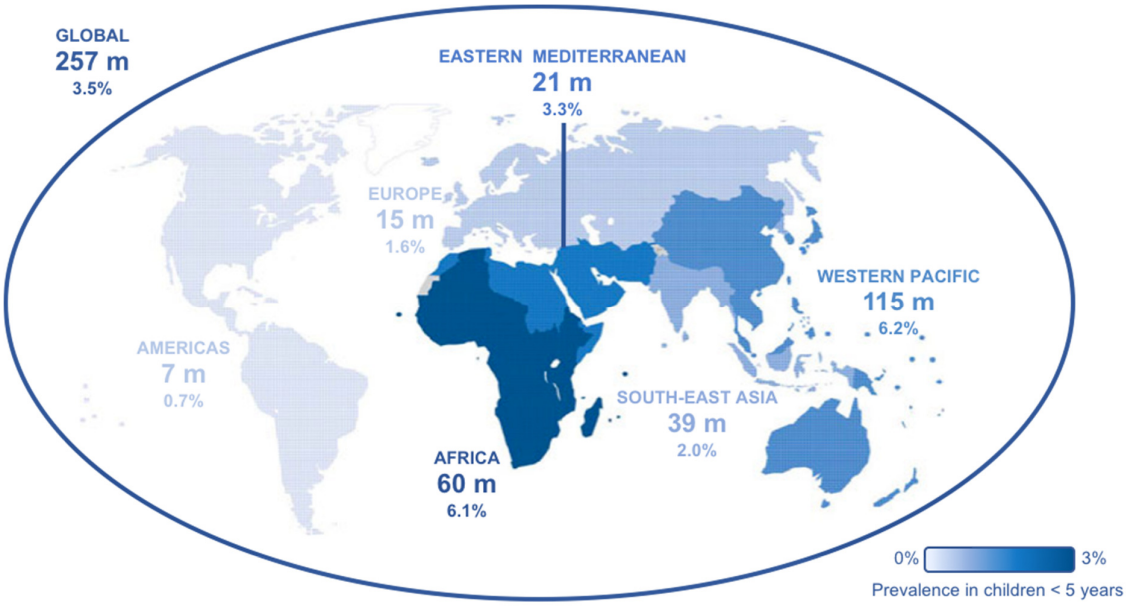

Figure 1: Global prevalence of HBV infection (HBsAg) by WHO regions in 2015. Indicated are estimated numbers of persons living with HBV in millions (m) and estimates of the prevalence of HBV infection in %. The color code depicts the incidence of chronic HBV infection in children under 5 years of age in 2015: the estimated global prevalence of HBV infection in this age group was about 1.3% (3% in the African region), compared to about 4.7% in the pre-vaccination era. HBsAg: HBV surface antigen, WHO: world health organization. Modified from WHO Global hepatitis report 2017: www.who.int/hepatitis/publications/global-hepatitis-report2017 (WHO, 2017).

Infection with HBV occurs through contact with infected body fluids like blood or semen. The vast majority of infections arise from three major modes of transmission (Trépo, Chan and Lok, 2014). The infection of embryos by their infected mothers before, during, or shortly after birth is called perinatal transmission. The risk for vertical transmission from an infected mother to her infant increases with maternal HBV DNA and HBV e antigen (HBeAg) serum levels (Umar, Umar and Khan, 2013). The transmission rate from HBeAg-positive mothers is very efficient and can reach up to 90% (Umar, Umar and Khan, 2013). The two major sources of horizontal HBV transmission are unsafe sexual contact and drug injection. Occasionally, HBV infection is acquired through contact with contaminated blood products, organ donations, medical instruments or unsafe medical practices (Trépo, Chan and Lok, 2014). Horizontal HBV infection via the bloodstream is very efficient as only a few number of HBV particles (< 20) seems to be sufficient to infect the liver (Candotti *et al.*, 2019). HBV infection only rarely leads to chronic infection with the main determinant for the likelihood of progression to chronicity being the age at infection. For infants infected at birth this likelihood is 90%, while children infected at the age between 1 and 5 years develop chronic infection in about 30% of cases (Edmunds *et al.*, 1993). After horizontal transmission in adults, acute infection is resolved spontaneously in over 90% of cases, while progression to chronicity occurs in only around 5% of cases (MacLachlan and Cowie, 2015; Petruzziello, 2018). The global distribution of chronic HBV carriers is not homogenous, with high

prevalence (> 8%) in highly endemic areas like sub-Saharan Africa and east Asia. In these regions vertical transmission dominates, while in low-endemic areas (prevalence of chronic infection ≤ 2%) transmission occurs mostly horizontally (Schweitzer *et al.*, 2015; Caballero *et al.*, 2018).

There exist different genotypes of HBV, that are classified according to phylogenic analysis of the viral genome. To date, ten genotypes (A-J) have been defined by more than 8% sequence variation across the genome (Okamoto *et al.*, 1988; Norder, Couroucé and Magnius, 1994; Stuyver *et al.*, 2000; Arauz-Ruiz *et al.*, 2002; Olinger *et al.*, 2008; Tatematsu *et al.*, 2009). There are differences among the genotypes in geographic distribution, transmission mode, and clinical outcomes (McNaughton *et al.*, 2019). For instance, genotypes A and D are prevalent in Europe, genotypes B and C in Asia, and genotype E in sub-Saharan Africa (Valaydon and Locarnini, 2017).

HBV natural history and pathology

Upon HBV infection, patients can develop acute hepatitis with or without symptoms and typically recover and acquire protective levels of antibodies targeting the HBV surface protein (anti-HBs) (Fattovich, 2003). In case patients do not recover, they develop chronic HBV infection, which is characterized by the persistence of HBsAg in serum longer than six months after infection (Trépo, Chan and Lok, 2014). In contrast to infants infected perinatally, adults infected with HBV only rarely develop chronic HBV infection, thanks to a functionally efficient antiviral T-cell response, which allows a persistent control of infection (Ferrari, 2015). The development of chronic HBV infection, however, is linked with a lack or an exhaustion of HBV-specific T-cell responses (Ferrari, 2015). Chronic HBV infection is a dynamic process determined by the interaction between HBV replication and the host immune response (EASL, 2017). The course of HBV infection is divided into five phases, which describe disease progression (see Figure 2) (Fattovich, Bortolotti and Donato, 2008). Each of them is characterized by distinctive serum levels of HBV markers (for HBV antigens see chapter HBV proteins) and the stage of liver inflammation (Terrault *et al.*, 2016; EASL, 2017). Another characteristic used to distinguish the phases is the serum activity of alanine aminotransferase (ALT), an enzyme found abundantly in the cytosol of hepatocytes (Kim *et al.*, 2008). Because serum ALT activity is increased in the case of hepatocellular injury or death, it is widely used as a sensitive marker for liver disease (Kim *et al.*, 2008). In 2017, a new nomenclature has been suggested by the EASL to emphasize the difference between chronic HBV infection (normal ALT, no liver inflammation) and chronic hepatitis B (elevated ALT, liver inflammation and fibrosis, accelerated progression of liver disease) (EASL, 2017):

1. HBeAg-positive chronic HBV infection (formerly immune tolerant phase) is characterized by high serum levels of HBe and HBV DNA, and normal ALT activity. In the liver, there are no signs of significant inflammation or fibrosis.

2. HBeAg-positive chronic hepatitis B (formerly immune active phase) is characterized by high serum levels of HBe and HBV DNA, in combination with elevated ALT activity and liver injury including moderate to severe inflammation and accelerated fibrosis progression.
3. HBeAg-negative chronic HBV infection (formerly inactive carrier phase) is induced by seroconversion from HBe to anti-HBe. It is characterized by high serum anti-HBe and low serum HBV DNA, and normal ALT activity. In the liver, there is minimal inflammation, however variable fibrosis following previous liver injury.
4. HBeAg-negative chronic hepatitis B (previously immune reactivation phase) is characterized by absent serum HBeAg, detectable anti-HBe, moderate to high HBV DNA, and elevated ALT activity. In the liver, there is inflammation and fibrosis.
5. The HBsAg-negative phase (previously occult HBV infection) is characterized by absent serum HBsAg, detectable anti-HBc, undetectable HBV DNA, and normal ALT activity. The stage of liver injury depends on previous phases.

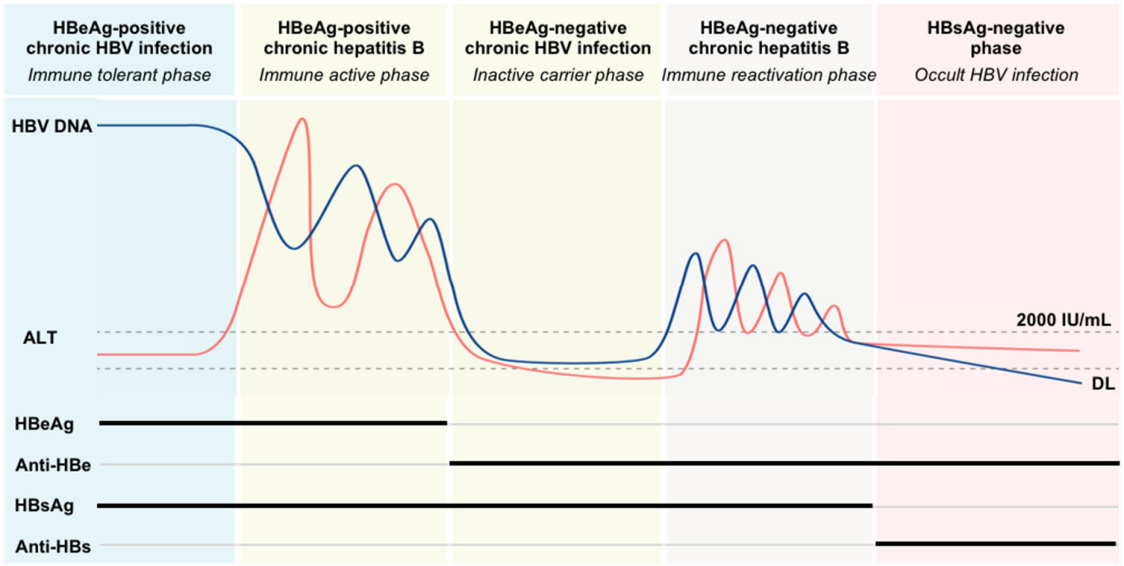

Figure 2: Natural history of chronic HBV infection. Schematic representation of five phases of the natural history of chronic HBV infection. Nomenclature as defined by the European Association for the Study of the Liver (EASL) in 2017 in bold type and former nomenclature in italic type. Serum HBV DNA levels (blue line) and ALT activity (red line) are indicated. The threshold for the definition of low HBV DNA is 2000 IU/mL. Presence of HBeAg, anti-Hbe, HBsAg, and anti-HBs are indicated by black lines. For details see chapter HBV natural history and pathology. ALT: alanine aminotransferase, HBeAg: HBV e antigen, anti-HBe: antibody targeting HBeAg, HBsAg: HBV surface antigen, anti-HBs: antibody targeting HBsAg, DL: detection limit. Modified from: (Fanning *et al.*, 2019).

These phases can last between weeks and decades, depending primarily on transmission mode and age at infection (Fattovich, 2003). Importantly, the succession of the phases of chronic HBV infection is not necessarily consecutive (EASL, 2017).

In patients with chronic hepatitis B (CHB), liver disease progresses through liver fibrosis and liver cirrhosis finally leading to HCC. Liver fibrosis is the excessive accumulation of extracellular matrix proteins in the liver including collagen, leading to a distortion of the hepatic structure (Bataller and Brenner, 2005). The cell type mainly responsible for the excess production of collagen are hepatic stellate cells, which change from a quiescent to an activated, collagen-producing state (Tsukada, Parsons and Rippe, 2006). To describe the severity of fibrosis, scoring systems based on histological staining of liver biopsy samples are used to discriminate five stages of fibrosis F0-F4 (Manning and Afdhal, 2008). The last stage of fibrosis describes the development of nodules of regenerating hepatocytes surrounded by fibrous bands, which is also defined as cirrhosis (Bataller and Brenner, 2005; Schuppan and Afdhal, 2008). The major clinical consequences of cirrhosis are hepatocellular dysfunction, an increased intrahepatic resistance to blood flow (portal hypertension) and the development of HCC (Bataller and Brenner, 2005; Schuppan and Afdhal, 2008). Reaching cirrhosis is a critical step, as HBsAg loss before the onset of cirrhosis is associated with a minimal risk of cirrhosis and HCC, and improved survival (EASL, 2017). Causing roughly 50% cases of total liver cancer mortality, chronic HBV infection is a leading risk factor for HCC (Lozano *et al.*, 2012). There are several risk factors that increase HCC risk among HBV carriers, including older age, male gender, high viral load, co-infection (HCV, HDV, HIV), exposure to aflatoxin, alcohol abuse, and cigarette smoking (Petruzziello, 2018). Three different mechanisms have been suggested to contribute to the multistep process of hepatocarcinogenesis in patients chronically infected with HBV, including indirect (inflammation-mediated) and direct (HBV DNA integration and HBV protein-induced) mechanisms (Bouchard and Navas-Martin, 2011). Hepatic inflammation caused by chronic HBV infection and resultant liver regeneration leads to an accumulation of genetic damage contributing to carcinogenesis (Bouchard and Navas-Martin, 2011; Teng *et al.*, 2018). The important role of liver disease in HCC development is highlighted by the fact that the vast majority (70-90%) of HBV-related HCC develops in cirrhotic livers (J. D. Yang *et al.*, 2011). Furthermore, the expression of the HBV X protein (HBx) that modulates several processes including transcription, cell cycle progression and apoptosis is thought to play a crucial role in HCC development (Ng and Lee, 2011). Finally, HBV DNA integration into the host genome may promote HCC development, as HBV integration is observed more frequently in tumors than in adjacent liver tissues, and the number of HBV integrations in HBV-derived HCC is associated with patient survival (Sung *et al.*, 2012). In this regard, HBV integration not only promotes genomic instability, but also directly mutagenizes cancer related genes, for instance the human telomerase reverse transcriptase gene (Paterlini-Bréchot *et al.*, 2003; Buendia and Neuveut, 2015; Levrero and Zucman-Rossi, 2016). Remarkably, HBV DNA integration starts occurring at an early stage of HBV infection, suggesting that hepatocarcinogenesis could be ongoing already during the HBeAg-positive chronic HBV infection (formerly immune tolerant phase) (EASL, 2017).

Treatment of HBV infection

Treatment of chronic HBV infection aims at the improvement of quality of life and survival of patients by preventing disease progression. The ideal clinical goal of HBV treatment is 'viral cure', the elimination of all forms of viral genome with the potential to replicate from the patient's liver (Liang *et al.*, 2015). However, this goal seems very ambitious, considering HBV DNA integration and the long lasting persistence of the viral maintenance reservoir cccDNA in hepatocytes (see chapter HBV genome organization), even in patients who recover from acute hepatitis (Michalak *et al.*, 1994; Rehermann *et al.*, 1996; Revill *et al.*, 2019). Therefore, alternative clinical endpoints of therapy are pursued. The more realistic 'functional cure' characterized by loss of HBsAg indicates a profound suppression of viral replication and protein expression and allows for safe discontinuation of antiviral therapy (EASL, 2017). Further endpoints of therapy include ALT normalization, HBeAg loss, and suppression of serum HBV DNA levels (EASL, 2017).

Currently, there are two classes of antivirals approved for the treatment of chronic HBV infection that suppress viral replication: interferon-α (IFN-α) based treatment and nucleos(t)ide analogues (NAs) (see Table 1) (Levrero *et al.*, 2018). IFN-α was the first approved treatment option for chronic hepatitis B (CHB) (Greenberg *et al.*, 1976). Today, IFN-α is used in a PEGylated form, PEGylated interferon-α (PegIFN-α), which improves its stability, half-life, and treatment response (Craxi and Cooksley, 2003). The effect of IFN-α is mainly through the induction of immunological control, but also through direct antiviral effect on HBV (Rijckborst and Janssen, 2010). NAs act as inhibitors of the reverse transcriptase activity of the HBV polymerase. Lamivudine, the first NA approved for the treatment of HBV infection was first approved for the treatment of the retrovirus HIV (Quercia *et al.*, 2018). Since then, second generation NAs with a higher barrier to resistance have been developed. Tenofovir alafenamide (TAF) was approved for the treatment of chronic HBV infection in 2016 and is now on the list of preferred HBV therapies, along with entecavir (ETV), tenofovir disoproxil fumarate (TDF), and PegIFN-α recommended by the European Association for the Study of the Liver (EASL) and the American Association for the Study of Liver Diseases (AASLD) (EASL, 2017; Terrault *et al.*, 2018). Both of these classes of antivirals allow improved quality of life and survival. However, both of them fail to systematically achieve functional cure. In addition, treatment with second generation NAs decreases the risk of HCC but is not sufficient to eliminate the risk (Papatheodoridis *et al.*, 2017). Since viral eradication with these medications is rare, lifelong therapy is required in most cases (Werle–Lapostolle *et al.*, 2004a). Therefore, alternative therapeutic strategies against chronic HBV infection are needed.

Several alternative treatment options directly targeting HBV proteins or genome intermediates have been suggested. Polymers of different chemical composition have broad spectrum antiviral activity, relying on their amphipathic (hydrophobic) character (Vaillant, 2016). For instance, sulfated polysaccharides interfere with the initial attachment of virions to the host cell surface by interaction with viral envelope proteins (Hosoya *et al.*, 1991). In addition to the entry inhibitory activity, single stranded nucleic acid polymers were shown to inhibit the release of HBsAg (Noordeen *et al.*, 2015; Quinet *et al.*,

2018). Compounds targeting the HBV core protein, named capsid assembly modulators (CAMs), were shown to have antiviral activity by interfering with capsid assembly (Deres *et al.*, 2003).

Two different classes of CAMs with distinct mechanisms have been developed. Compounds belonging to the group of phenylpropenamides and sulfamoylbenzamides increase the rate of capsid assembly, leading to the formation of empty capsids without the polymerase-pgRNA complex (Katen *et al.*, 2010; Campagna *et al.*, 2013). The second class of CAMs, the heteroaryldihydropyrimidines, cause abnormal aggregation of core protein units leading to the formation of capsid-like structures (Stray *et al.*, 2005; Wang *et al.*, 2012). More recently, CAMs were shown to have a dual mechanism of action, also interfering with early steps of the viral life cycle by disruption of intact capsid (Berke *et al.*, 2017; Schlicksup *et al.*, 2018).

Table 1: Features of currently available classes of antivirals for the treatment of chronic hepatitis B. Modified from: (EASL, 2017)

	NAs (ETV, TDF, TAF)	***PegIFN-α***
Strategy	Inhibition of viral replication	Induction of immune control
Administration	Oral	Subcutaneous injection
Treatment duration	Long-term until HBsAg loss	48 weeks
Tolerability	High	Low
Contraindications	None	Many
Resistance development risk	Minimal	No
Viral suppression level	High	Moderate
Effect on HBsAg levels	Low	Higher than NAs

The development of new powerful genome engineering tools, in particular the exploitation of the RNA-guided Cas9 nuclease from the microbial clustered regularly interspaced short palindromic repeats (CRISPR) adaptive immune system, have opened new treatment options (Hsu *et al.*, 2013). In this regard, it has been shown that the Cas9 nuclease can be recruited to HBV DNA and efficiently cleave cccDNA and integrated HBV DNA (see chapter HBV genome organization) (Seeger and Sohn, 2014; Ramanan *et al.*, 2015; H. Li *et al.*, 2017). The removal of all forms of viral DNA from infected hepatocytes is essential for viral cure. In another approach, RNA interference (RNAi) has been used to target cccDNA-derived viral RNA transcripts with high specificity (Schluep *et al.*, 2017; Wooddell *et al.*, 2017).

Novel therapeutic strategies include not only direct-acting antiviral (DAAs) that target viral products, but also host-targeting agents (HTA) that modify the host cell function to prevent viral replication.

Host-targeting-agents (HTAs) for the treatment of viral infection

Advantages of HTAs are the higher genetic barrier to resistance compared to DAAs and potential broad-spectrum antiviral effects by targeting host proteins required by several viruses (Bekerman and Einav, 2015). For instance, targeting cyclophilins has broad-spectrum antiviral activity against several viruses including HCV and HIV by different mechanisms including the modulation of protein folding and immune responses (Frausto, Lee and Tang, 2013; Lin and Gallay, 2013; Gallay *et al.*, 2015). Furthermore, inhibitors of cyclin G-associated kinase interfere with trafficking of numerous viruses including HCV, Dengue virus and Ebola virus (Pu *et al.*, 2018). Arbidol, a broad-spectrum antiviral drug approved for the treatment of influenza in Russia and China, acts as fusion inhibitor of enveloped and unenveloped viruses (Kadam and Wilson, 2017). It has also been shown to be effective against HCV, Ebola virus, and flaviviruses including Zika, however at high doses and in a cell-type specific manner (Boriskin, Pécheur and Polyak, 2006; Borisevich *et al.*, 2016; Fink *et al.*, 2018; Haviernik *et al.*, 2018). Obviously, essential cellular factors cannot be targeted as the regular functioning of the cell has to be maintained to avoid toxicity.

Given the tiny size of its genome and the small number of encoded proteins, HBV relies on many host functions for its life cycle (see chapter HBV host interactions). Therefore, HTAs have emerged as novel antiviral strategy for the treatment of HBV infection (Baumert *et al.*, 2015). The discovery of the sodium taurocholate co-transporting polypeptide (NTCP) as functional receptor for HBV infection has stimulated the development of several entry inhibitors for treatment of HBV infection targeting the receptor (Volz *et al.*, 2013; Nkongolo *et al.*, 2014; Watashi *et al.*, 2014; Donkers *et al.*, 2017; Shimura *et al.*, 2017; Kaneko *et al.*, 2018). Myrcludex B (MyrB) is a myristoylated 47-amino acid peptide derived from the preS1-domain of the large HBV surface protein. It is a competitive inhibitor of HBV and HDV entry, as it specifically binds to NTCP and thereby blocks entry of both viruses in cell lines, PHH, and humanized mice at nanomolar concentrations (Gripon *et al.*, 2002; Volz *et al.*, 2013; Ni *et al.*, 2014). Remarkably, the inhibitory effect of MyrB on HBV entry was already discovered before the identification of its binding receptor NTCP (Gripon *et al.*, 2002). In phase II clinical trials, monotherapy with MyrB of patients with chronic hepatitis B and D was well tolerated. While HBsAg levels were not affected and HBV DNA declined insignificantly, MyrB monotherapy lead to a reduction of serum HDV RNA levels and alanine aminotransferase (ALT) normalization (Bogomolov *et al.*, 2016; Wedemeyer *et al.*, 2018). Another known inhibitor of NTCP is the cyclic eleven amino acid peptide cyclosporin A (CsA) (Schreiber and Crabtree, 1992; Azer and Stacey, 1993; Mita *et al.*, 2006; Dong, Ekins and Polli, 2013). Clinically, it is used as immunosuppressive drug after kidney transplantation and for other applications (e.g. in eye drops) (Calne *et al.*, 1978; Lim, Kohli and Bloom, 2017; Nebbioso *et al.*, 2019). Shortly after the identification of NTCP as HBV/HDV receptor, CsA was shown to inhibit infection of both viruses by blocking NTCP-mediated entry (Nkongolo *et al.*, 2014; Watashi *et al.*, 2014). Because CsA also interferes with the transporter function of NTCP and impairs bile acid uptake, CsA derivatives have been generated that prevent HBV entry while maintaining the bile acid transporter function of

NTCP (Shimura *et al.*, 2017). In a repurposing approach, HBV entry inhibitors targeting NTCP were identified by screening of EMA/FDA-approved drugs for reduction of taurocholic acid uptake and MyrB-binding (Donkers *et al.*, 2017). Although NTCP remains a promising target, other host functions have been identified as potential targets for HTAs against HBV infection. For instance, an inhibitor of clathrin-mediated endocytosis, chlorpromazine, might inhibit HBV infection (Huang *et al.*, 2012). Furthermore, cellular permissiveness to HBV replication may be impaired through depolymerization of cellular microtubules by the compound nocodazole (Iwamoto *et al.*, 2017). Another potential target for HTAs against HBV might be the hepatocyte nuclear factor 4 alpha (HNF4α), which is known to play a critical role in HBV replication (Raney *et al.*, 1997; Tang and McLachlan, 2001). In this respect, knockdown of HNF4α by RNAi was shown to inhibit HBV transcription and replication in a cell line and a mouse model (He *et al.*, 2012). The identification of further proviral factors involved in the HBV life cycle may lead to the development of novel treatment strategies including HTAs. While central interactions with hepatocyte functions remain unknown, the HBV genome and structure are established.

HBV virology

Hepadnaviridae

The *hepadnaviridae* family includes small enveloped hepatotropic DNA viruses belonging to the pararetrovirus group. All members of this family, including HBV, have a narrow host range and share comparable genome structure and replication strategy involving reverse transcription (Nassal, 2015). HBV infection is restricted to humans and non-human primates. Although chimpanzees can experimentally be infected with HBV derived from human plasma, there exist HBV strains indigenous to chimpanzees and other primates (Vaudin *et al.*, 1988; Norder *et al.*, 1996; Lanford *et al.*, 1998; Warren, Heeney and Swan, 1999; Wieland, 2015). The tree shrew tupaia belangeri can also experimentally be infected with HBV, but natural infection in this species has not been identified (Walter *et al.*, 1996). Until recently, only two species-specific genera were known infecting mammals (*orthohepadnaviruses*) or birds (*avihepadnaviruses*). In addition those infecting primates, there exist other *orthohepadnaviruses* infecting ducks, woodchucks, and squirrels (Summers, Smolec and Snydert, 1978; Marion *et al.*, 1980; Mason, Seal and Summers, 1980). Notably, *avihepadnaviruses* are missing a functional X protein present in viruses infecting mammals (van Hemert *et al.*, 2011). In addition to the discovery of further *ortho-* and *avihepadnaviruses*, putative hepadnaviruses infecting other groups of vertebrates like fish (*metahepadnaviruses*) and amphibians (*herpetohepadnaviruses*) have been identified within the last years (Hahn *et al.*, 2015; Dill *et al.*, 2016; Jo *et al.*, 2017; Lauber *et al.*, 2017; Gogarten *et al.*, 2019). Moreover, a hepadna-like family of non-enveloped fish viruses has been described and named *nackednaviridae*. In contrast to hepadnaviruses, this new family is missing a

PreS/S ORF for envelope proteins (Lauber *et al.*, 2017). Furthermore, the identification of this virus family gives new insights into the phylogenetic origin of HBV. In contrast to the previous phylogenetic hypothesis suggesting a more recent origin of *orthohepadnaviruses,* HBV might have an ancient origin and might descend from non-enveloped progenitors in fishes (Lauber *et al.*, 2017). The ancient origin of mammalian HBV is corroborated by the discovery of shrew viruses (Rasche *et al.*, 2019).

HBV genome organization

HBV virions carry a partially double-stranded DNA genome and virus replication involves the reverse transcription of a viral RNA intermediate called pregenomic RNA (pgRNA) (Summers and Mason, 1982). The replication via reverse transcription is usually applied by retroviruses that have RNA genomes. Therefore, HBV is considered a pararetrovirus, despite its DNA genome. HBV has a remarkably small and compact genome of about 3.2 kb (dependent on the genotype) of which each nucleotide has coding capacity (Tong and Revill, 2016). Four viral genes are encoded by four overlapping and frame-shifted open reading frames (ORFs) and are called Surface (S), Core (C), Pol (P), and X (see Figure 3) (McNaughton *et al.*, 2019). Seven distinct viral proteins are translated from four 5' capped and 3' polyadenylated viral mRNAs. All four viral mRNAs and the fifth transcript pgRNA are encoded by the negative DNA strand and have all the same 3' end with polyadenylation site (McNaughton *et al.*, 2019). Transcription initiation at different promoter sites therefore leads to five viral transcripts of different lengths (Nassal, 2015). The longest viral RNA is a 3.5-kb and hence greater-than-genome length RNA, which serves as precore mRNA which is the template for the translation of the soluble HBeAg (Quarleri, 2014). The slightly shorter 3.5-kb pregenomic RNA (pgRNA) is the template for viral replication, which also serves as bicistronic template for the transcription of the core protein and the polymerase (Sells *et al.*, 1988; Quarleri, 2014). The preS1 and preS2/S mRNAs of 2.4 and 2.1 kb, respectively, function as templates for the transcription of three different forms of surface protein (Cattaneo, Will and Schaller, 1984; Sells *et al.*, 1988). The shortest viral RNA transcript of 0.7 kb serves as mRNA for the translation of the X protein (Valaydon and Locarnini, 2017). Additionally, the HBV genome contains several regulatory elements that control gene expression and viral replication. Besides four promoter regions for transcription initiation, there exist two enhancers (En1 and En2) that are bound by transcription activators to promote gene transcription. Furthermore, *cis*-elements act as binding sites for transcription factors and two direct repeats (DRs) DR1 and DR2 of about 11 nucleotides are required for viral DNA synthesis (Nassal, 2015; Valaydon and Locarnini, 2017). Throughout the viral life cycle, the HBV genome assumes different shapes, which all contribute to infection and pathogenesis.

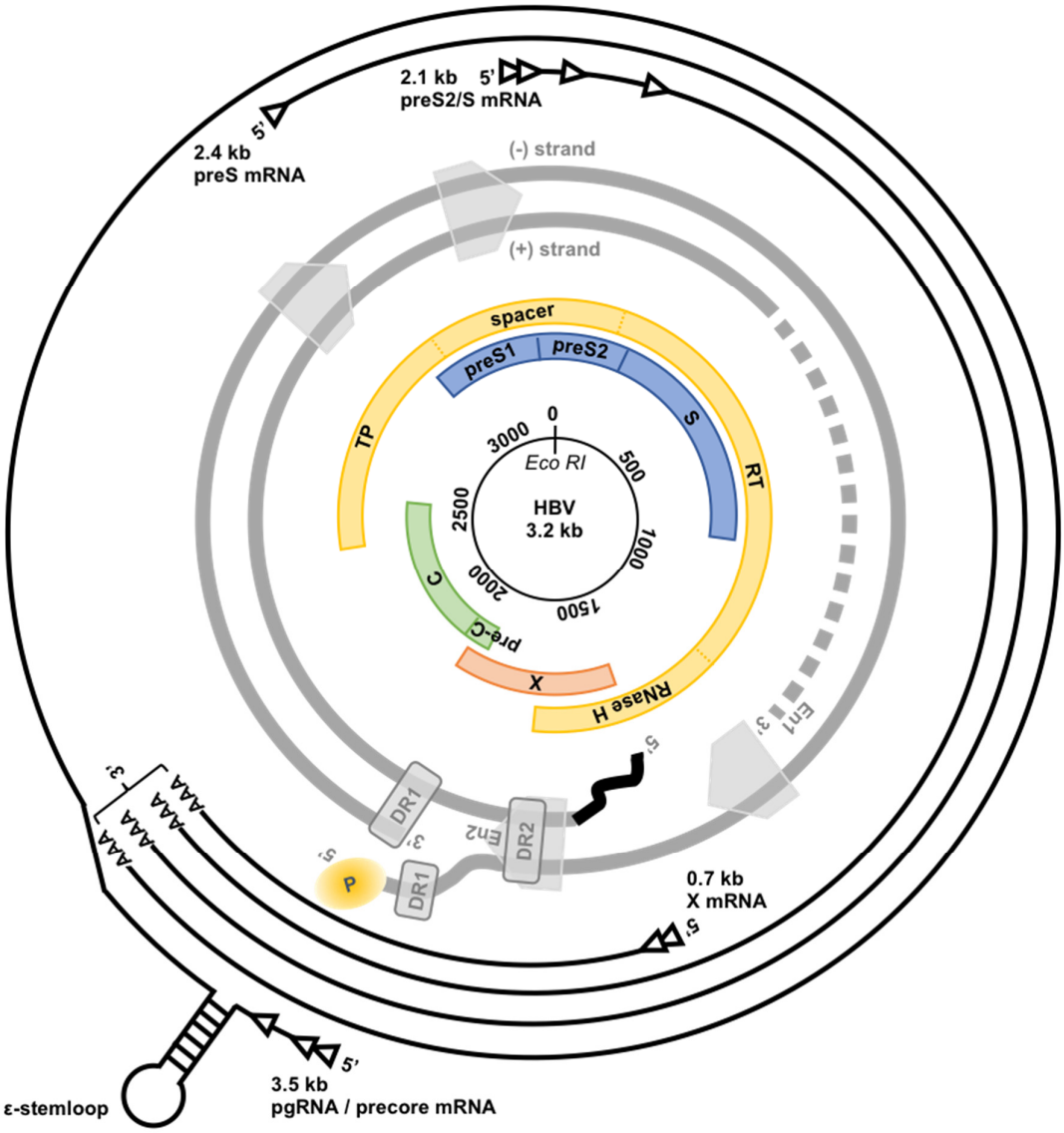

Figure 3: Organization of the HBV genome. The HBV genome has a small size of only 3.2 kb. A unique EcoRI restriction site is used for the definition of the EcoRI numbering convention (Ono *et al*., 1983). In color are shown the overlapping four open reading frames (ORFs) and seven encoded proteins. Four functional domains of the HBV polymerase are shown (yellow). Three surface proteins are encoded, all of which contain the S domain (blue). The HBV core protein lacks the pre-C domain, which is present in the HBeAg. In grey is the structure of HBV relaxed circular DNA (rcDNA) which is transformed into covalently closed circular DNA (cccDNA) in the nucleus of infected hepatocytes. The 5' end of the (-) strand is covalently bound to the viral polymerase (P). The 5' end of the (+) strand carries a 19 nt RNA primer (black line) and its 3' end varies in length. Following regulatory elements are indicated: promoters (grey pentagons), transcriptional enhancers 1 and 2 (En1/2), direct repeats 1 and 2 (DR1/2). Outermost in black are shown the viral mRNAs and pregenomic RNA (pgRNA) that are transcribed from cccDNA. Small arrowheads depict transcription starts, the ε-stemloop at the 5' end of pgRNA is shown. Figure inspired from: (Nassal, 2015; McNaughton *et al.*, 2019).

HBV rcDNA

Inside infectious virions, the HBV genome exists in the form of a partially double-stranded relaxed circular DNA (rcDNA) (Summers, O'Connell and Millman, 1975). Two linear DNA strands with

overlapping 5' ends of about 250 nucleotides form a circular structure in which the negative (-) strand (complementary to the mRNA transcripts) covers the whole genome while the positive (+) strand lacks sequences of varying lengths at its 3' end (Summers and Mason, 1982). The single stranded region arising from the incomplete positive strand can span more than 50% of the genome (Delius *et al.*, 1983). Another peculiarity of the HBV genomic structure is the covalent link between the viral DNA polymerase and the 5' end of the (-) strand and the presence of a 19-nucleotide RNA primer at the 5' end of the (+) strand (Gerlich and Robinson, 1980; Lien, Aldrich and Mason, 1986).

HBV cccDNA

Inside the nuclei of chronically infected hepatocytes, the HBV genome exists in form of a double-stranded covalently closed circular DNA (cccDNA). In a multi-step-process, it is formed from incoming rcDNA. cccDNA is associated to histones and has a chromatin-like structure of typical nucleosomes in a "beads-on-a-string" formation (Bock *et al.*, 1994; Newbold *et al.*, 1995). In addition to histones, the HBV core protein is another structural component of the viral mini-chromosome and causes an additional compaction on the viral chromatin (Bock *et al.*, 2001). The major role of cccDNA in the life cycle of HBV is its role as template for the transcription of all viral RNAs for replication and translation. Despite being a key intermediate, the median intrahepatic cccDNA level in HBV infected patients of only 0.01 to 1 cccDNA copies per cell is very low (Werle–Lapostolle *et al.*, 2004a). In chronic HBV carriers, cccDNA resides in the nucleus of infected cells and gives rise to progeny virus. It is therefore considered the virologic key to persistence of HBV infection (Nassal, 2015). The rebound of viral replication upon withdrawal of currently available antiviral therapy indicates that cccDNA can persist for decades (Rehermann *et al.*, 1996). To achieve HBV cure, the elimination of cccDNA from infected hepatocytes will be essential (Lucifora and Protzer, 2016).

pgRNA

For HBV replication, a longer than genome size RNA is transcribed from cccDNA within infected hepatocytes. It is called pregenomic RNA (pgRNA) and serves as template for reverse transcription by the viral polymerase and as bicistronic mRNA for the translation of polymerase and precore/core proteins. Like all other viral mRNAs, pgRNA is 5'-capped and 3'-polyadenylated (Nassal, 2015). As an additional structural element, it carries a secondary structure called epsilon (ε) stem-loop close to the 5' end (Junker-Niepmann, Bartenschlager and Schaller, 1990). This *cis*-acting element serves as encapsidation signal and recognizes the viral polymerase for the initiation of reverse transcriptase (Knaus and Nassal, 1993; Nassal and Rieger, 1996).

Linear HBV DNA

In addition to infectious virions containing HBV rcDNA, enveloped nucleocapsids can contain HBV double stranded linear DNA (dslDNA). This form of HBV DNA is generated during reverse

transcription through *in situ* priming of the positive strand primer (Staprans, Loeb and Ganem, 1991). HBV dslDNA is 18 nt longer than genome length and represents typically between 5% and 10% of encapsidated HBV DNA in patients with chronic HBV infection (Zhao *et al.*, 2016; Caballero *et al.*, 2018; Tu and Urban, 2018). In the nucleus, dslDNA can give rise to defective cccDNA by non-homologous recombination (NHEJ) or can be integrated into the host genome (Bill and Summers, 2004; Caballero *et al.*, 2018).

Integrated HBV DNA

In chronic HBV patients as well as in animal and cell culture models, HBV DNA can be integrated into the host genome. This occurs during early infection in a frequency of approximately 1 in 10^2 - 10^4 cells (Yang and Summers, 1999; Summers *et al.*, 2003; Mason *et al.*, 2016; Tu and Urban, 2018). HBV dslDNA in incoming virions is a precursor of integrated HBV DNA (Yang and Summers, 1995). Integration into the host genome occurs at DNA double strand breaks via NHEJ (Bill and Summers, 2004). Typical HBV genome breakpoints for integration are located in a region where the viral enhancer 2 and the ORFs X and C are located (Sung *et al.*, 2012). In this case, the expression of all ORFs except the surface ORF is affected (Caballero *et al.*, 2018). Therefore, integrated HBV DNA is mainly a source of HBV surface antigen (Wooddell *et al.*, 2017). Furthermore, HBV DNA integration is a cause of HCC-development (Sung *et al.*, 2012) (see chapter HBV natural history and pathology).

HBV proteins

The HBV genome encodes seven proteins namely three distinct surface proteins, core, precore, polymerase, and X protein.

Surface proteins

The HBV large (L), middle (M), and small (S) surface (HBs) proteins are encoded by the S ORF, which has a length of about 1100 base pairs and entirely overlaps with the P ORF. Two mRNAs serve as templates for translation of the three surface proteins, with a longer mRNA for L and a shorter mRNA for M and S (Heermann *et al.*, 1984). One single stop codon is used during translation of all surface proteins, giving rise to three glycoproteins with different amino-terminal (N-ter) ends (Heermann *et al.*, 1984). The common carboxy-terminal (C-ter) S domain consists of 226 amino acids that form four transmembrane domains which are connected through loops on both sides of the membrane (Valaydon and Locarnini, 2017). This is the only domain of the S surface protein. The M surface protein contains an additional 55-amino acid N-ter domain called preS2. The L surface protein comprises, in addition to the C-ter S and central preS2-domains, the 108-amino acid preS1 domain at its N-terminus (Barrera *et al.*, 2005). The S, M and L surface proteins are synthesized at the endoplasmic reticulum and maturation occurs at the Golgi apparatus. Secretion of enveloped particles containing HBV surface proteins can be

secreted via multivesicular bodies (MVBs) or the cellular secretory pathway (Watanabe *et al.*, 2007). The HBV surface proteins, especially the S and L forms, play critical roles in HBV infection and secretion (Sureau, Guerra and Lanford, 1993; Abou Jaoude and Sureau, 2007). The S and preS1 domains of the HBV surface proteins each contain a determinant of infectivity required for viral attachment and binding. The antigenic loop (AGL) within the S domain mediates HBV low-affinity interactions with heparan sulfate proteoglycans (HSPGs) present on the cell surface of hepatocytes (Sureau and Salisse, 2013). The N-ter 75 amino acids of the preS1 domain of the L surface protein is crucial for the binding to the hepatocyte surface preceding entry (Le Seyec *et al.*, 1999; Blanchet and Sureau, 2007). This N-ter extremity of the preS1 domain is post-translationally modified by addition of a myristic acid, and this myristylation is indispensable for HBV infectivity (Persing, Varmus and Ganem, 1987; Gripon *et al.*, 2002). More recently, the binding receptor of the preS1 domain on the hepatocyte surface has been identified to be NTCP (Yan *et al.*, 2012; Ni *et al.*, 2014). In addition to role of HBs in infectivity, the L surface protein is involved in nucleocapsid encapsidation for the formation of infectious virions (Valaydon and Locarnini, 2017). HBs was the first HBV protein discovered in sera from Australian aborigines, and is therefore also called Australia antigen (Blumberg, 1964; Blumberg and Alter, 1965). Its detection triggered the discovery of HBV as hepatitis virus (Prince, 1968; Dane, Cameron and Briggs, 1970; Millman *et al.*, 1970).

Core

The HBV core (HBc) and precore proteins are encoded by the C ORF, which has a length of approximately 650 base pairs and partially overlaps with the X and P ORFs. It contains two start codons, of which the second AUG gives rise to the 183 amino acid and 21 kDa core protein, the building block of the viral capsids (Cohen and Richmond, 1982; Standring *et al.*, 1988; Zlotnick *et al.*, 2015). The N-ter 149 residues form the assembly domain, which is sufficient for assembly competence. The arginine-rich C terminal domains (CTDs) are not required for assembly of empty capsids, but confer RNA-binding and contain nuclear localization signals (Birnbaum and Nassal, 1990; Nassal, 1992; Li *et al.*, 2010). The basic soluble unit for capsid assembly is a HBcAg dimer, which consists of two monomeric α-helical hairpins forming a four-helix bundle (Boettcher, Wynne and Crowther, 1997; Conway *et al.*, 1997; Wynne, Crowther and Leslie, 1999). One hundred twenty HBcAg dimers self-assemble to form an icosahedral shell (Crowther *et al.*, 1994; Wynne, Crowther and Leslie, 1999). In addition to its role as structural protein, HBc modulates several other steps of the HBV life cycle. For instance, it binds to HBV cccDNA and plays a role in epigenetic regulation, it is involved in the regulation of reverse transcription and virus secretion, and it carries HBs binding sites (Guo *et al.*, 2011; Zlotnick *et al.*, 2015). Due to its divers functions in the HBV life cycle, HBc might be a suitable target for the development of direct-acting antivirals (Diab *et al.*, 2018).

Precore

Translation of the entire C ORF leads to the formation of the precore protein or HBeAg, an accessory protein without functions in capsid assembly (Zlotnick *et al.*, 2015). Maturation of the HBe involves post-translational proteolytical modifications at N and C termini (Takahashi *et al.*, 1983; Standring *et al.*, 1988). Compared to the core protein, mature HBeAg contains an additional 10-amino-acid sequence at its N-terminus called pre-C (Wasenauer, Köck and Schlicht, 1992). This sequence not only acts as signal sequence for secretion via the cellular secretory pathway but also determines the biophysical and antigenic properties of the precore protein (Standring *et al.*, 1988; Schlicht and Wasenauer, 1991). A cysteine within this region is essential for formation of an intramolecular disulfide bond, which is critical for correct dimerization and secretion (Wasenauer, Köck and Schlicht, 1992; Nassal and Rieger, 1993). In HBV infected patients, HBe dimers are secreted in a huge excess compared to infectious virions (Dimattia *et al.*, 2013). Clinically, HBe is used as serological marker, as high serum HBeAg levels indicate active replication (Valaydon and Locarnini, 2017).

Polymerase

The polymerase is encoded by the P ORF, which is the longest ORF covering about 70% of the viral genome and overlapping with all other ORFs (Valaydon and Locarnini, 2017). It is a multifunctional enzyme synthesizing new rcDNA from pgRNA before degrading its template within progeny virions. The polymerase is composed of four domains with different functions (Radziwill, Tucker and Schaller, 1990). The N-ter domain, which is also called terminal protein (TP) domain, is involved in pgRNA packaging and a tyrosine residue within this domain serves as primer for the synthesis of the viral (-) DNA strand (Bartenschlager and Schaller, 1988; Zoulim and Seeger, 1994). Via a spacer region, the TP domain is linked to the reverse transcriptase (RT) domain, which is the catalytic center of the enzyme and comprises the YMDD consensus sequence required for retroviral RT activity (Radziwill, Tucker and Schaller, 1990). It functions as RNA-dependent polymerase during reverse-transcription of pgRNA and subsequently as DNA-dependent polymerase using the synthesized (-) strand DNA as template for (+) strand DNA synthesis. At its C-terminus, the polymerase carries a RNase H domain, which degrades the complete pgRNA template except the pgRNA 5′ end, which subsequently serves as primer for (+) strand DNA synthesis (Clark and Hu, 2015). Since P lacks a proofreading capacity, HBV reverse transcription is fairly error-prone (Park *et al.*, 2003). The consequence is a high mutation rate, which allows the virus to evolve and adapt to its environment. Currently used antivirals for the treatment of patients chronically infected with HBV target the viral polymerase (see chapter Treatment of HBV infection) (Terrault *et al.*, 2018).

X protein

The HBV X protein (HBx) is encoded by the smallest ORF of the viral genome, the X ORF, which is transcribed independently from all other viral transcripts under the control of En1 and X promoter

(Tang *et al.*, 2006). HBx comprises 154 amino acids and has a molecular mass of approximately 17 kDa (Bouchard and Schneider, 2004). HBx is a multifunctional regulatory protein modulating the expression of several viral and cellular proteins, which are involved in various processes including cell signaling, transcription, cell proliferation, DNA repair, and apoptosis (Belloni *et al.*, 2009; Ng and Lee, 2011). For instance, HBx induces normally quiescent hepatocytes to exit G0 and enter G1 phase of the cell cycle by regulating expression levels and activity of cell cycle regulating proteins, including the cyclin-dependent kinase 4 (CDK4) (Gearhart and Bouchard, 2010b). The extensive involvement of HBx in a wide field of cellular processes is reflected by its differential distribution in the cell, as HBx is found both in the cytoplasm and in the nuclei of infected hepatocytes. More precisely, HBx stimulates signaling pathways in the cytoplasm and transactivates transcription elements in the nucleus (Doria *et al.*, 1995). In addition to its transcriptional activity via protein-protein interactions, HBx also binds to HBV cccDNA and modifies its epigenetic regulation (Belloni *et al.*, 2009; Rivière *et al.*, 2015). Moreover, HBx has been implicated in hepatocarcinogenesis (Ng and Lee, 2011).

Structure of infectious virions and subviral particles

Infectious HBV virions, also referred to as Dane particles, are small spherical particles with a diameter of 42 nm (Dane, Cameron and Briggs, 1970). The outer envelope is composed of a lipid bilayer embedding viral S, M and L surface proteins in a S:M:L ratio of 4:1:1 (Seitz *et al.*, 2007). The inner nucleocapsid of icosahedral symmetry contains the polymerase-coupled genome in the form of rcDNA (Gerlich and Robinson, 1980; Boettcher, Wynne and Crowther, 1997; Conway *et al.*, 1997). A small fraction of complete HBV virions contains the viral genome in the form of dslDNA instead of rcDNA (Zhao *et al.*, 2016).

During natural infection, subviral particles (SVP) are formed in a very high excess of up to 100,000-fold more than complete virions (Hu and Liu, 2017). They are assembled from different forms of HBsAg and form filamentous or spherical particles with diameters of a bit more than 20 nm (Huang *et al.*, 1972). Filamentous and spherical SVPs differ from each other with respect to composition and secretion mode.

In contrast to spheres, which mainly contain S and M surface proteins, filamentous SVPs also contain L surface proteins (Heermann *et al.*, 1984). Also, they are secreted via MVBs along with complete virions, while spherical SVPs are secreted through the cellular secretory pathway (Watanabe *et al.*, 2007; Jiang *et al.*, 2016). Bearing no DNA, SVPs are not infectious. Anyhow, they have an influence on the course of infection by binding neutralizing antibodies and reducing the immune response against infectious particles (Rydell *et al.*, 2017). Clinical implications of SVPs include HBV diagnosis and vaccination and HDV infection. Other viral products that are secreted from infected hepatocytes, along with Dane particles and HBs-composed SVPs, are virion-like particles of enveloped nucleic-acid free capsids and HBe (Caballero *et al.*, 2018).

HBV life cycle

The first step of HBV infection is virion attachment and subsequent binding to cellular factors presented on the hepatocyte surface (see Figure 4). In a first step, low-affinity attachment of HBs to heparan sulfate proteoglycans (HSPGs) allows the enrichment of virions at the host cell surface (Schulze, Gripon and Urban, 2007; Verrier *et al.*, 2016a). In a second step, high-affinity binding of the myristoylated preS1 domain of HBs to the functional receptor NTCP allows internalization of the virus (Sureau, Guerra and Lanford, 1993; Gripon *et al.*, 1995; Yan *et al.*, 2012; Ni *et al.*, 2014). For the functional role of NTCP in HBV infection see review in the annex (Eller *et al.*, 2018).

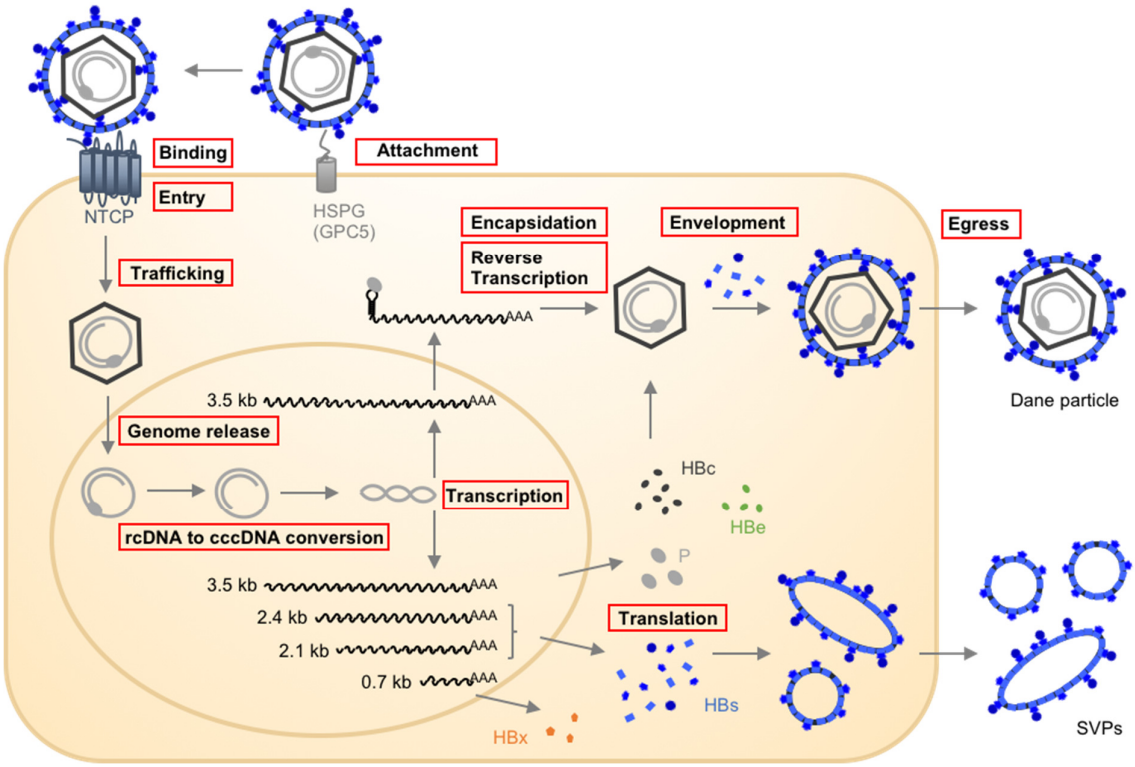

Figure 4: The HBV life cycle. Following attachment to HSPG (GPC5) HBV enters hepatocytes via the receptor NTCP. Within the nucleus, rcDNA is transformed into cccDNA in a multistep process. All viral RNAs are transcribed from cccDNA. HBV pgRNA is exported to the cytosol and co-packaged with the Polymerase (P) into the newly forming nucleocapsid. Inside the capsid, P reverse transcribes pgRNA into rcDNA. Infectious HBV virions are also called Dane particles. Subviral particles (SVPs) are produced and secreted in a large excess as compared to Dane particles. For details see chapters HBV proteins and HBV life cycle.

Further steps of HBV entry into hepatocytes following internalization are poorly understood. The HBV capsid is released into the cytoplasm and thought to be actively transported towards the nucleus (Rabe *et al.*, 2006; Li *et al.*, 2010). Once at the nuclear pore, the viral capsid might disassemble and release HBV rcDNA into the nucleus (Schmitz *et al.*, 2010). Inside the nucleus, the protein-linked rcDNA is converted into the viral histone-associated minichromosome cccDNA (Lucifora and Protzer, 2016). This key step of establishment of HBV infection is thought to require several proviral host factors (see chapter HBV host interactions) but the process remains largely unknown (Nassal, 2015). The

episomal cccDNA persists in the nucleus as central transcription template for all viral RNAs. cccDNA transcription by the host RNA polymerase II is regulated by cellular transcription factors, the viral regulatory protein HBx and chromatin modification (Levrero *et al.*, 2009). All five viral RNAs are 5' capped and 3' polyadenylated and exported into the cytosol, where viral proteins are produced. Cytosolic pgRNA then binds to the viral polymerase, which recognizes the ε stem-loop close to the 5' end of pgRNA. Binding triggers the co-packaging of pgRNA and polymerase into newly forming nucleocapsids (Nassal, 2015). Reverse transcription is primed by the formation of a covalent link between a tyrosine residue within the TP domain of P and the first nucleotide of the growing (-) DNA strand (Nassal and Rieger, 1996). Further steps of reverse transcription include transfers of primers from one end of a strand to another, giving rise to rcDNA. Occasional *in situ* priming gives rise to double stranded linear HBV DNA (dslDNA) (Staprans, Loeb and Ganem, 1991). Inside the nucleocapsid, the synthesis of the (-) strand DNA is associated with the appearance of a signal for nucleocapsid envelopment (Gerelsaikhan and Tavis, 1996). Mature rcDNA containing capsids are secreted via budding of MVBs (Watanabe *et al.*, 2007). As an alternative to nucleocapsid envelopment, the capsids containing rcDNA can re-enter the nucleus for intracellular recycling. Within the nucleus, the incoming rcDNA molecule is then transformed into a new cccDNA molecule, thus increasing the cccDNA pool inside the cell (Tuttleman, Pourcel and Summers, 1986; Wu *et al.*, 1990). As a byproduct of infectious virions containing rcDNA, virions containing dslDNA can be secreted from infected cells, representing precursors for viral DNA integration into the host genome (Yang and Summers, 1999). Several of these steps in the HBV life cycle rely on the function of pro-viral host factors. While some HBV host interactions have already been established (see chapter HBV host interactions), the vast majority of putative pro-viral host factors remain obscure. This is in parts due to the long-term lack of suitable cell culture models supporting the entire viral life cycle.

HBV model systems

Many early studies investigating the structure and genome of HBV were performed using biological material from infected patients and basic biochemical and biomolecular methods. The study of virus-host interactions and the development of antivirals, however, require appropriate experimental model systems that allow viral replication. For the investigation of the full viral life cycle, infectious models are necessary. Different animal and cell culture models are available for diverse experiments from high throughput screenings to pre-clinical studies.

Animal models

A characteristic trait of HBV is its remarkably narrow species tropism, restricting its natural hosts to humans, non-human primates, and tree shrews as the only susceptible non-primate (Ortega-Prieto *et al.*, 2019). This severely limits the options for *in vivo* studies. Despite major ethical concerns,

chimpanzees have been used as immunocompetent model for HBV research, being fully susceptible to HBV infection (Maynard *et al.*, 1972; Pancholi *et al.*, 2001; Shata *et al.*, 2006; Wieland, 2015). Indeed, as little as one genome equivalent of virus per animal can cause HBV infection in a chimpanzee (Asabe *et al.*, 2009). Studies using chimpanzees have allowed the development and safety/efficacy testing of a vaccine, and the investigation of host responses to infection and mechanisms of cccDNA persistence (Ortega-Prieto *et al.*, 2019). Surrogate models like woodchuck hepatitis B virus (WHBV) and duck hepatitis B virus (DHBV) allow the study of related viruses (Summers, Smolec and Snydert, 1978; Mason, Seal and Summers, 1980). However, the molecular virology of these viruses and the genetic background and immune responses of their hosts differ from those of HBV (Prince, Vnek and Stephan, 1983; Lelie *et al.*, 1987; Ortega-Prieto *et al.*, 2019). Small-animal models, like mice, are widely used in biomedical research. However, mice are not susceptible to infection with HBV or any other known virus belonging the *hepadnaviridae* family. Even transgenic mice (and mouse hepatocyte derived cell lines) expressing human NTCP, despite supporting HDV infection, are not susceptible to HBV infection (Li *et al.*, 2014; He *et al.*, 2015). Transgenic mice expressing viral proteins support production of infectious HBV virions and can be used to study immune responses. Mice transfected with HBV DNA using hydrodynamic injection of vectors or adeno-associated virus-based vectors are available and allow HBV replication and Dane particle production (Hu *et al.*, 2019; Ortega-Prieto *et al.*, 2019). For the study of HBV infection, there exist humanized chimeric mouse models with human hepatocytes, which equally support the formation of cccDNA. To overcome immune deficiency as a major limitation of human liver chimeric mice, dual chimeric mice with human liver cells and a human immune system are developed (Sun and Li, 2017).

Cell culture models

Primary hepatocytes

Human hepatocytes are the natural host cells of HBV infection and, therefore, cultured primary human hepatocytes (PHHs) are the most physiological *in vitro* model system for HBV infection (see Figure 5). In the presence of dimethyl sulfoxide (DMSO), PHHs support the complete life cycle of HBV (Gripon *et al.*, 1988). However, their use is hampered by high costs, limited availability, high donor variability, and absent proliferation and limited life span in culture. Moreover, due to quick de-differentiation and loss of polarization in culture, the infectivity declines after plating and viral spread is limited (Verrier *et al.*, 2016b; Hu *et al.*, 2019). Primary hepatocytes from susceptible animals are an alternative to human hepatocytes. Hepatocytes from the tree shrew species *tupaia belangeri* in culture support HBV infection (Walter *et al.*, 1996). For instance, primary tupaia hepatocytes (PTH) were utilized for the identification of NTCP as HBV receptor (Yan *et al.*, 2012). Ectopic expression of human NTCP in primary hepatocytes from macaques and pigs renders these cells susceptible to HBV infection

(Burwitz *et al.*, 2017; Lempp *et al.*, 2017). Considerable effort is currently made for the development of novel technologies to improve culture systems of primary hepatocytes. These include the isolation of human hepatocytes from chimeric mice and methods to achieve proliferation, expansion and long-term cultivation of human hepatocytes (Ishida *et al.*, 2015; Branche *et al.*, 2016; Zhang *et al.*, 2018; Kim *et al.*, 2019; Unzu *et al.*, 2019). Although PHHs mimic best the natural host cell of HBV, other infectious cell culture systems are used for the study of different phases of the HBV life cycle. However, discoveries made in alternative model systems are commonly verified using PHHs.

Human hepatoma-derived cell lines

Human hepatoma-derived cell lines are widely used as surrogate models for hepatocytes in different fields including liver metabolism, development, oncogenesis, and hepatotoxicity (Lopez-Terrada *et al.*, 2009). Despite only partially mimicking physiological hepatic functions, they have also been used to study HBV replication (Verrier *et al.*, 2016b). Widely employed cell lines are the hepatoblastoma-derived HepG2 cells and the hepatocellular carcinoma-derived Huh7 cells. HepG2 cells originate from a 15-year-old Caucasian male and Huh7 cells from a 57-year-old Japanese male (Aden *et al.*, 1979; Nakabayashi *et al.*, 1982; Lopez-Terrada *et al.*, 2009). Lacking the HBV receptor NTCP, these cell lines are not susceptible to HBV infection (Yan *et al.*, 2012). Nevertheless, they have been widely used for the investigation of late steps of the viral life cycle by transfection with HBV DNA (Hu *et al.*, 2019). Transfected HBV DNA can replace cccDNA as template for viral replication and allows the expression of viral gene products and the assembly and secretion of infectious virions and subviral particles (Sureau *et al.*, 1986; Tsurimoto, Fujiyama and Matsubara, 1987). Stable integration of HBV DNA into the host cell genome allowed the generation of HepAD38 and Hep2.2.1.5 cell lines (Sells, Chen and Acs, 1987; Ladner *et al.*, 1997). Both HepG2-derived cell lines are commonly used as tools to produce infectious HBV particles. Cell lines stably producing HBV also represent suitable models for the investigation of many steps of the HBV life cycle, virus-host interactions, and for drug screenings (Königer *et al.*, 2014; van de Klundert, Zaaijer and Kootstra, 2016). However, because of unfunctional viral entry due to the absence of the receptor NTCP, these models do not allow the study of early stages of the viral life cycle like entry and trafficking to the nucleus.

HepaRG

HepaRG cells are a liver progenitor cell line derived from a HCV-associated hepatocarcinoma of a female patient (Gripon *et al.*, 2002). Despite the origin of this cell line, the HCV genome is absent (Gripon *et al.*, 2002). Upon treatment with DMSO and hydrocortisone, the bipotent progenitor cells differentiate into hepatocyte-like and biliary-like epithelial cells (Guillouzo *et al.*, 2007). HepaRG cells differentiated into hepatocyte-like cells (dHepaRG) express NTCP and support HBV infection (Gripon *et al.*, 2002; Kotani *et al.*, 2012). The susceptibility of dHepaRG cells to HBV infection correlates to their differentiation (Gripon *et al.*, 2002). However, in HBV infected HepaRG cells, there is little or no

amplification of cccDNA via intracellular recycling of encapsidated rcDNA (Hantz *et al.*, 2009). In contrast to other human hepatoma-derived cell lines, HepaRG cells retain several physiological hepatic functions. In particular, expression profiles of cytochrome P450 and components required for innate immune responses are comparable to those found in cultured PHHs (Guillouzo *et al.*, 2007; Luangsay *et al.*, 2015). Also, HBV-infected dHepaRG cells, and not HepG2-NTCP cells, are able to mount an innate immune response that is able to suppress HBV replication (Lucifora *et al.*, 2010). Despite inefficient infection and the necessity for a lengthy differentiation process, HepaRG cells represent a suitable infectious cell culture model for the investigation of immune response and host interactions of HBV (Allweiss and Dandri, 2016). HepaRG cells have allowed the discovery of several host factors, including the heparan sulfate proteoglycans (HSPGs) for attachment, NTCP for binding, and the Serine/Threonine Polo-like-kinase 1 (PLK1) for viral replication (Schulze, Gripon and Urban, 2007; Ni *et al.*, 2014; Diab *et al.*, 2017).

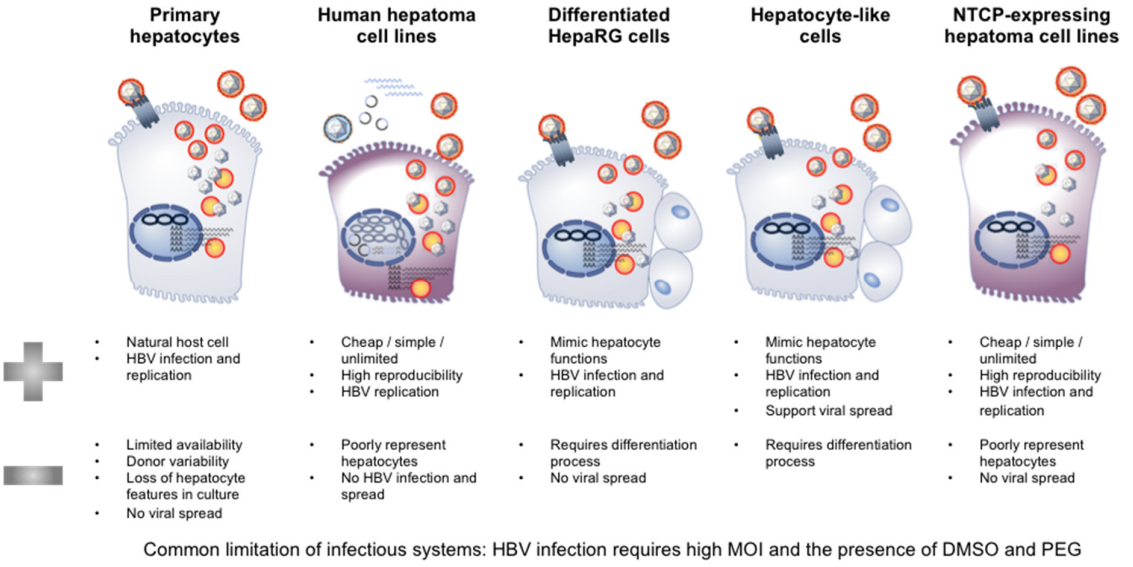

Figure 5: Cell culture model systems for HBV infection and replication. The key advantages and limitations of available systems are indicated. MOI: multiplicity of infection, DMSO: dimethyl sulfoxide, PEG: polyethylene glycol. Modified from (Allweiss and Dandri, 2016).

Hepatocyte-like cells

Another possibility to generate cells mimicking hepatic functions, including susceptibility to HBV infection, is the differentiation of pluripotent cells into hepatocyte-like (HL) cells. Due to ethical concerns regarding the use of embryonic stem cells, the access to human pluripotent cells was restricted until the discovery of induced pluripotent stem (iPS) cells (Takahashi and Yamanaka, 2006). These cells can be differentiated into HL cells, which exhibit hepatic morphology and hepatic markers (Sullivan *et al.*, 2010). Moreover, throughout the differentiation process from iPS cells into HL cells, NTCP

expression increases (Shlomai *et al.*, 2014). Consistently, iPS cell-derived HL cells support HBV infection and further steps of the viral life cycle as cccDNA formation, viral replication, and virus production (Shlomai *et al.*, 2014; Kaneko *et al.*, 2016; Sakurai *et al.*, 2017; Xia *et al.*, 2017). HL cells susceptible to HBV infection can also be derived from hepatocyte-derived liver progenitor-like cells (Fu *et al.*, 2019). In contrast to PHHs, HL cells maintain their permissiveness to HBV infection for up to four weeks after reaching the differentiated state and support viral spread (Xia *et al.*, 2017). Therefore, HL cells represent a suitable tool to investigate virus-host interactions and for drug discovery (Xia *et al.*, 2017). Besides, differentiation of iPS cells allows the generation of personalized infectious cell culture models from patients with specific genetic polymorphisms (Ni and Urban, 2017). However, human biological material and differentiation procedures are required to obtain HL cells.

NTCP-overexpressing human hepatoma-derived cell lines

Hepatoma-derived cells can become permissive to HBV infection when transgenically expressing human NTCP. For instance, HepG2 cells constitutively expressing NTCP (HepG2-NTCP) support viral entry and represent an infectious cell culture model system (Yan *et al.*, 2012; Ni *et al.*, 2014). Infection, however, requires a high multiplicity of infection (MOI) and addition of DMSO and polyethylene glycol (PEG) 8000 during inoculation (Ni *et al.*, 2014). Also, viral spread is limited or absent in HBV infected HepG2-NTCP cells and can be increased by maintaining PEG in the culture medium after infection (Michailidis *et al.*, 2017). Very recently, a HepG2-NTCP clone supporting the full HBV life cycle including viral spread has been introduced (König *et al.*, 2019). Despite their necessity to non-physiological conditions during infection, HepG2-NTCP cell lines have emerged as suitable model systems for the investigation of virus-host interactions during early events of the life cycle and for the discovery of novel antivirals targeting viral entry (Qi *et al.*, 2016; Shimura *et al.*, 2017; Kitamura *et al.*, 2018; Iwamoto *et al.*, 2019). In contrast to HepG2-NTCP cells, Huh7 cells constitutively expressing human NTCP (Huh7-NTCP) are only very weakly permissive to HBV infection (Ni *et al.*, 2014). Interestingly, both cell lines support HDV infection (Ni *et al.*, 2014). This is of importance as both viruses share the same envelope and NTCP-mediated HDV entry serves as surrogate model for HBV entry. In this way, GPC5 was identified as a common host cell entry factor for HBV and HDV using Huh7 cells overexpressing NTCP (Verrier *et al.*, 2016a). Within a few years, NTCP-overexpressing hepatoma-derived cell lines have contributed to an increased understanding of viral entry and virus-host interactions (Verrier *et al.*, 2016c, 2018, 2019; Shimura *et al.*, 2017; Iwamoto *et al.*, 2019) Thanks to their abundance, proliferation under convenient culture conditions and nearly unlimited life span, they are suitable models for high throughput screenings. However, they only partially mimic human hepatocytes and lack the ability to mount an immune response (Hu *et al.*, 2019).

HBV: Host interactions

Many steps of the HBV life cycle are known to be mediated by proviral host factors. While some host functions involved in HBV infection have been characterized, other virus-host interactions remain to be discovered. Selected host factors modulating viral entry, cccDNA formation, and further steps of the viral life cycle are presented below.

On the surface of hepatocytes, the carbohydrate side chains of heparan sulfate proteoglycans (HSPGs) serve as attachment receptors for initial attachment and enrichment of HBV virions (Schulze, Gripon and Urban, 2007). This interaction is mediated by positively charged residues within the antigenic loop (AGL) of the HBV surface protein (Sureau and Salisse, 2013). Especially, glypican 5 (GPC5), a member of a HSPG family associated with proteoglycans, serves as entry factor for HBV and HDV infection (Verrier *et al.*, 2016a). For a long time, the identity of the functional receptor for HBV/HDV infection, the binding partner of the HBV preS1 domain, was unknown and several potential receptor candidates have been proposed (Neurath, Strick and Sproul, 1992; Pontisso *et al.*, 1992; Ryu *et al.*, 2000; Falco *et al.*, 2001; Li *et al.*, 2005) Only in 2012, NTCP was identified as receptor for HBV/HDV entry into hepatocytes (Yan *et al.*, 2012). To date, NTCP remains the only known cellular receptor for HBV and HDV infection (Yan *et al.*, 2012; Ni *et al.*, 2014). Its discovery allowed the development of novel infectious culture systems (see chapter HBV model systems) and antiviral entry inhibitors for the treatment of HBV or HDV infected patients (Ni *et al.*, 2014). For a detailed description of the functional role of NTCP in HBV and HDV infection see the review article in the annex (Eller *et al.*, 2018). The HBV receptor activity of NTCP may depend on a functional interaction with the co-host entry factor epidermal growth factor receptor (EGFR), as HBV is internalized through the translocation of the NTCP-EGFR complex between the plasma membrane and intracellular vesicles (Iwamoto *et al.*, 2019). HBV nucleocapsid de-envelopment and trafficking to the nucleus are not well characterized but thought to exploit host membrane trafficking pathways. HBV entry may be assisted by clathrin-mediated endocytosis (CME), as the HBV preS1-domain interacts with clathrin heavy chain and clathrin adaptor protein and a compound interfering with CME was shown to inhibit HBV infection (Huang *et al.*, 2012; Umetsu *et al.*, 2018). Post-internalization, transport of HBV virions may occur in a Rab5- and Rab7-dependent manner (Macovei *et al.*, 2013). Subsequently, nuclear import of the HBV genome is thought to involve interactions of the HBV capsid with nucleoporin 153, an essential protein of the nuclear basket which participates in nuclear transport via importin β (Schmitz *et al.*, 2010). Inside the nuclei of infected hepatocytes, the rcDNA to cccDNA conversion involves several host factors, including members of the DNA repair machinery (Nassal, 2015). The first step of cccDNA formation is the removal of the viral P protein, which is covalently linked to the (-) strand of rcDNA via a tyrosine residue. Although the cellular tyrosyl-DNA-phosphodiesterase 2 has been shown to cleave this link, this enzyme might not be required for cccDNA formation *in vivo* (Königer *et al.*, 2014; Cui *et al.*, 2015). Furthermore, cellular DNA polymerases (Pol) κ and λ are required for cccDNA formation in *de novo* HBV infection (Qi *et al.*, 2016). Interestingly, Pol α, but not Pol κ and Pol λ, contribute to the conversion

of rcDNA to cccDNA during intracellular amplification of cccDNA in human hepatoma cells (Tang *et al.*, 2019). Other cellular enzymes that may be required for cccDNA formation are the flap-like structure specific endonuclease 1 and the DNA ligase 1 and 3 (Long *et al.*, 2017; Kitamura *et al.*, 2018). Moreover, cellular topoisomerases 1 and 2 might regulate viral DNA supercoiling (Sheraz *et al.*, 2019). Finally, histones are associated to cccDNA, which leads to the formation of the viral mini-chromosome (Bock *et al.*, 1994; Newbold *et al.*, 1995). The host cell RNA polymerase II, which is also responsible for cellular mRNA synthesis, recognizes HBV cccDNA as template and transcribes all viral RNAs (Beck and Nassal, 2007). Efficient cccDNA transcription is regulated by liver-enriched transcription factors, for instance hepatocyte nuclear factor 4 (HNF4), retinoid X receptor α plus peroxisome proliferator-activated receptor α, and hepatic leukemia factor (HLF) (Ishida *et al.*, 2000; Tang and McLachlan, 2001). Indeed, HNF4α was shown to bind to various HBV enhancer regions and to promote viral transcription (Moolla, Kew and Arbuthnot, 2002; Quasdorff *et al.*, 2008). Besides several other cellular transcription factors binding HBV promoters and enhancers, also epigenetic modifications are involved in the regulation of cccDNA transcription (Hong, Kim and Guo, 2017; Mitra *et al.*, 2018). For instance, the acetylation status of cccDNA-bound histones H3 and H4 and the epigenetic suppression of cccDNA transcription via methylation of histones by the protein arginine methyltransferases 1 and 5 regulate HBV replication (Pollicino *et al.*, 2006; Benhenda *et al.*, 2013; Zhang *et al.*, 2017). The viral regulatory protein HBx also affects cccDNA transcription by interaction with cellular factors, as for example the structural maintenance of chromosomes 5/6 (Smc5/6) complex, which blocks extrachromosomal DNA transcription. For this, HBx mediates the degradation of the restricting Smc5/6 complex, via the recruitment of a complex formed by the cellular DNA damage-binding protein 1 and E3 ubiquitin ligase cullin 4A (DDB1-CUL4A), to allow cccDNA transcription (Angers *et al.*, 2006; Decorsière *et al.*, 2016). After translation, the proper folding of the HBV polymerase and its binding to pgRNA are assisted by host chaperons including the heat shock protein 90 (Hu *et al.*, 2002). Furthermore, the interaction between P and the ε stem-loop of pgRNA, which triggers nucleocapsid assembly and RT priming, is mediated by the cellular RNA-Binding Motif Protein 24 (Yao *et al.*, 2019). Cellular kinases modulate pgRNA encapsidation and capsid maturation, as they phosphorylate serine-phosphorylation sites within the HBc CTD. While phosphorylation triggers pgRNA encapsidation, the subsequent dephosphorylation is associated with capsid maturation, envelopment and egress (Mitra *et al.*, 2018). Different host kinases are involved, including cyclin-dependent kinase 2 (CDK2) and Polo like-kinase 1 (Ludgate *et al.*, 2012; Diab *et al.*, 2017). Envelopment and secretion of mature nucleocapsids relies on a mechanism involving host cell multivesicular bodies (Watanabe *et al.*, 2007).

Also, the cell cycle of the host cell in general has an effect on HBV infection, as HBV is thought to preferentially replicate in non-dividing cells (Aden *et al.*, 1979; Sureau *et al.*, 1986). In addition, HBV replication was shown to be inversely correlated with cellular DNA synthesis and to be enhanced in quiescent hepatocytes (Ozer *et al.*, 1996). Actually, effective HBV infection in cell culture requires the presence of DMSO, which is known to reduce cell proliferation (Urban *et al.*, 2014; De Abreu Costa *et*

al., 2017). Furthermore, modulation of CDK function is thought to impact HBV infection. In human hepatoma cell lines, inhibition or knockout of CDK2 was shown to enhance HBV replication by phosphorylation and deactivation of the host restriction factor SAMHD1 (Hu *et al.*, 2018). Although the link between host cell cycle and HBV replication has been made, it remains controversial whether HBV favorably replicates in a specific cell cycle phase and involved key mechanisms and players remain obscure (Gearhart and Bouchard, 2010a; Xia *et al.*, 2018).

As it has been established here, HBV relies on a variety of host factors throughout its life cycle, some of which contribute to it species and tissue tropism. While some virus host interactions have been identified, many further host functions promoting the HBV life cycle remain unknown. A better understanding of proviral and restrictive hepatocyte factors interacting with HBV is fundamental for the development of improved infectious model systems and novel therapeutic strategies (see chapters HBV model systems and HTAs for the treatment of viral infections).

Functional genomics screens for the identification of virus-host interactions

Viruses depend on the host cell machinery for infection and replication. The discovery of which specific host functions are exploited by a virus was difficult until technological advances allowed the performance of unbiased genome-scale high throughput screenings. In 2008, the first genome-wide RNAi screens for the identification of host factors required for HIV replication were performed (Brass *et al.*, 2008; Zhou *et al.*, 2008). Arrayed genome-scale small interfering RNA (siRNA) libraries were used to identify previously uncharacterized host factors for HIV, illustrating the power of functional genomics screening in discovering host-pathogen interactions (Brass *et al.*, 2008; Zhou *et al.*, 2008) The common principal of these screens is the transfection of cells with siRNAs, the infection with the studied virus, and the subsequent evaluation of viral replication. Similar approaches allowed the identification of host factors involved in the life cycle of multiple other viruses including HCV, flaviviruses, influenza virus, and HDV (Li *et al.*, 2009; Krishnan *et al.*, 2011; Cheng *et al.*, 2015; Verrier *et al.*, 2019). However, the common limitation for RNAi based screens is the limited knockdown efficiency which determines the sensitivity of a screen. Thanks to its ability to achieve complete knockouts, the development of the CRISPR/Cas9 system opened vast possibilities for genome-wide loss-of-function studies (Hsu, Lander and Zhang, 2014; Shalem *et al.*, 2014; Taylor and Woodcock, 2015). For the identification of Flavivirus-host interactions, Cas9-overexpressing cells were transduced with pooled lentiviral libraries encoding single-guide RNAs (sgRNAs) and subsequently infected with the studied virus. After extraction of genomic DNA, amplification of sgRNAs, and next-generation sequencing of amplicons, the enrichment of each sgRNA was determined by comparing the abundance in both selected and uninfected control cells (Marceau *et al.*, 2016; Savidis *et al.*, 2016; Zhang *et al.*, 2016). Other genome-scale CRISPR screens allowed the identification of host dependency factors for diverse other viruses including HCV, HIV and Influenza virus (Marceau *et al.*, 2016; Park *et al.*, 2017; Han *et al.*, 2018). Common limitations of loss-of-function screens are the identification of false-positives due to potential off-target effects and

the requirement for robust infectious cell culture models. In 2011, Yang *et al.* announced the generation of a genome-scale expression collection of over 16,100 human open-reading frames (ORFs) in a lentiviral vector, enabling overexpression high-throughput screens (X. Yang *et al.*, 2011). This library has allowed the identification of genes that cause chromosome instability in a step towards the selective targeting of tumor cells (Duffy *et al.*, 2016). Very recently, a genome-wide gain-of-function screen allowed the identification of host factors required for the replication of dengue virus, zika virus and yellow fever virus (Petrova *et al.*, 2019). As functional genomics screens have been a valuable tool for the identification of host factors of diverse viruses, and HBV also depends on several host functions (see chapter HBV host interactions), similar approaches can be used to identify novel host factors involved in the HBV life cycle. This is demonstrated by the work presented here.

OBJECTIVES

Half a century after the identification of HBV, the viral genome and structure are well known. While the virus itself is well characterized, the interactions with its host cells remain only partially understood. Although knowledge about virus-host interactions during HBV infection is limited, the importance of host factors for the viral life cycle is evident. In this regard, restriction factors as well as pro-viral host factors are thought to modulate the HBV life cycle. The missing knowledge about virus-host interactions is closely linked to the lack of robust *in vitro* and *in vivo* model systems that support HBV infection, originating from the narrow host and tissue tropism of HBV for human hepatocytes. While limited infectious model systems have hampered the study on host factors involved in HBV infection, their discovery might allow the development of improved infectious model systems. Hepatoma-derived HepG2 and Huh7 cell lines are widely used as surrogate models for hepatocytes, however both cell lines are not susceptible to HBV or HDV infection. The discovery of NTCP as functional receptor for HBV and HDV in hepatocytes allowed the development of infectious tissue culture systems based on hepatoma-derived cell lines (Yan *et al.*, 2012; Ni *et al.*, 2014). On the one hand, overexpression of NTCP in HepG2 cells renders these cells susceptible to HBV and HDV infection confirming NTCP as essential host factor. On the other hand, NTCP-overexpressing Huh7 cells are susceptible to HDV infection but, interestingly, only very weakly to HBV infection (Ni *et al.*, 2014). This indicates that one or more proviral host factor(s) are missing in Huh7-NTCP cells or one or more restriction factor(s) are overexpressed in these cells. Here, we hypothesized missing host factors being responsible for the poor permissiveness of Huh7 cells overexpressing human NTCP. Aim of this project was the identification and characterization of HBV host factors using a functional genomics approach. For this we took advantage of the poor susceptibility of NTCP-overexpressing Huh7 cells in combination with a gain-of-function screen.

The aim of this thesis consisted in the validation of potential candidate host factors identified in the gain-of-function screen and the characterization of selected virus-host interactions. A more profound knowledge about these interactions might finally lead to the establishment of improved infectious model systems and the development of new antiviral strategies.

The work performed during my thesis led to the shared first-authored scientific paper “A genome-wide gain-of-function screen identifies CDKN2C as a HBV host factor” submitted to Nature Communications. For clarity reasons, preliminary results obtained before my arrival at the Inserm Unit 1110 and my contribution to this project will be highlighted subsequently. For detailed methods and results, I refer to the manuscript added at the end of this chapter.

RESULTS

Preliminary results

Before my arrival at the Inserm Unit 1110, a genome-wide gain-of-function screen was performed to identify host factors that allow HBV infection in Huh-106 cells, a Huh7-derived cell line which constitutively expresses hNTCP under the control of a cytomegalovirus promoter (Verrier *et al.*, 2016a). First, the HBV life cycle block in Huh-106 cells was further characterized by comparing it to NTCP-overexpressing HepG2 cells. Infection of both cell lines and visualization of intracellular HBsAg by immunofluorescence (IF) 10 days post infection (dpi) revealed only very weak infection rates in Huh-106 cells, confirming the results obtained by Ni *et al*. in our systems (Ni *et al.*, 2014). In a binding assay, HBV was shown to bind to Huh-106 cells and HepG2-NTCP cells in a comparable manner. This indicates that the life cycle block in Huh-106 cells might be due to the lack of essential host factors involved in a step post-binding. Therefore, this cell line was considered suitable for the identification of novel host factors using a gain-of-function approach. For the screen, Huh-106 cells were transduced with a pooled lentiviral ORF library provided by the Broad Institute (X. Yang *et al.*, 2011). Transduced cells were infected with recombinant HBV and cultivated in Williams medium E containing 2% DMSO. 10 days post inoculation the cells were sorted for phenotype (infected/non-infected) using immunostaining of HBsAg and fluorescence activated cell sorting (FACS). Finally, ORF abundance in infected and non-infected cells was quantified using next-generation sequencing. The enrichment of certain ORFs in the infected population was determined by calculation of a post-sort/pre-sort *log2 fold change* (Log2FC) value. The candidates yielding the highest Log2FC values (Log2FC ≥ 1.5) were then chosen as potential host factors, leading to 90 candidates. Based on sequence analyses of multiple ORFs per gene and the assessment of candidate gene expression in the liver, 47 ORFs where chosen for individual validation. Lentiviruses were purchased for the individual overexpression of these 47 candidates, of which 35 met internal quality control based on lentivirus titers. In addition, lentiviruses for the overexpression of *GFP*, *KRT80* and *CPA1* cDNA sequences were obtained as negative controls. For validation, Huh-106 cells were transduced with lentiviral vectors containing one of the chosen hits and inoculated with recombinant HBV four days after transduction. Cells were examined for HBV infection 10 days post infection. For each ORF, secreted HBsAg and HBeAg in the cell culture supernatant was quantified by chemiluminescence immunoassay (CLIA). Overexpression of certain candidate genes lead to increased HBsAg and HBeAg concentrations compared to the controls. These ORFs were considered candidate host factors for HBV infection in Huh-106 cells. Among the ORFs increasing HBe and HBs antigen concentration in infected Huh-106 cells, there is *HNF4A* encoding the transcription factor hepatocyte nuclear, which has already been described to regulate HBV gene

expression (Raney *et al.*, 1997; Quasdorff *et al.*, 2008). This endorses the ability of the screen performed here to distinguish HBV host factors.

Results

Characterization of different aspects of HBV infection in Huh-106 and HepG2-NTCP

By the time I got involved into the project, the genome-wide gain-of-function screen described above had been performed based on the hypothesis that missing host factor(s) are responsible for the poor susceptibility of Huh-106 cells to HBV infection. For the validation of candidate host factors and the characterization of their potential roles in the HBV life cycle, we urged to better understand the restriction of HBV infection in Huh-106 cells compared to HepG2-NTCP cells. To further localize the HBV life cycle block in Huh-106, the capacity of both cell lines to support different steps of the life cycle was assessed. As cccDNA is a key intermediate of the life cycle, we analyzed if and to which extent the studied cell lines support cccDNA formation using Southern Blot. For this, a large number of cells were infected with HBV and DNA was extracted from infected and non-infected cells 10 dpi using the phenol/chloroform extraction method. Equal amounts of extracted DNA were loaded to a 1.2% agarose gel for electrophoresis and subsequent Southern blotting. With probes specifically detecting HBV DNA, three different forms of HBV DNA (rcDNA, dslDNA and cccDNA) can be distinguished. In both cell lines, HBV cccDNA was detected in HBV-infected cells. Interestingly, cccDNA levels in infected Huh-106 cells are markedly reduced compared to infected HepG2-NTCP cells (Eller, Heydmann *et al.* Fig. 1b). This indicates, that host factors involved in cccDNA formation or earlier steps of the life cycle might be weakly expressed in Huh-106. To assess whether rcDNA to cccDNA conversion was slowed down or reduced in Huh-106 cells, further Southern blot analyses were performed to assess the kinetics of cccDNA formation in both cell lines. Cells were lysed for DNA extraction two, five, and nine days after infection with HBV. In both cell lines, cccDNA was already detected two days post infection, indicating that cccDNA formation occurs at the same pace in Huh-106 and HepG2-NTCP cells. The specificity of infection in Huh-106 cells was confirmed by pre-treating cells with the entry inhibitor preS1 peptide prior to HBV infection, as cccDNA levels are strongly reduced under this condition. (Eller, Heydmann *et al.* Fig. 1c, d). Also, intracellular pgRNA levels and secreted HBe and HBs antigen concentrations in the cell culture supernatant were found to be markedly higher in infected HepG2-NTCP cells than in infected Huh-106 cells. These results indicate that HBV infection in Huh-106 cells is constrained in a step between viral entry and cccDNA-mediated transcription.

Validation of CDKN2C as host factor for HBV infection

For the validation and selection of candidate host factors from the primary screen for the analysis of their mechanism of action, further validation experiments were pursued. For this purpose, lentiviral vectors bearing single candidate ORFs and a selection marker compatible to Huh-106 cells were produced. In addition, lentiviruses for the overexpression of *HNF4A*, *GFP*, *KRT80* were made as positive control, transduction control or negative control from the primary screen, respectively. For validation experiments, Huh-106 cells were transduced with lentiviruses for overexpression of candidate and control ORFs. Transduction efficiency was controlled by fluorescence microscopy of cells overexpressing GFP three days post transduction. Cells were then infected with HBV and infection was assessed after 10 days. As determined by quantification of pgRNA by qRT-PCR, transduction of Huh-106 cells with empty control lentivirus (ctrl) or lentivirus for overexpression or *KRT80* did not significantly alter HBV infection levels. Cells overexpressing *HNF4A*, *CDKN2C* or *ESRP1* displayed the highest pgRNA levels after HBV infection (Eller, Heydmann *et al.* Fig. 4a). Detection of intracellular HBsAg by IF and flow cytometry confirmed increased infection levels in cells overexpressing *HNF4A*, *CDKN2C* or *ESRP1*. Interestingly, co-overexpression of *CDKN2C* and *ESRP1* resulted in higher infection levels than overexpression of single candidate host factors (Eller, Heydmann *et al.* Fig. 5a, b). These results indicate that *CDKN2C* and *ESRP1* are factors involved in HBV infection in Huh-106 cells, acting though distinct mechanisms. *CDKN2C* encodes the cyclin dependent kinase inhibitor 2C (CDKN2C), a member of the inhibitor of CDK4 (INK4) family of CDK inhibitors (CKIs) and regulator of cell cycle G1 progression (Guan *et al.*, 1994). *ESRP1* encodes the epithelial splicing regulatory protein 1 (ESRP1), an epithelial cell-type-specific splicing regulator (Warzecha *et al.*, 2009). Because ESRP1 is only very weakly expressed in hepatocytes, we focused on CDKN2C.

As mentioned above, the lack of a proviral host factor in Huh-106 was hypothesized to be responsible for the poor susceptibility to HBV infection compared to HepG2-NTCP cells. Therefore, differential expression of *CDKN2C* between both cell lines was assessed. Detection of *CDKN2C* mRNA by qRT-PCR revealed decreased expression levels of this gene in Huh-106 cells (Eller, Heydmann *et al.* Fig. 3d). This indicates that the lack of *CDKN2C* expression may contribute to the limited susceptibility to HBV infection of Huh-106 cells.

To further corroborate the role of CDKN2C as host factor for HBV infection, the effect of small interfering RNAs (siRNAs) specifically targeting *CDKN2C* on HBV infection in HepG2-NTCP cells was evaluated. For this, HepG2-NTCP cells were transfected with siRNA and infected with HBV two days post transfection. As determined by IF 10 dpi, intracellular HBsAg was reduced in cells treated with siRNA targeting *CDKN2C* or *SLC10A1* (NTCP) before HBV infection compared to cells treated with non-targeting control siRNAs (Eller, Heydmann *et al.* Fig. 4b, c). To rule out unspecific effects of siRNAs, we used the CRISPR-Cas9 system to produce HepG2-NTCP CDKN2C knockout (KO) cells. Via clonal selection, four HepG2-NTCP KO-CDKN2C cell lines were generated with absent CDKN2C protein expression. HBV infection of these cells and detection of intracellular pgRNA by qRT-PCR and

secreted HBeAg by CLIA 10 dpi, revealed decreased infection levels in all four KO-CDKN2C cell lines compared to naïve HepG2-NTCP cells (Eller, Heydmann *et al.* Fig. 4d, e). These results support the role of CDKN2C for HBV infection in HepG2-NTCP cells.

Functional role of CDKN2C and cell cycle arrest on HBV infection

After confirming its role as host factor for HBV infection, we next analyzed at which step of the viral life cycle CDKN2C mediates infection. To assess whether cccDNA formation is affected by CDKN2C, Southern Blot detection of HBV DNA was performed in Huh-106 cells. Cells overexpressing *CDKN2C* did not display higher cccDNA levels than cells overexpressing *GFP* or un-transduced cells. This indicates that CDKN2C modulates the HBV life cycle in a step after rcDNA to cccDNA conversion. To evaluate the effect of CDKN2C on the concentration of HBV transcripts, Northern Blot was performed for the detection of viral RNAs in HBV infected Huh-106 cells. For this, equal amounts of total RNA extracted from HBV infected cells overexpressing *CDKN2C* or control genes were loaded to a 1% agarose gel containing 2.2 M formaldehyde followed by Northern Blot transfer. The 3.5 kb pgRNA/precore mRNA and 2.1/2.4 surface mRNAs were detected using ^{32}P-labeled RNA probes. The signal for all detected forms of HBV RNA was stronger in CDKN2C overexpressing cells. These results indicate a role of CDKN2C in a step of the HBV life cycle between cccDNA formation and translation of viral proteins. To determine whether CDKN2C has a direct effect on HBV RNA formation, nascent HBV RNAs were quantified via incorporation of labelled uridine. Newly synthesized HBV RNA levels were 3-fold higher in Huh-106 cells overexpressing *CDKN2C.* These results suggests a role for CDKN2C in transcription of HBV RNAs.

CDKN2C is a key player in cell cycle regulation and interacts with CDK4/6 to block cell cycle G1 progression. To assess whether induction of cell cycle arrest is responsible for its role for HBV infection, functional studies were performed using two clinical small molecule CDK inhibitors. For this, two specific inhibitors of CDK4/6, Palbociclib (PD-0332991) and Ribociclib (LEE011) were used. (Fry *et al.*, 2004; Kim *et al.*, 2013). Both of them are approved for the treatment of metastatic hormone receptor positive (HR+) breast cancer and are evaluated in clinical trials for the treatment of other malignancies (Vijayaraghavan and Moulder, 2018). First, the toxicity profile of both compounds was tested in Huh-106 and HepG2-NTCP cells to identify a suitable working concentration for functional studies. The capacity of Palbociclib to induce cell cycle G1 arrest in Huh7 and Huh-106 cells was assessed by analysis of the cell cycle using propidium iodide (PI) and flow cytometry for the quantification of DNA content of cells. Cell populations treated with 100 nM Palbociclib for three or ten days contained a higher percentage of cells in the G0/G1 phase of the cell cycle than untreated cells. To analyze the effect of CDK4/6 inhibitor-induced G1 cell cycle arrest on HBV infection in Huh-106 cells, we treated cells with Palbociclib or Ribociclib for one day before HBV infection. CDK4/6 inhibitors were again added to the medium after removal of the inoculum and HBV infection was assessed 10 dpi. Visualization of cytosolic HBsAg in HBV infected Huh-106 cells using IF, revealed

increased antigen levels in cells treated with either of the CDK4/6 inhibitors. As detected by Southern Blot, treatment of Huh-106 cells with the same concentration of Palbociclib did not alter cccDNA levels. The significant increase of HBV infection rates in Huh-106 cells treated with 100 nM of Palbociclib or Ribociclib was subsequently confirmed by detection of pgRNA by qRT-PCR and by quantification of intracellular HBsAg using flow cytometry (Eller, Heydmann *et al.* Fig. 6). These results indicate, that induction of a cell cycle G1 arrest by CDK4/6 inhibitors is favorable for HBV infection and modulates the life cycle in a step after cccDNA formation.

Publication of results

These results were integrated in the manuscript Eller, Heydmann *et al.* "A genome-wide gain-of-function screen identifies CDKN2C as a HBV host factor" which is in revision in *Nature Communications*.

A genome-wide gain-of-function screen identifies CDKN2C as a HBV host factor

Carla Eller[1,#], Laura Heydmann[1,#], Che C. Colpitts[1,2], Houssein El Saghire[1], Federica Piccioni[3], Frank Jühling[1], Karim Majzoub[1], Caroline Pons[4], Julie Lucifora[4], Joachim Lupberger[1], Michael Nassal[5], Glenn S. Cowley[3], Naoto Fujiwara[6], Sen-Yung Hsieh[7], Yujin Hoshida[6], Emanuele Felli[1,8], Patrick Pessaux[1,8], Camille Sureau[9], Catherine Schuster[1], David E. Root[3], Eloi R. Verrier[1,$*], Thomas F. Baumert[1,8,10,$*]

#Contributed equally as first authors

$Contributed equally as last authors

[1]Université de Strasbourg, Inserm, Institut de Recherche sur les Maladies Virales et Hépatiques UMR_S1110, F-67000 Strasbourg, France; [2]Department of Biomedical and Molecular Sciences, Queen's University, Kingston, Canada; [3]Broad Institute of Massachusetts Institute of Technology and Harvard, Cambridge, MA, USA; [4]Inserm, U1052, Cancer Research Center of Lyon (CRCL), Université de Lyon (UCBL1), CNRS UMR_5286, Centre Léon Bérard, Lyon, France [5]Department of Internal Medicine II/Molecular Biology, University Hospital Freiburg, Freiburg, Germany; [6]Liver Tumor Translational Research Program, Simmons Comprehensive Cancer Center, Division of Digestive and Liver Diseases, Department of Internal Medicine, University of Texas Southwestern Medical Center, Dallas, Texas; [7]Department of Gastroenterology and Hepatology, Chang Gung Memorial Hospital, Taipei, Taiwan; [8]Institut Hospitalo-Universitaire, Pôle Hépato-digestif, Nouvel Hôpital Civil, 67000 Strasbourg, France; [9]INTS, Laboratoire de Virologie Moléculaire, Paris, France, [10] Institut Universitaire de France (IUF), Paris , France.

*Corresponding authors: Prof. Thomas F. Baumert, MD and Dr Eloi R. Verrier; Inserm U1110, Institut de Recherche sur les Maladies Virales et Hépatiques, Université de Strasbourg, 3 Rue Koeberlé, 67000 Strasbourg, France; Phone: +33 3 68 85 37 03, Fax: +33 3 68 85 37 24, e-mail: thomas.baumert@unistra.fr; e.verrier@unistra.fr

ABSTRACT (149/150 words)

Chronic HBV infection is a major cause of liver disease and cancer worldwide. While current therapies can suppress viral replication, approaches for cure are lacking, and the knowledge of virus-host interactions is still limited. Here, we performed a genome-wide gain-of-function screen, using a poorly permissive hepatoma cell line named Huh-106, to uncover host factors that enhance HBV infection. Validation studies in primary human hepatocytes identified CDKN2C as an important host factor for the HBV life cycle. Interestingly, CDKN2C is overexpressed in highly permissive cell lines and HBV-infected patients. Mechanistic studies unraveled a role of CDKN2C in inducing cell cycle G1 arrest through inhibition of CDK4/6 and stimulating HBV replication. A correlation between CDKN2C expression and disease progression in HBV-infected patients suggests a functional role in HBV-induced liver disease. Taken together, we identified a previously undiscovered clinically relevant HBV host factor, allowing the development of improved infectious model systems for drug discovery and the study of the HBV life cycle.

Keywords: HBV, Genome-wide screen, host-dependency factors

INTRODUCTION

Chronic infection by hepatitis B virus (HBV) is a major health problem and the leading cause of hepatocellular carcinoma (HCC) worldwide [1]. The global HBV burden persists despite the availability of an effective preventative vaccine and it is estimated that HBV chronically infects 250 million people. While current therapies based on nucleot(s)ide analogs (NUCs) suppress viral replication and reduce progression of liver disease, treatment is lifelong and viral cure is extremely rare [2]. Different curative strategies are urgently needed to address this global medical burden.

HBV is a small enveloped DNA virus in the *Hepadnaviridae* family [3]. The HBV surface antigen (HBsAg) mediates entry of the virus into hepatocytes via primary low-affinity interactions with heparan sulfate proteoglycans [4–6] and secondary specific binding to the sodium taurocholate cotransporting polypeptide (NTCP) [7,8], ultimately leading to fusion and release of the viral capsid into the cytoplasm. The capsid delivers the viral genome to the nucleus, where HBV relaxed circular DNA (rcDNA) is converted into episomal covalently closed circular DNA (cccDNA), in a process thought to be mediated by host DNA repair enzymes, such as tyrosyl-DNA-phosphodiesterase 2 (TDP2) [9] and DNA Polymerase kappa (POLK) [10]. The cccDNA is the reservoir for viral persistence and serves as a template for all viral transcripts. CccDNA is not affected by the NUC-based treatments targeting the viral reverse transcriptase, which converts viral pre-genomic RNA (pgRNA) into *de novo* genomic DNA, within newly formed nucleocapsids prior to virion budding [11].

Currently available drugs for the treatment of chronic HBV infection, such as NUCs, are direct-acting antiviral (DAAs) and allow the suppression of viral replication, but viral cure is rarely achieved.

Innovative therapeutic strategies, such as host targeting agents (HTAs), have emerged as novel candidates for the treatment of viral infections, including hepatotropic viruses [12–15]. However, this strategy requires a comprehensive understanding of virus-host interactions at the molecular level. In the context of HBV infection, the limited access to robust infection models has restrained for a long time the characterization of host factors involved in the viral entry process. The discovery of NTCP as a receptor for HBV has allowed the development of cell culture models suitable for the study of the full life cycle [7,16]. Indeed, exogenous expression of NTCP in human hepatoma cell lines (such as HepG2 and Huh7) confers susceptibility to HBV infection. However, NTCP-overexpressing Huh7 cells remain poorly permissive to HBV infection but support infection by hepatitis D virus (HDV), an HBV-satellite virus carrying HBV envelope proteins [16]. This suggests that after HBV entry, additional key factors are still limiting in these cells. Therefore, we hypothesized that characterization of differences between the two cell lines should allow the identification of previously undiscovered HBV host factors. Discovery of such host factors in human hepatoma cells would open avenues to develop new infection models, such as immunocompetent transgenic animal models that are fully susceptible to HBV. Indeed, a previous study suggests that the limited ability of HBV to replicate in mouse cells is caused by the lack of a host cell dependency factor [17]. Here, we performed a genome-wide gain-of-function screen using a weakly permissive NTCP-overexpressing Huh7-derived cell line termed Huh-106 cells [5] and a genome-scale lentiviral open reading frame (ORF) library [18], aiming to uncover HBV-related host-dependency factors. We expect that the identification of these previously undiscovered HBV factors will facilitate the development of improved infectious cell culture systems for the identification of innovative antiviral molecules.

RESULTS

A high-throughput screening strategy for the identification of HBV host factors by functional genomics. To characterize HBV infection in different hepatoma cell lines, we compared the susceptibility of two NTCP-overexpressing cell lines (Huh7-derived Huh-106 [5] and HepG2-NTCP) to HBV and HDV infection. Both cell lines were similarly susceptible to HDV infection, suggesting equivalent virus entry in both cell lines **(Fig. 1a)**. However, in contrast to HepG2-NTCP cells, Huh-106 cells appear poorly permissive to HBV infection **(Fig. 1a)**, despite their ability to bind HBV particles **(Fig. 1b)**. Furthermore, Huh-106 cells support the conversion of incoming HBV rcDNA to cccDNA, although to a much lesser extent than HepG2-NTCP cells **(Fig. 1c, d)**. Interestingly, the kinetics of cccDNA formation are similar in both cell lines **(Fig. 1e)**. Moreover, quantification of intracellular pgRNA and secreted antigens (HBsAg and HBeAg) during the course of infection revealed a severe restriction of the HBV life cycle in Huh-106 cells at different steps **(Fig. 1f-h)**. Taken together, these findings suggest that HBV infection is constrained in Huh-106 cells in a step between NTCP-mediated entry and cccDNA-mediated transcription.

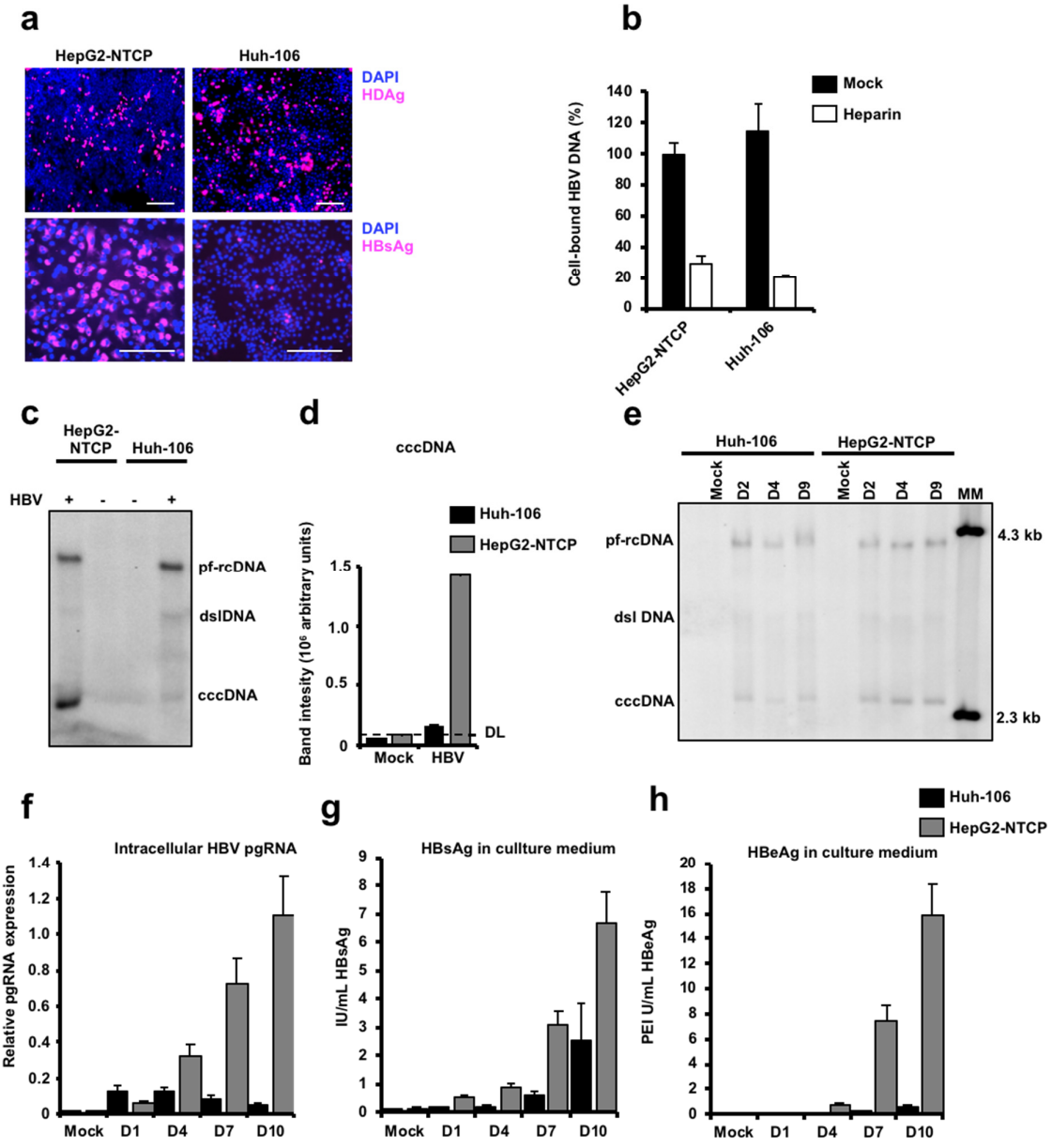

Figure 1. Huh-106 are less permissive to HBV infection than HepG2-NTCP. a HBV and HDV infection of HepG2-NTCP and Huh-106 cells and detection of HBsAg and HDAg by IF after 10 dpi. One representative experiment is shown. Scale bars: 100 µm. **b** Binding of HBV particles to HepG2-NTCP and Huh-106 cells**.** Results are expressed as means +/- SEM bound HBV genome copies (%) from 3 independent experiments (n=8). **c** Comparison of HBV cccDNA levels in HepG2-NTCP and Huh-106 cells detected by Southern blot. Protein-free relaxed circular DNA (pf-rcDNA), double stranded linear DNA (dsl DNA) and covalently closed circular DNA (cccDNA) are indicated. One representative experiment out of four is shown. **d** Quantification of cccDNA band intensity. Dashed line indicates the detection limit (DL). Results are expressed as means +/- SEM 10^6 band intensity (arbitrary units) from 4 independent experiments. **e** Time course experiment of HBV infection in Huh-106 and HepG2-NTCP. DNA was extracted from cells 2 (D2), 4 (D4) or 9 (Mock, D9) days post HBV infection and detected by Southern blot. Bands of pf-rcDNA, dsl DNA, and cccDNA were identified using a molecular marker (MM). One experiment is out of three shown. Quantification of cccDNA band intensities in Figure S5a. **f-h** Quantification of intracellular pgRNA by qRT-PCR (f) and secreted HBsAg (g) and HBeAg (h) by CLIA in Huh-106 and HepG2-NTCP cells 1 (D1), 4 (D4), 7 (D7) or 10 (Mock, D10) days post HBV infection. **f** Results are expressed as means +/- SEM relative pgRNA expression from 4 experiments (n=13). **g** Results are expressed as means +/- SEM IU/mL HBsAg from 4 experiments (n=12). **h** Results are expressed as means +/- SEM PEI U/mL HBeAg from 4 experiments (n=12).

Assuming that this restriction is due to the lack of key host factor(s) for HBV infection, we pursued a functional genomics approach to screen for factors that increase the susceptibility of Huh-106 cells to HBV infection. To this end, we performed a gain of function screen for HBV infection using Huh-106 cells and a genome scale lentiviral expression library of more than 16,000 human ORFs [18]. Huh-106 cells were first transduced with the lentiviral hORFeome V8.1 [18], and then inoculated with HBV **(Fig. 2a)**. Sorting for HBsAg-positive cells by fluorescence-activated cell sorting (FACS) 10 days post-infection allowed the collection of HBV-infected cells only (HBV sorted) for subsequent analysis to identify factors conferring susceptibility to HBV infection. Using Illumina next-generation sequencing (NGS) and deconvolution using PoolQ, we compared the infected pool of cells (HBV sorted, **Fig. 2a, b**) to the control population (HBV pre-sort, **Fig. 2a, b**) to determine which ORFs were enriched in HBs-positive cells. Candidate HBV host factors were identified based on an enrichment threshold of log2 fold change (Log2FC) > 1.5 **(Fig. 2c, d)**. Following an algorithm based on liver expression and the number of sequences per candidate to further filter the list (see Methods), 47 candidate genes were selected for validation **(Table S1)**. Among them was *HNF4A*, a gene encoding a transcription factor previously known to enhance HBV replication [19], supporting the ability of our screen to identify HBV host factors. Interestingly, another transcription factor stimulating HBV replication, *HLF* [20], scored a Log2FC = 1.49 just below the selection threshold. The remaining candidates therefore represent a list of putative new factors for HBV infection for further validation and study.

Identification of CDKN2C as an HBV host factor differentially expressed in HepG2- and Huh7-derived cell lines. To validate the candidate host factors identified above, we individually overexpressed the candidate ORFs in Huh-106 cells before infection with HBV for 10 days. Of the 47 identified ORFs, 35 were evaluated (see Methods), along with lentiviruses encoding *GFP*, *KRT80* and *CPA1* as negative controls **(Table S1)**. HBV infection was assessed by quantification of secreted HBV antigens in the cell culture supernatant of infected cells, indicating increased HBV infection versus controls for a majority of the candidates. Several had large effects on both secreted HBeAg and HBsAg, including the top scorers *ESRP1, SPATA24, U2AF1*, *CDKN2C*, and *GPR27* **(Fig. 3a)**. Importantly, the top candidate ESRP1 was not detected at the protein level in our systems (data not shown), suggesting a non-physiological effect on HBV infection. However, this construct was used as a technical positive control in further experiments. To systematically identify genes that are differentially expressed in the studied cell lines, we performed transcriptomic analyses using microarrays for gene expression profiling in HepG2-NTCP and Huh-106 cells. Pathway enrichment analysis identified a small number of signaling pathways that exhibited significantly different expression patterns between the two cell lines, although the vast majority of pathways were similarly expressed **(Fig. 3b)**. Notably, IFNα response gene expression was higher in HepG2 cells, consistent with previous observations that HepG2 cells are more competent for mounting an efficient innate immune response following viral infection compared to Huh7-derived cells [21,22].

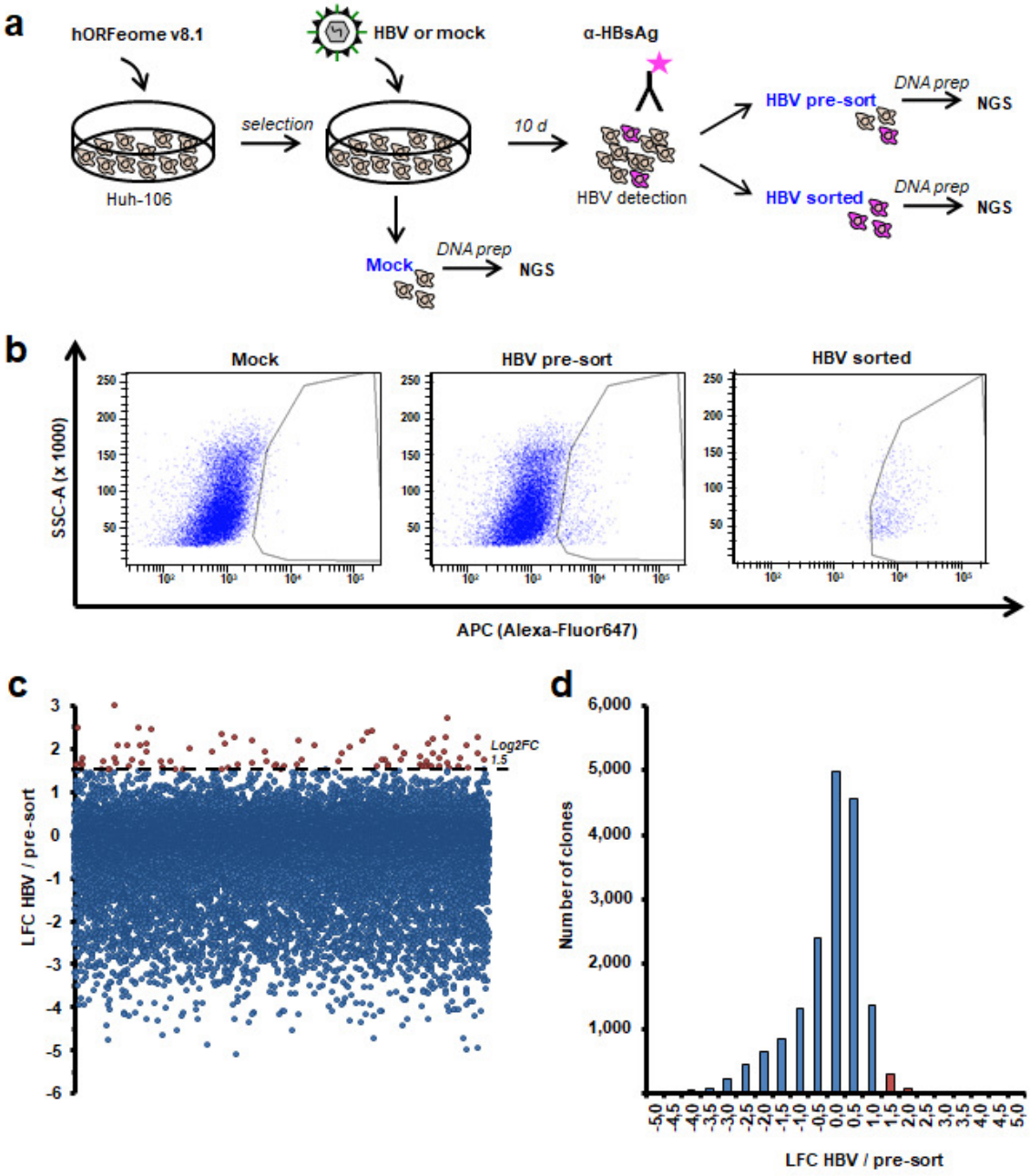

Figure 2. Gain-of-function (GOF) screen in Huh-106 cells for the identification of HBV host factors. **a** Schematic workflow of GOF-screen. **b** FACS for HBsAg-positive cells in Huh-106 transduced with an ORF-library (hORFeome v8.1) 10 days after HBV infection (HBV pre-sort). Flow-cytometric analysis of uninfected cells as gating control (Mock) and of the HBsAg-positive sorted population as sorting control (HBV sorted). **c-d** Primary screen candidates. ORFs with Log2FC > 1.5 were selected for validation.

Comparing the expression of primary screen candidate genes from the microarray data, we identified *CDKN2C* and *SPATA24* as highly expressed genes in HepG2-NTCP versus Huh-106 cells **(Fig. 3c)**. Given the specific previously annotated function of SPATA24/T6441 in spermiogenesis [23] we focused instead on *CDKN2C* for further characterization. The higher expression of *CDKN2C* in HepG2-

NTCP versus Huh-106 cells was confirmed by qPCR and Western blot (**Fig. 3d, e**). The involvement of *CDKN2C* in HBV infection in Huh-106 cells was confirmed by a 6-fold increase in viral pgRNA levels following overexpression of *CDKN2C* when compared to the empty control vector **(Fig. 4a)**.

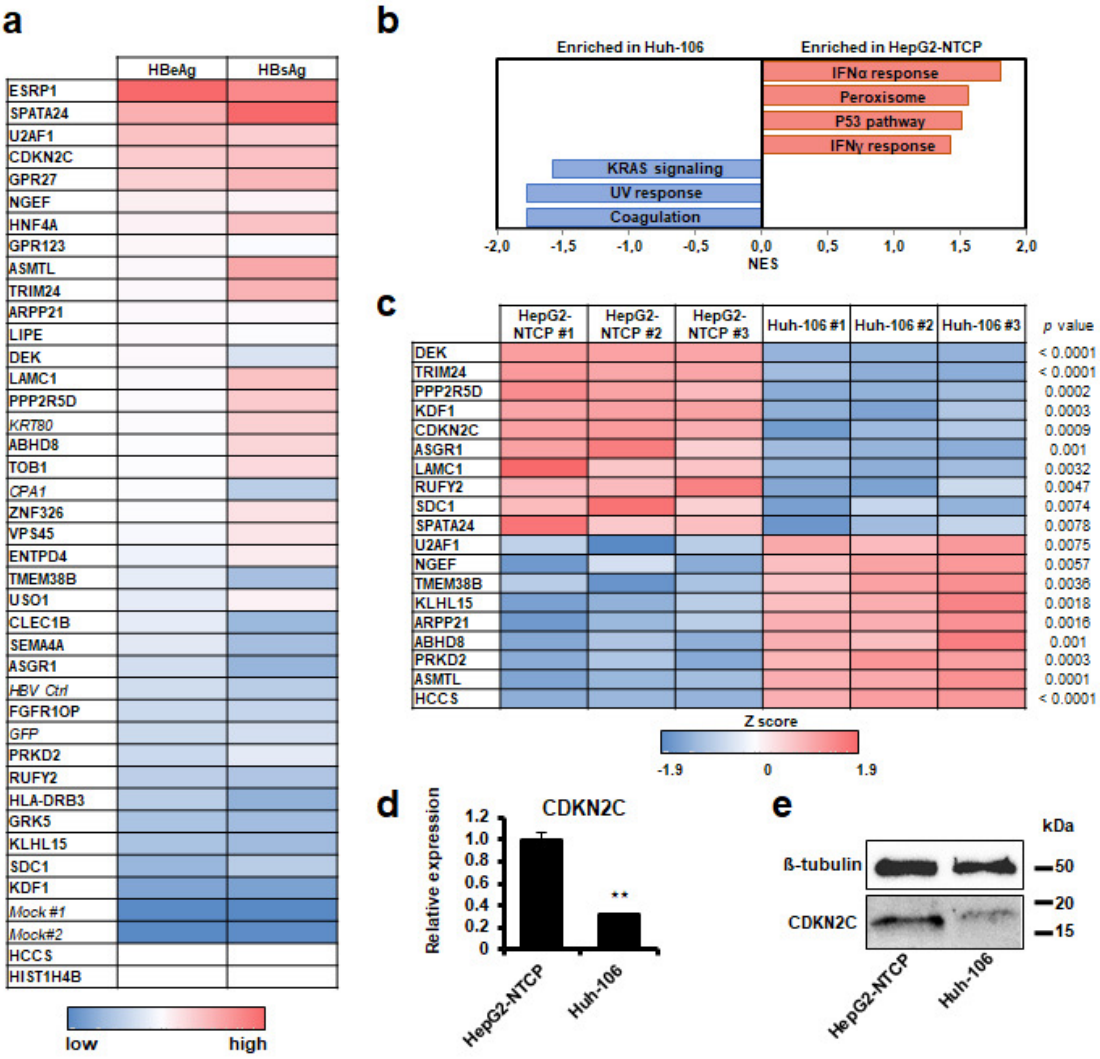

Figure 3. CDKN2C is differentially expressed in HepG2-NTCP and Huh-106 cells. a Heatmap of candidate validation. Huh-106 cells were transduced with the indicated ORF and infected with HBV. HBV infection was assessed at 10 dpi by CLIA quantification of secreted HBeAg and HBsAg. Results are expressed as means concentration of secreted HBeAg or HBsAg from 1 experiments (n=2). **b-c** Microarray for comparison of gene expression in HepG2-NTCP and Huh-106 cells. Analysis of differentially expressed pathways (**b**) and candidate host factors from the primary screen through Z score transformation (**c**) are presented. **d-e** CDKN2C is upregulated in HepG2-NTCP compared to Huh-106 cells. **d** *CDKN2C* mRNA expression in HepG2-NTCP and Huh-106 cells quantified by qRT-PCR. Results are expressed as means +/- SEM *CDKN2C* relative expression compared to HepG2-NTCP (set to 1) from 3 independent experiments (n=6). **e** Endogenous CDKN2C protein expression in HepG2-NTCP and Huh-106 cells detected by Western Blot. One representative experiment out of two is shown. ★★ $p < 0.01$.

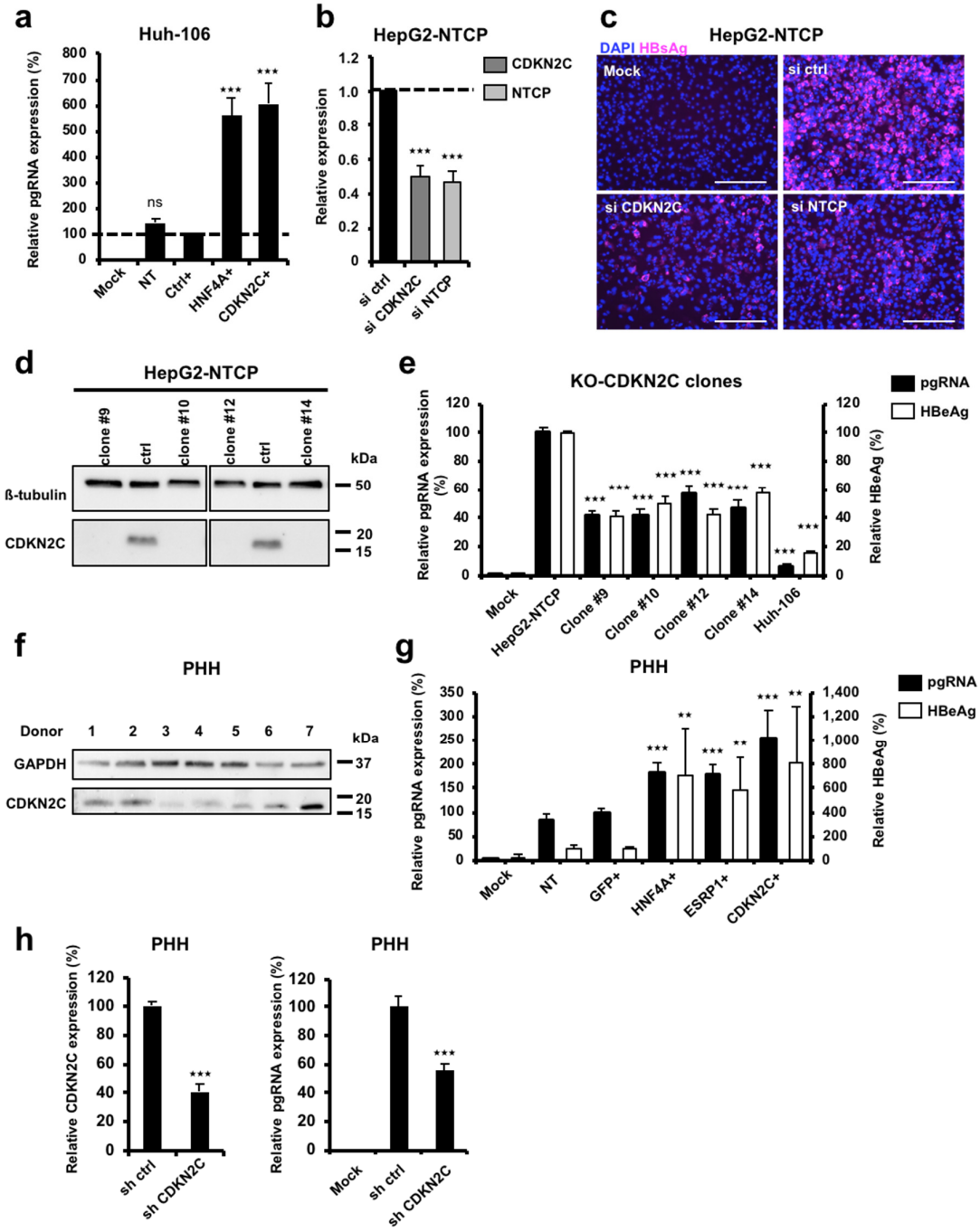

Figure 4. CDKN2C is a HBV host factor. a Individual ORF-overexpression in Huh-106 and HBV infection 3 days after transduction. Detection of HBV pgRNA by qRT-PCR 10 dpi. Results are expressed as means +/- SEM relative pgRNA expression (%) compared to ctrl (set as 100%) from 8 independent experiments (n=21). **b-c** Transfection of HepG2-NTCP cells with siRNAs targeting CDKN2C, NTCP or non-targeting control (si ctrl). **b** mRNA expression was quantified by qRT-PCR 2 days post transfection. Results are expressed as means +/- SEM relative expression compared to si ctrl (set to 1) from 4 independent experiments (n=8). **c** HBV infection of HepG2-NTCP cells 2 days post transfection and detection of HBsAg by IF 10 dpi. Scale bars: 100 µm. **d** Knockout of CDKN2C in HepG2-NTCP and clonal selection for production of KO-CDKN2C cell lines. CDKN2C expression was controlled by Western Blot for in HepG2-NTCP (ctrl) and KO-CDKN2C clones. **e** HBV infection of HepG2-NTCP, KO-CDKN2C clones and Huh-106. HBV infection was assessed at 10 dpi by quantification of HBV pgRNA by qRT-PCR (black) and quantification of secreted HBeAg by CLIA (white). Results are expressed as means +/- SEM % HBV infection compared to HepG2-NTCP (set as 100%) from 3 independent experiments

(n=9 for pgRNA and n=12 for HBe CLIA). **f** Western Blot for detection of endogenous CDKN2C expression in primary human hepatocytes (PHH) from 7 different donors (1-7). One experiment is shown. **g** Validation studies in PHH from 3 different donors transduced with ORF lentivirus for 3 days and infected with HBV. HBV markers are detected 10 dpi. Total RNA was extracted and pgRNA was quantified by qRT-PCR (black). Concentration of secreted HBeAg in cell supernatant was assessed by CLIA (white). Results are expressed as means +/- SEM % HBV infection compared to ctrl (GFP) (set to 100%) from 3 independent experiments from different donors (n=12 for pgRNA; n=6 for HBeAg). **h** Silencing of *CDKN2C* expression decreases HBV infection in PHH. PHH from 3 independent donors were transduced with lentiviruses containing CDKN2C-targeting shRNA or non-targeting shRNA control (sh ctrl). Silencing efficacy was assessed after 3 days by qRT-PCR. Results are expressed as means +/- SEM % gene expression compared to sh ctrl (set to 100%) from 3 independent experiments from different donors (n=9). PHH were then infected with HBV and HBV infection was assessed by qRT-PCR quantification of HBV pgRNA 8 dpi. Results are expressed as means +/- SEM relative pgRNA expression compared to sh ctrl (set to 100%) from 3 independent experiments from different donors (n=9). ★ $p < 0.05$; ★★ $p < 0.01$; ★★★ $p < 0.001$.

Taking advantage of high infection levels in HepG2-NTCP cells, we aimed to confirm the phenotypic effect of *CDKN2C* on HBV infection by a loss-of-function approach, using siRNA specifically targeting *CDKN2C* or *SLC10A1* (the gene encoding the HBV receptor NTCP) in susceptible HepG2-NTCP cells, as shown in **Figure 4b-c**. We observed a marked decrease in HBV infection in cells with silenced *CDKN2C* or *SLC10A1* expression. To rule out off-target effects, we used CRISPR-Cas9 to generate and clonally select four independent HepG2-NTCP CDKN2C knockout (KO) cell lines **(Fig. 4d)**. Functional analysis confirmed a marked decrease in both HBV pgRNA and secreted HBe antigen levels in HepG2-NTCP KO-CDKN2C cells compared to naïve HepG2-NTCP cells **(Fig. 4e)**. Finally, to validate the relevance of *CDKN2C* in a physiological model, we investigated CDKN2C-HBV interactions in primary human hepatocytes (PHH), the natural target cells for HBV infection, which express the protein at varying levels comparable to HepG2-NTCP cells **(Fig. 4f)**. Consistent with our previous observations, the overexpression of *HNF4A* and *CDKN2C* in PHH resulted in a significant and marked increase in HBV infection **(Fig. 4g)**. Moreover, the silencing of *CDKN2C* expression using target-specific shRNA induced a significant and robust decrease in HBV infection **(Fig. 4h)**. Taken together, our data support a role for *CDKN2C* in HBV infection. Therefore, the differential expression of this gene between the two cell lines suggests that a lack of *CDKN2C* expression may contribute to the limited susceptibility of Huh-106 cells to HBV infection.

CDKN2C stimulates HBV cccDNA-mediated transcription. To address the mechanism by which *CDKN2C* contributes to HBV infection, we performed additional experiments using alternative read-outs to identify the steps of the viral life cycle that may be affected by *CDKN2C* expression. Transduction efficacy was assessed by quantification of GFP expression in HBV-infected *GFP*-transduced cells after 10 days **(Fig. S2)**. Detection of intracellular HBsAg by immunofluorescence (IF) **(Fig. 5a)** and its quantification by flow cytometric analysis **(Fig. 5b)** revealed a significant increase in HBV infection levels in Huh-106 cells overexpressing *HNF4A, ESRP1* and *CDKN2C*. Notably, co-overexpression of *CDKN2C* and *ESRP1* leads to an even higher percentage of HBsAg positive cells **(Fig. 5b),** suggesting that the two factors affect HBV infection through independent pathways.

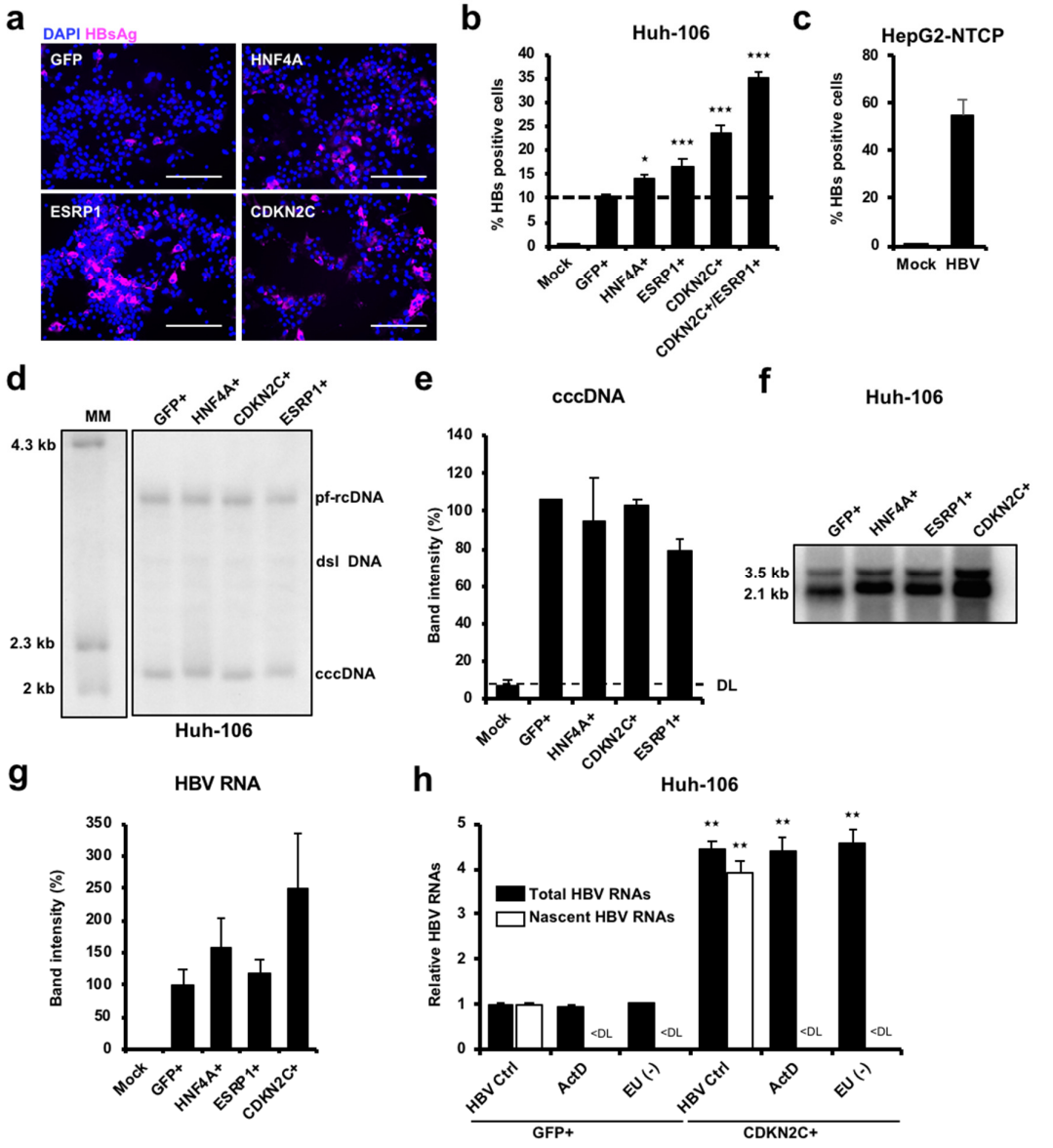

Figure 5. CDKN2C stimulates HBV cccDNA-mediated transcription. a-b, d-g Validation studies in Huh-106 overexpressing individual ORFs and infected with HBV for 10 days. **a** Detection of HBsAg by IF. Scale bars: 100 µm. **b** Flow-cytometric analysis for quantification of HBsAg-positive cells. Results are expressed as means +/- SEM % HBsAg positive cells compared to GFP from 5 independent experiments (n=13, n=11 for HNF4A) and 3 independent experiments (n=8) for CDKN2C+ESRP1 **c** Flow-cytometric analysis for quantification of HBsAg-positive cells in HBV-infected HepG2-NTCP cells. Results are expressed as means +/- SEM % HBsAg positive cells from 4 independent experiments (n=4) **d-e** Detection of HBV DNAs by Southern Blot in transduced and HBV infected Huh-106 4 dpi. **d** Southern Blot with indicated bands of HBV pf-rcDNA, dsl HBV DNA and HBV cccDNA. One representative experiment out of two is shown. **e** Quantification of cccDNA using Image Lab Version 5.2.1 (Bio-Rad). Results are expressed as means +/- SEM % band intensity compared to GFP (set to 100%) from 3 independent experiments (n=2). **f** Detection of HBV RNAs by Northern blot. The pgRNA (3,5 kb) and surface mRNAs of 2.1 to 2.4 kb (2.1 kb) are detected. One representative experiment out of two is shown. **g** Quantification of HBV RNA band intensity. Results are expressed as means +/- SEM % band intensity compared to GFP (set to 100%) from 4 independent experiments. **h** Analysis of nascent HBV RNA synthesis. Quantification of total HBV RNAs (4 dpi) and nascent HBV RNAs (d4pi, 120 minutes) in Huh-106 cells overexpressing CDKN2C using labelled uridine (EU). Actinomycin D (ActD) was used as negative control. Results are expressed as means +/- SEM % relative HBV RNAs compared to HBV Ctrl (Huh-106 GFP+ - set to 1) from 2 independent experiments (n=6). ★ $p < 0.05$; ★★★ $p < 0.001$

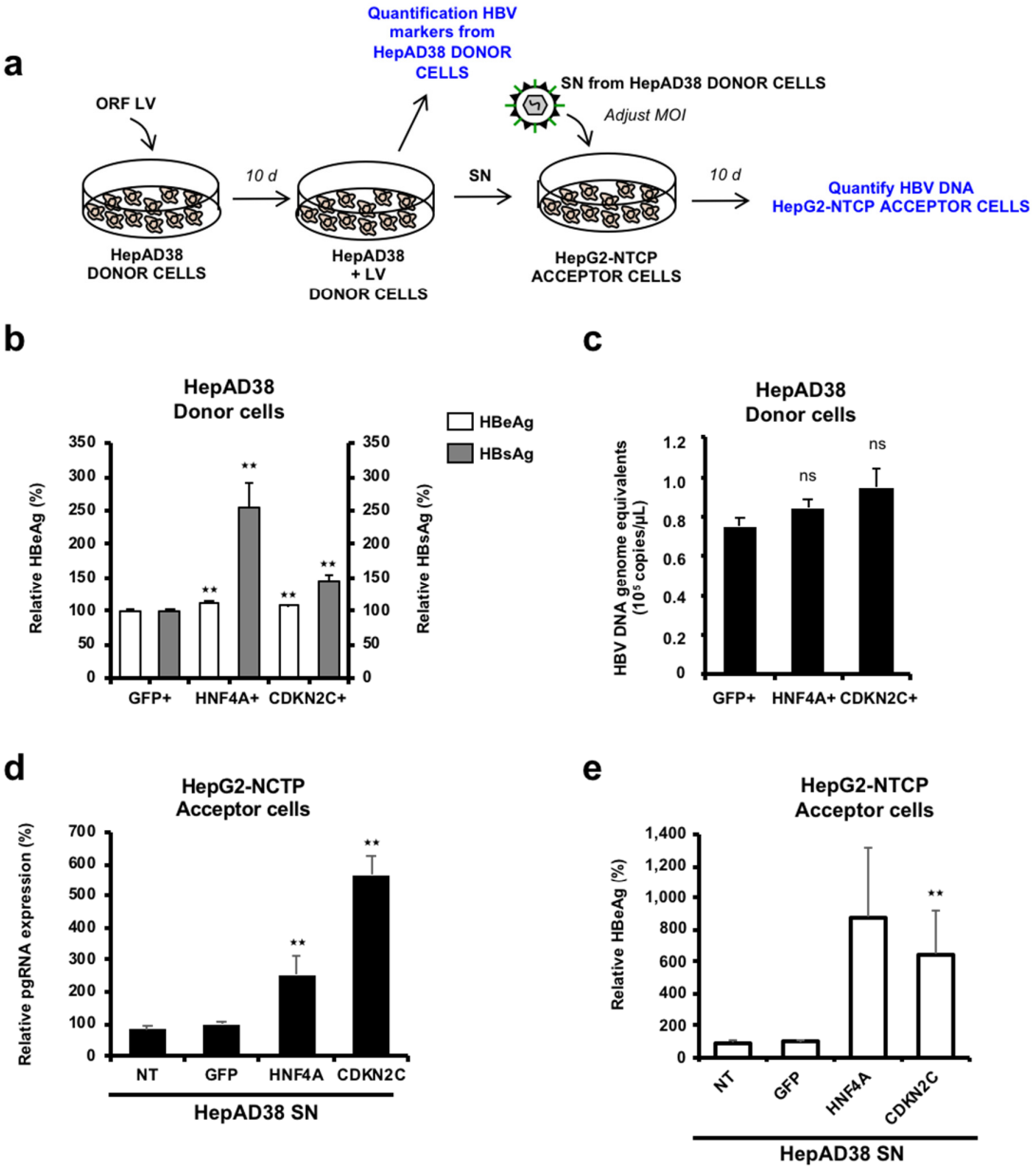

Figure 6. *CDKN2C* overexpression improves quality but not quantity of HBV virion production in HepAD38 cells. a Schematic workflow of experiments. HepAD38 cells in production medium (Donor cells) were non-transduced (NT) or transduced with ORF lentivirus for 10 days. **b-c** Supernatant (SN) from HepAD38 donor cells was harvested and HBV markers were quantified from SN. **b** HBeAg and HBsAg secretion was quantified by CLIA. Results are expressed as means +/- SEM % secreted HBeAg or % secreted HBsAg compared to NT (set to 100%) from 3 independent experiments (n=6). **c** HBV DNA level in the supernatant was determined by qPCR. Results are expressed as means +/- SEM HBV DNA genome equivalents from 3 independent experiments (n=6). **d-e** HepG2-NTCP (Acceptor cells) were infected with adjusted MOI from supernatant from HepAD38 donor cells. **d** HBV pgRNA expression was quantified by qRT-PCR 10 dpi. Results are expressed as means +/- SEM % relative pgRNA expression compared to NT (set at 100%) from 3 independent experiments (n=6). **e** HBeAg secretion was quantified by CLIA 10 dpi. Results are expressed as means +/- SEM % relative secreted HBeAg compared to NT (set at 100%) from 3 independent experiments (n=6). ★★ $p < 0.01$

Interestingly, overexpression of both factors in Huh-106 cells markedly increased HBV infection but failed to reach levels observed in HepG2-NTCP cells (**Fig. 5b, c**), suggesting the existence of additional differentially expressed factors in the two cell lines. To determine the step of the HBV life cycle affected by *CDKN2C* expression, we detected HBV DNA genome intermediates by Southern blot and HBV RNA levels by Northern blot. As shown in **Figure 5d-e**, no marked change in HBV cccDNA levels was observed when *CDKN2C* was overexpressed, suggesting no effect on HBV replication before cccDNA formation. Detection of viral RNAs by Northern blot revealed increased HBV RNA levels in cells overexpressing *HNF4A* and *CDKN2C* compared to *GFP*-overexpressing cells **(Fig. 5f-g)**. To determine whether CDKN2C has a direct effect on HBV RNA formation, we quantified nascent HBV RNAs using labelled uridine. Huh-106 cells overexpressing *CDKN2C* displayed a 3-fold increased level of newly synthesized HBV RNA **(Fig 5h)**. This suggests a role for CDKN2C in cccDNA-mediated transcription of HBV RNAs.

***CDKN2C* overexpression improves quality but not quantity of HBV virion production in HepAD38 cells.** To investigate whether modification of *CDKN2C* expression modulates the production of virus particles in HBV-expressing cells, we overexpressed *CDKN2C* and *HNF4A* in HepAD38 donor cells. 10 days after ORF-lentivirus transduction, we harvested supernatants and infected HepG2-NTCP acceptor cells with an adjusted MOI from supernatant from HepAD38 donor cells containing HBV particles **(Fig. 6a)**. While a slight increase in the secretion of HBsAg and HBeAg is observed, *CDKN2C* overexpression in HepAD38 donor cells did not affect the secretion of HBV DNA in the supernatant **(Fig. 6b, c)**. Interestingly, overexpression of *CDKN2C* in HepAD38 increased infection of HepG2-NTCP acceptor cells 3-fold, suggesting a role for CDKN2C in enhancing the formation of high-quality neo-virions **(Fig. 6d, e),** in line with the recent observation that virion production is enhanced in slow-proliferating HBV-replicating cells [24]. Collectively, our data identify CDKN2C as a previously undiscovered HBV host factor, which activates transcription from cccDNA in human hepatocytes.

CDKN2C-mediated stimulation of HBV transcription is correlated with a cell cycle arrest. *CDKN2C* encodes the cyclin dependent kinase inhibitor 2C (CDKN2C), a regulator of G1 cell cycle progression through interaction with cyclin dependent kinases 4 and 6 (CDK4/6). In fact, overexpression of *CDKN2C* induces G1 cell cycle arrest in Huh-106 cells **(Fig. S3)**. To determine if this known function of CDKN2C is responsible for enhancing HBV infection, we performed functional studies using two clinical CDK4/6-specific small molecule inhibitors, Palbociclib [25] and LEE011 [26]. We first confirmed that Palbociclib treatment of Huh7 and Huh-106 cells at non-toxic concentrations induced G1 cell cycle arrest as demonstrated by the accumulation of cells in G1 phase **(Fig. S4a-b)**. We then determined HBV infection levels in Huh-106 cells treated with either of the inhibitors before and after HBV infection **(Fig. 7a, e)**.

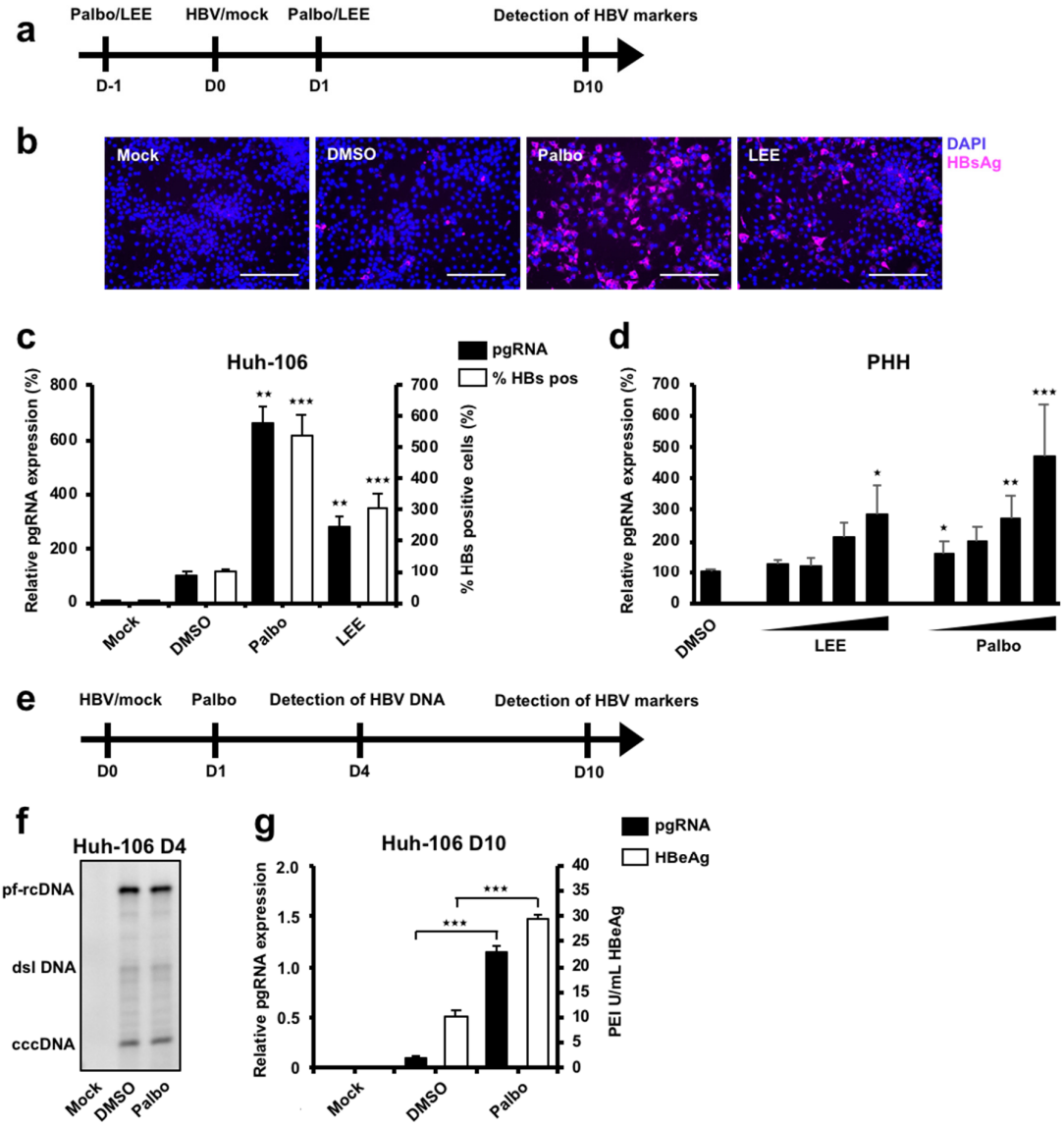

Figure 7. CDKN2C-mediated stimulation of HBV transcription is correlated with a cell cycle arrest. Effect of treatment with CDK4/6 inhibitors Palbociclib (Palbo) and LEE011 (LEE) on HBV infection. **a** Schematic workflow of experiments b-d. Detection of HBV markers in mock/HBV infected Huh-106 cells or PHH treated with DMSO or Palbo/LEE before (D-1 to D0) and after (D1 to D10) HBV infection 10dpi. **b-c** Detection of HBV markers 10 dpi in mock-treated of HBV infected Huh-106 cells treated with DMSO or 100 nM Palbo/LEE. **b** Detection of HBsAg by IF 10 dpi. Scale bars: 100 µm. **c** Quantification of HBV pgRNA by qRT-PCR (black). Quantification of HBsAg-positive cells by flow cytometric analysis (white). Results are expressed as means +/- SEM % HBV infection compared to DMSO (set to 100%) from 3 independent experiments (n=5) for pgRNA and from 4 independent experiments (n=12) for % HBs pos. **d** Quantification of HBV pgRNA10 dpi in mock-treated of HBV infected PHH treated with DMSO or 1-1000 nM Palbo/LEE. Results are expressed as means +/- SEM % relative pgRNA expression compared to DMSO (set to 100%) from 3 independent donors (n=9). **e** Schematic workflow of experiments f-h. Treatment of mock/HBV infected Huh-106 or HepG2-NTCP cells with 0 nM (DMSO) or 100 nM Palbociclib (Palbo) after HBV infection. **f** Detection of HBV DNA by Southern blot in Huh-106 cells 4 dpi. HBV pf-rcDNA, dsl DNA cccDNA bands are indicated. One representative experiment out of 3 is shown. Quantification of cccDNA bands in Figure S5b. **g** Detection of HBV markers in Huh-106 10 dpi. Quantification of HBV pgRNA by qRT-PCR (black) and of secreted HBeAg by CLIA (white). Results are expressed as means +/- SEM relative pgRNA expression (pgRNA) or as means +/- SEM PEI U/mL HBeAg from 3 independent experiments (n=9) for pgRNA and from 3 independent experiments (n=12) for HBeAg. ★ $p < 0.05$; ★★ $p < 0.01$; ★★★ $p < 0.001$

Visualization of intracellular HBsAg revealed a marked increase in HBV infection levels after treatment with Palbociclib or LEE011 **(Fig. 7b)**. Furthermore, quantification of HBV pgRNA and HBsAg-positive cells revealed a significant increase in HBV infection upon both Palbociclib and LEE011 treatment **(Fig. 7c).** Similar results were obtained in PHHs treated with CDK4/6 inhibitors at different concentrations (1, 10, 100 and 1,000 nM) confirming the proviral effect of Palbociclib and LEE011 **(Fig. 7d)**. To investigate whether Palbociclib-mediated enhancement of infection is dependent on HBV entry, we treated HBV infected Huh-106 cells with 100 nM Palbociclib following removal of the HBV inoculum **(Fig. 7e)**. As shown in **Figure 7f**, Palbociclib treatment did not affect HBV cccDNA levels, suggesting no effect on the viral entry steps including cccDNA formation. However, pgRNA and secreted HBeAg levels were significantly increased in Palbociclib-treated cells, indicating that CDKs are important for post-entry steps of the viral life cycle **(Fig. 7g)**. Collectively, our data identify *CDKN2C* as a previously undiscovered HBV host factor, most likely acting through inhibition of CDK4/6, triggering a cell cycle G1 arrest and enhancing HBV transcription **(Fig. 8)**.

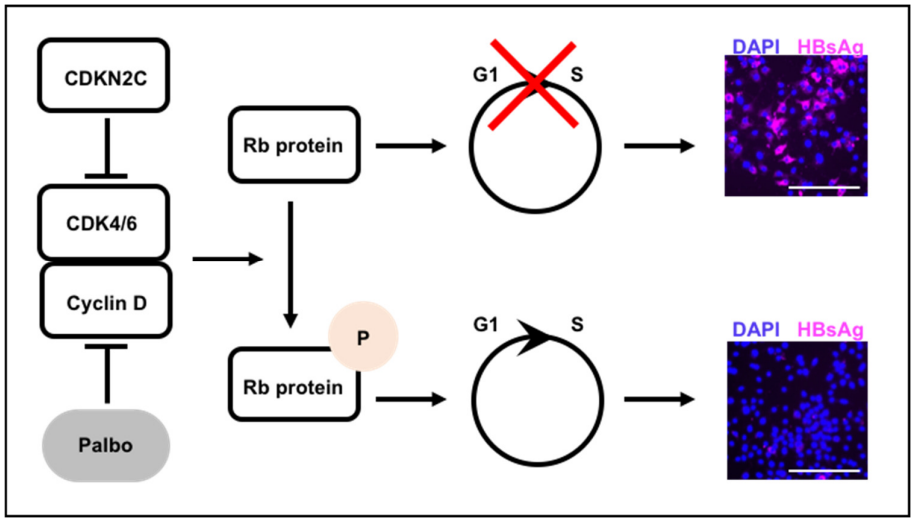

Figure 8. Schematic model of the effect of CDKN2C expression and Palbociclib (Palbo) treatment on HBV infection. CDKN2C and Palbociclib inhibit the CDK4/6 and Cyclin D mediated phosphorylation of Rb protein, leading to an accumulation of Rb protein in its unphosphorylated state. Unphosphorylated Rb protein induces a cell cycle G1 arrest resulting in increased HBV infection rates. Illustrative HBV infection pictures come from Fig. 7. Scale bars: 100 µm.

CDKN2C expression is associated with HBV-related chronic liver disease and survival in patients. To assess whether HBV infection directly affects *CDKN2C* expression, we infected PHH with HBV and evaluated *CDKN2C* gene expression. Interestingly, *CDKN2C* expression was upregulated upon HBV infection **(Fig. 9a)**. In line with this observation, the analysis of *CDKN2C* expression from patient liver tissues retrieved from the Gene Expression Omnibus database revealed an upregulation of *CDKN2C* in patients with active replication compared to patients with undetectable viral load and healthy patients **(Fig. 9b)**. Moreover, a correlation was observed between HBV viral load and *CDKN2C*

expression in liver tissues from 9 HBV-infected patients **(Fig. S6a)**. Finally, *CDKN2C* expression appeared to be modulated in different stages of HBV infection **(Fig. 9c)**.

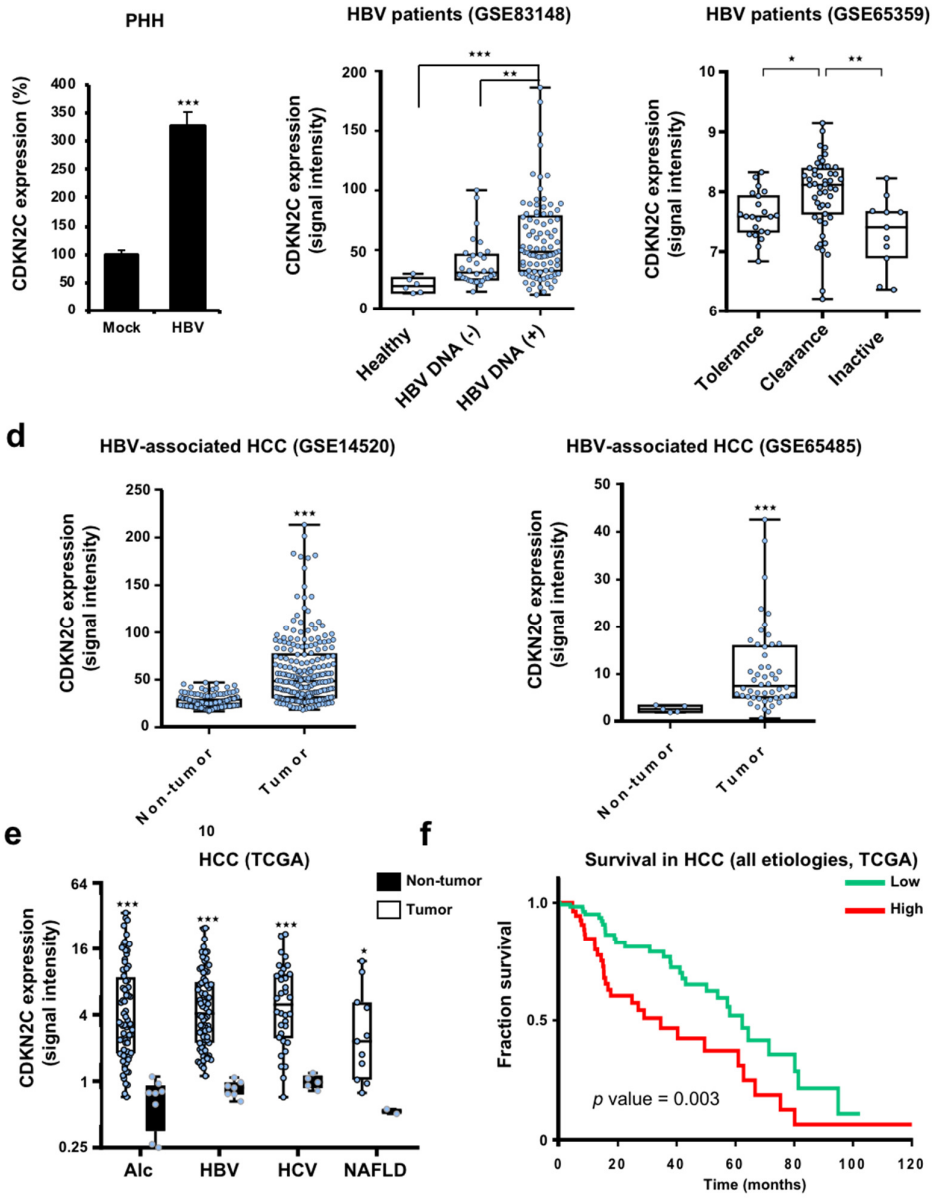

Figure 9. CDKN2C expression is associated with HBV infection, liver disease and survival in patients. **a** *CDKN2C* mRNA expression in HBV infected PHH from 3 different donors quantified by qRT-PCR. Results are expressed as means +/- SEM % relative CDKN2C expression compared to Mock (set to 100%) from 3 independent experiments (n=9). **b** *CDKN2C* expression in HBV-infected patients with undetectable (HBV DNA(-)) or detectable (HBV DNA(+)) HBV DNA compared to healthy patients(cohorts described in Methods). **c** *CDKN2C* expression in HBV-infected patients depending on the stage of virus infection (cohorts described in Methods). **d** *CDKN2C* expression in tumor and adjacent tissues in HCC patients from two independent cohorts (see Methods)**.** **e** *CDKN2C* expression in tumor and non-tumor (normal) liver tissue from HCV-infected patients, HBV-infected patients, patients with alcoholic liver disease (Alc) and patients with non-alcoholic fatty liver disease (NAFLD) extracted from TCGA database as described in Methods. **f** Survival analysis for HCC patients with low or high CDKN2C expression (cohort see Methods). ★ $p < 0.05$; ★★ $p < 0.01$; ★★★ $p < 0.001$.

Taken together, these data suggest that HBV infection modulates *CDKN2C* expression in chronically infected patients. To evaluate whether *CDKN2C* expression is associated with the development of virus induced liver disease, we analyzed *CDKN2C* expression in HBV patients with advanced liver disease and HCC. We first observed that patients with advanced fibrosis (F3) exhibit higher *CDKN2C* mRNA levels compared to patients with F1 or F2 fibrosis *CDKNC2* expression **(Fig. S6b)**. Moreover, *CDKN2C* expression was significantly higher in tumor tissues from HBV-derived HCC compared to adjacent tissue **(Fig. 9d)**. To assess the specificity of this correlation, we analyzed *CDKN2C* expression in HCC patients regardless the etiology. *CDKN2C* levels were markedly elevated in the tumor liver tissue of patients chronically infected with HCV or HBV and patients with alcoholic liver disease (Alc) or non-alcoholic fatty liver disease (NAFLD) as compared with non-tumor tissue **(Fig. 9e)**, suggesting that *CDKN2C* expression is upregulated in HCC in an etiology-independent manner. Finally, higher expression of *CDKN2C* in HCC patients was associated with significantly lower long-term overall survival **(Fig. 9f)**. Taken together, our data suggest that HBV infection modulates *CDKN2C* expression and that *CDKN2C* expression is associated with liver disease progression and poor survival.

DISCUSSION

Chronic hepatitis B is the most common form of severe viral hepatitis worldwide and a leading cause of hepatocellular carcinoma. To date, molecular details of HBV-host interactions are not fully understood. Using a functional genomics approach, we identified *CDKN2C* as a previously undiscovered host factor for HBV infection. The functional impact of this finding is confirmed by: (1) a marked increase or decrease in HBV infection after *CDKN2C* overexpression or knockout, respectively; (2) an increase in HBV markers following *CDKN2C* overexpression and (3) a significant pro-viral effect of CDK4/6 inhibitors correlated with cell cycle G1 arrest.

The role of *CDKN2C* as an HBV host factor was identified in a gain-of-function approach combining a cell-based model system [5] with a genome-scale ORF library [18]. The ability of our screen to discover HBV host factors promoting different steps of the HBV life cycle is supported by the identification of *HNF4A* in the primary screen. *HNF4A* encodes a liver-specific transcription factor, hepatocyte nuclear factor 4 (HNF4), that has been shown to be important for HBV replication by enhancing transcription from the promoters of HBV core [27], major surface antigen and large surface antigen [19]. Hence, HNF4A is likely to be a key transcription factor that regulates the HBV replication cycle and contributes to hepatotropism [28,29]. Notably, the hepatic leukemia factor (HLF), another transcription factor playing a role in the regulation of the HBV core promoter via interaction with sites other than HNF4 [20], scored with a Log2FC value of 1.49 just below our threshold for selection of candidate host factors. This supports the ability of our screening strategy to detect HBV host factors. Notably, the screen and validation experiments identified *ESRP1* as the top candidate HBV host factor. *ESRP1* encodes a splicing regulator especially involved in a large splicing program critical for the

development in mammals [30]. Importantly, ESRP1 protein expression was not detected in our systems, suggesting no or weak expression in hepatocytes. It is however likely that the splicing regulation of hepatocyte factors or the virus transcripts themselves (as it has been described, see [31]) explain the observed effect, even if not physiologically relevant.

While some pro- and anti-viral host factors have been described, many aspects of virus-host interactions remain poorly understood. Importantly, the correlation between HBV replication and cell cycle progression has long been a topic of investigation. For instance, in 1979, Aden *et al*. [32] demonstrated increased HBV antigen production in non-dividing tumor-derived cells with integrated HBV DNA sequences. Similar observations were made in an HBV-transfected hepatoma derived cell line [33]. Later, HBV replication was found to be inversely correlated to cellular DNA synthesis and to be enhanced in quiescent hepatocytes [34]. In fact, effective *in vitro* infection with HBV requires the presence of dimethyl sulfoxide (DMSO), known to enhance and prolong HBV infection by several mechanisms [16,35] and to decrease cell proliferation [36]. It has been previously described that HBV preferentially infects resting cells and that the virus is able to deregulate the infected cell cycle to favor its replication [37,38]. However, it remains unclear which host factors are involved in that process and whether cells arrested in G0/G1 or G2/M phase are more prone to HBV infection. Our data support the hypothesis that G1 cell cycle arrest is favorable for HBV replication and that CDKN2C is a key host factor mediating this virus-host interaction. A comparison of the proliferative ability of HepG2 cells with that of HepG2.2.15 (constitutively expressing HBV from integrated viral DNA), indicated that HBV induces a G1 phase arrest [37]. It has also been shown in PHH that HBV arrests infected cells in the G2/M phase and replicates more favorably during this cell cycle phase [38]. In eukaryotic cells, cyclin dependent kinases (CDKs) are key components of cell cycle regulation machinery. They form complexes with cyclins to control the transition through cell cycle phases and therefore allow cell division of healthy cells [39]. Interactions of HBV with certain CDKs have been shown. For example, CDK2 is involved in the phosphorylation of HBc and might be incorporated into viral capsids [40]. Moreover, inhibitors of CDKs have been shown to modulate HBV infection with different outcomes. On the one hand, knockout or inhibition of CDK2 enhances HBV replication by phosphorylation and deactivation of the host restriction factor SAMHD1 [41]. On the other hand, the CDK9-inhibitor FIT039 prevents replication of HBV and other DNA viruses and is under consideration as an antiviral candidate against HBV [42,43]. These data suggest a link between the CDK-cyclin pathway and the HBV life cycle. However, the key components and mechanisms remain unclear.

Here, we identify CDK4/6 as additional players in the regulation of HBV infection and show that CDK4/6 inhibitors are beneficial for the viral life cycle. CDK4/6 promote the cell cycle G1/S transition by phosphorylating the retinoblastoma (Rb) protein, the gene product of a tumor suppressor gene, and a central regulator of cell cycle progression [44]. The cyclin dependent kinase inhibitor 2C (CDKN2C) interacts with CDK4/6 to block cell cycle G1 progression via Rb protein phosphorylation [45]. Here, we find that CDKN2C overexpression in HBV infected hepatocytes enhances replication in both

NTCP-overexpressing hepatoma derived cell line and in PHH. Our results suggest an effect of CDKN2C on host cellular factors that are instrumental in HBV transcription. Overall, we identify CDKN2C as HBV host factor, acting through inhibition of CDK4/6 and prevention of G1 cell cycle progression.

The identification of *CDKN2C* as a host factor for HBV infection not only improves our understanding of the virus-host interactions, but also contributes to the explanation of the poor permissivity of NTCP-overexpressing Huh7 cells to this virus **(Fig. 1a)**. A detailed understanding of the molecular mechanisms underlying the varying susceptibility of currently available HBV infection models to HBV infection is crucial for the development of improved infectious cell culture models. The weak permissivity of Huh7-NTCP compared to HepG2-NTCP cells to HBV infection could be partially explained by lower *CDKN2C* expression levels **(Fig. 3d-e)**. However, the lower cccDNA levels in Huh7-NTCP compared to HepG2-NTCP are not caused by the lack of *CDKN2C* as its overexpression does not affect cccDNA formation **(Fig. 5d, e)**. Huh7-NTCP cells might therefore be useful to identify additional missing pro-viral host factors or restriction factors involved in cccDNA formation. Overall, a better understanding of virus-host interactions will facilitate the development of improved infectious model systems for drug discovery.

In patients, *CDKN2C* expression is accompanied with progression of HBV-associated fibrosis and is higher in HBV-associated cirrhotic and HCC tissues compared to tumor-adjacent tissues. In fact, *CDKN2C* is an etiology-independent marker of liver disease **(Fig. 9)** and part of a regulatory signature involved in liver regeneration [46]. This might explain the association of higher *CDKN2C* expression in HCC patients with lower long-term survival **(Fig. 9)**. While the upregulation of CDK inhibitors in cancer cells may appear counterintuitive, our consistent observations of an association between *CDKN2C* expression and progressive liver disease and hepatocarcinogenesis in several independent cohorts **(Fig. 9)** are in line with a recent observation that the expression of the tumor suppressor and *CDKN2C* effector Rb, which is regularly inactivated in human cancer, was inversely correlated with *CDKN2A*, another CDK4/6 inhibitor [47].Given the positive correlation of CDKN2C expression and survival, it is likely that CDKN2C rather has procarcinogenic properties than a tumor suppressive function in HCC **(Fig. 9)**. It is also interest to note that a recent study showed that pgRNA-positive HCCs were characterized by low levels of cell cycle and DNA repair markers and that pgRNA and cccDNA in tumors was correlated to the absence of tumorous microvascular invasion and to better patient survival [48]. Collectively, these observations provide a starting point for further studies investigating the functional role of CDKN2C is HBV-induced liver disease.

Interestingly, chemotherapeutic agents for cancer treatment cause immunosuppression and can lead to HBV reactivation in asymptomatic HBV carriers or patients with resolved HBV infection [49,50]. The list of chemotherapeutic agents associated with HBV reactivation is growing and includes anthracyclines, corticosteroids, platinum, vinca alkaloid, other small molecule agents, monoclonal antibodies and immune modulators [49]. Therefore, several professional societies, including AASLD and EASL, recommend HBV screening in all cancer patients undergoing chemotherapy and immunization

with HBV vaccine or prophylactic antiviral therapy [49]. However, awareness of this serious clinical problem is limited [49] and needs to be considered in clinical trials for new treatments. CDK-inhibiting drugs are a novel class of cancer therapeutics and three CDK4/6 inhibitors, palbociclib, ribociclib (LEE011), and abemaciclib are FDA and EMA approved for the treatment of advanced hormone receptor positive (HR+) breast cancer and in clinical trials for other non-breast malignancies [51]. Palbociclib (PD-0332991) is now under evaluation for the treatment of different Rb protein positive cancers [52–54] and most importantly in clinical trials for the treatment of HCC [55,56]. Chronic HBV infection accounts for approximately 50% of cases of HCC worldwide [1]. In this study, we show that CDK4/6 inhibition by palbociclib enhances HBV replication by arresting cells in the G0/G1 phase of the cell cycle. Therefore, caution is warranted in the use of such agents for HCC treatment. Our findings have important clinical implications as they indicate that there might be a potential risk of HBV reactivation during therapy with a CDK4/6 inhibitor, such as palbociclib, currently evaluated for HCC treatment.

Taken together, our gain-of-function screening approach allowed the identification of key HBV host factors, such as CDKN2C, with clinical implications in patients. Our data pave the way for the development of more permissive infection systems for the study of virus host interactions and the identification of previously undiscovered antiviral targets urgently needed for viral cure.

METHODS

Human subjects. Human serum from patients with chronic HBV/HDV infection followed at the Strasbourg University Hospitals, Strasbourg, France was obtained with informed consent. PHH were obtained from liver tissue from patients undergoing liver resection for liver metastasis at the Strasbourg University Hospitals with informed consent. Protocols were approved by the local Ethics Committee of the Strasbourg University Hospitals (CPP) and the Ministry of Higher Education and Research of France (DC-2016-2616). Human samples from HBV infected patients followed at the Chang Gung Memorial Hospital (Taipei, Taiwan) were obtained with informed consent. Protocols were approved by the local Ethics Committee (Institutional Review Board 102-3825C).

Cell lines and viruses. NTCP-overexpressing Huh-106 and HepG2-NTCP cell lines [5,57] as well as human embryonic kidney 293T (HEK 293T) [58] cell line have been described. PHH were isolated and cultured as described [58]. Recombinant HDV production [5,57] as well as purification of infectious HBV particles from the inducible human hepatoblastoma HepAD38 has been described [5,59,60].

Reagents and plasmids. DMSO, polybrene and PEG 8000 (polyethylene glycol) were obtained from Sigma-Aldrich (Merck). DNA and RNA transfection at the indicated concentrations was performed using CalPhos Mammalian Transfection Kit (Clonetech) and Lipofectamine RNAiMAX (Thermo Scientific) according to the manufacturer's instructions, respectively. The ORF-encoding lentivirus constructs for validations were obtained from the RNAi Platform, Broad Institute of MIT and

Harvard (Cambridge, MA, USA). Cell viability was assessed using PrestoBlue Cell Viability Reagent (Invitrogen) according to the manufacturer's instructions. Palbociclib and LEE011 (Ribociclib) were obtained from Synkinase and Sellekchem, respectively.

HBV binding. The binding of HBV virions at the cell surface was assessed as described [5]. In brief, cells were incubated with pretreated HBV in the presence of 4% PEG for 24 hours at 16 ºC. Unbound virions were removed by three washes with PBS, and cells and bound virions were lysed. HBV total DNA was quantified by qPCR using a standard curve generated from known HBV genome copies.

HBV and HDV infections. For HBV infection, NTCP-overexpressing cell lines and PHH were infected by recombinant HBV in presence of 4% of PEG-8000 (GEq 500 or 1000 per cell) [5,60]. After infection, Huh7-106 and HepG2-NTCP cells were washed and culture in PMM medium with 2% or 3.5% of DMSO respectively for 10 days. HBV infection was assessed 10 days post infection (dpi) by immunofluorescence (IF) using a mouse monoclonal antibody targeting HBsAg (Bio-Techne, clone 1044/329) and Alexa Fluor 647-labelled secondary antibody targeting mouse IgGs (Jackson Research). Cell nuclei were stained with DAPI. Fluorescent imaging was performed using an Axio Observer Z1 microscope (Carl Zeiss, Germany). Alternatively, cells were lysed and total RNA was extracted using the ReliaPrep RNA Miniprep Systems (Promega) and qRT-PCR quantification of HBV pregenomic RNA (pgRNA) was assessed as described [5,60,61]. HBsAg and hepatitis B e antigen (HBeAg) secretion were quantified by chemiluminescence immunoassay (CLIA, Autobio) following the manufacturer's instructions. Southern blot detection of HBV cccDNA was performed using digoxigenin (DIG)-labeled (Roche) specific probes as described [62]. Total DNA from HBV-infected cells was extracted using the Hirt method as described [63]. Specific DIG-labeled probes for the detection of HBV and mitochondrial probes for the detection of HBV and mitochondrial DNAs were synthetized using the PCR DIG Probe Synthesis Kit (Roche) and the primers as described [60]. HBV total RNAs were detected by Northern blot. Total RNA was purified using ReliaPrep RNA Miniprep Systems (Promega). 5 µg of total RNA was subjected to electrophoresis through a 2.2 M formaldehyde, 1% agarose gel and transferred to a nylon membrane positively charged (Roche). The membrane-bound RNA was hybridized to a ^{32}P-labeled RNA probe specific for detection of HBV RNA of 1200 to 1944 bp of viral genome (3.5 kbp to 2.1 kbp). For HDV infection, NTCP-overexpressing cell lines were infected with recombinant HDV (GEq 100 per cell) as described [5,60]. HDV infection was assessed 7 days after infection by IF using an antibody targeting the hepatitis delta antigen (HDAg) purified from serum of an HBV/HDV co-infected patient [64] and AF647-labeled secondary antibody targeting human IgGs (Jackson Research) as described [5,65].

Genome-scale lentiviral expression library and gain-of function screen. hORFeome V8.1 library (Broad Institute of MIT and Harvard, Cambridge, MA, USA) containing a pool of 16,172 clonal ORFs (mapping 13,833 human genes) was cloned into a pLX_TRC317 vector. The establishment of the

genome-scale ORFeome library has been described [18]. 30 million Huh-106 cells were transduced with the lentiviral ORFeome library in duplicate in the presence of polybrene (4 µg/ml). To avoid a cumulative effect of multiple ORFs, the LV volume was optimized to obtain 30% of transduced cells. Cells were then selected with puromycin (0.9 µg/ml) for 3 days. After amplification, transduced cells were infected with recombinant HBV at a MOI of 1000 GE/cell or mock-infected. At 10 days post infection, cells were stained for HBsAg expression and sorted by flow cytometry.

Gene expression analysis in HBV-infected Huh-106 after ORFeome transduction. HBV-infected cells were fixed in 100% methanol for at least 20 minutes at -20°C. Cells were then blocked and permeabilized using PBS 0.5% BSA and 0.05% saponin for 1 hour at RT. Cells were stained using an AF647-conjugated mouse monoclonal anti-HBsAg Ab (Bio-Techne, clone 1044/329) and resuspended in 0.5% BSA. HBsAg positive cells were sorted by Fluorescence Activating Cell Sorting (FACS) (BD FACSAria Flow Cytometer). 20 million cells were taken from HBV-infected sample as pre-sort control and total genomic DNA (gDNA) was extracted from cell pellets using Qiagen kits according to the manufacturer's protocol (Qiagen). Additionally, gDNA was extracted from 20 million HBV positive sorted cells from two biological replicates, named HBV sorted. Extracted DNA was used as a template for PCR to amplify the barcode sequences that accompany every ORF in the library. The unique barcode associated with each ORF construct was determined by Sanger sequencing in an arrayed collection of all the ORF constructs prior to pooling. PCR and sequencing were performed as previously described [66,67]. The details of the PCR primers and conditions can be found here: https://portals.broadinstitute.org/gpp/public/resources/protocols. Samples were sequenced on a HiSeq2000 (Illumina). The resulting reads were matched to their barcodes and their associated ORFs using PoolQ (see https://portals.broadinstitute.org/gpp/public/resources/protocols for more information on PoolQ). For analysis, the read counts were normalized to reads-per-million and then log2 transformed. The log2 fold-change (Log2FC) of each ORF was determined relative to the initial time point for each biological replicate. 90 hits with Log2FC values above the threshold set at 1.5 were selected as candidates.

Flow cytometry. For further flow cytometry analysis of HBV-infected cells, cells were fixed in 100% methanol for at least 20 minutes at -20 ºC. Cells were then blocked and permeabilized using PBS 1% FBS, 0.05% saponin for 30 min at RT. HBsAg was stained using a mouse monoclonal anti-HBsAg Ab (Bio-Techne, clone 1044/329) for 30 minutes at 4 ºC and then with an AF647-labelled secondary antibody targeting mouse IgGs (Jackson Research) for 30 minutes at 4 ºC. For flow cytometry analysis of DNA content, cells were fixed in ice-cold 75% ethanol in water for 30 minutes at 4ºC. Cells were washed and resuspend and incubated in PBS 50 µg/mL propidium iodide (Invitrogen) and 50 µg/mL Ribonuclease A (Sigma) for 30 min at RT. Cells were subsequently washed and resuspended in PBS 5 µM EDTA prior to sorting through a CytoFLEX flow cytometer system (Beckman Coulter).

Candidate selection from the primary screen. The impact of gene over-expression on HBV infection was defined by a specific enrichment in cDNA sequences in HBV-positive sorted cells compared to the pre-sort population. For hit selection, a functional threshold of Log2FC = 1.5 compared to pre-sorted cells was applied, leading to a total of 90 candidates **(Table S1, Figure 2c-d)**. As multiple ORF sequences for one given gene are sometimes present in the library, individual sequences were analyzed. Candidate genes with multiple associated ORFs were selected only if clones presented significant differences in their sequences (truncations in Cter or Nter of the proteins) or if a at least two identical ORFs exhibited a Log2FC > 1. Candidate gene expression in the liver was then assessed through the Human Protein Atlas (available from www.proteinatlas.org) [68]. Candidates with liver expression < 0.1 transcript per million (TPM) were removed from the analysis, leading to a final selection of 47 candidates **(Table S1)**. 47 ORF-containing lentiviruses were then obtained for individual validations, 35 of which met internal quality control based on lentiviral titration. In addition, lentiviruses encoding *GFP*, *KRT80* and *CPA1* cDNA sequences were obtained as negative controls from the primary screen.

Hit validation in Huh-106 cells and PHH. Individual ORFs were expressed from pLX-Blast-V5 (lentiviral) expression plasmids. Lentivirus particles were produced in HEK 293T cells by co-transfection of plasmids expressing the human immunodeficiency virus (HIV) gap-pol, the vesicular stomatitis virus glycoprotein (VSV-G) and the pLX-Blast-V5-ORF plasmids in the ratio of 10:3:10, using the CalPhos Mammalian Transfection kit as described [58]. Three days after transfection, supernatants were collected, pooled and clarified using 0.45 µm pore filters. Huh-106 were individually transduced with the 38 ORF-expressing lentivirus constructions and selected with 6 µg/mL of blasticidin 48 hours prior to HBV infection. HBV infection was assessed after ten days by quantification of HBeAg and HBsAg expression in the supernatant of infected cells as described above. For further validations, PHH and Huh-106 were transduced with individual ORF-containing lentivirus prior to HBV infection. Infection was assessed after ten days by Southern blot detection of HBV DNA, Northern blot and qRT-PCR detection of HBV RNAs, immunodetection of HBsAg, and quantification of HBeAg as described above.

CDKN2C HepG2-NTCP knockout generation. To generate clonal HepG2-NTCP CDKN2C knock-outs, the following primers corresponding to guide RNAs targeting CDKN2C exons were cloned into the Zhang lab generated Cas9 expressing pX458 plasmid (Addgene plasmid #48138):

guide 1;

Forward primer: CACCGACACCGCCTGTGATTTGGCC,

Reverse primer: AAACGGCCAAATCACAGGCGGTGTC.

guide 2;

Forward primer: CACCGCACAGGCGGTGTCCCCCTTA,

Reverse primer: AAACTAAGGGGGACACCGCCTGTGC.

pX458 plasmids encoding guide RNAs against CDKN2C were transfected into HepG2-NTCP cells using lipofectamine 3000 (Life technologies) according to manufactures guidelines. Transfected cells were single cell sorted based on + GFP expression into 96 well plates using the SONY SH800S cell sorter. Individual clones were expanded and four clonal cell lines were eventually selected for further characterization.

RNAi loss-of-function studies. ON-TARGETplus small interfering RNA (siRNA) pools (Dharmacon) targeting the transcripts of *CDKN2C* and *SLC10A1* (NTCP) were reverse-transfected into HepG2-NTCP cells with Lipofectamine RNAiMAX (Invitrogen) as described. RNA was purified from cells harvested 2 days after transfection, and gene expression was analyzed by qRT-PCR. For silencing of *CDKN2C* expression in PHH, PHH were transduced with lentiviral vectors containing *CDKN2C*-targeting shRNA (target sequence: GATGTTAACATCGAGGATAAT) or a scrambled shRNA control (target sequence: CCTAAGGTTAAGTCGCCCTCG) obtained from VectorBuilder. RNA was purified from PHH harvested 3 days after transduction, and gene expression was analyzed by qRT-PCR.

Comparative analysis of gene expression in Huh-106 and HepG2-NTCP cells. Huh-106 and HepG2-NTCP cells were lysed and total RNA from three biological replicates per cell line was then extracted as described above. Microarray analysis of gene expression in both cell lines was performed at the IGBMC GenomEast platform (Illkirch, France). Biotinylated single strand cDNA targets were prepared from 200 ng of total RNA using the Ambion WT Expression Kit (Cat # 4411974) and the Affymetrix GeneChip® WT Terminal Labeling Kit (Cat # 900671) according to Affymetrix recommendations. Following fragmentation and end-labeling, 3 µg of cDNAs were hybridized for 16 hours at 45°C on GeneChip® Human Gene 2.0 ST arrays (Affymetrix) interrogating over 40 0000 RefSeq transcripts and ~ 11000 LncRNAs. The chips were washed and stained in the GeneChip® Fluidics Station 450 (Affymetrix) and scanned with the GeneChip® Scanner 3000 7G (Affymetrix) at a resolution of 0.7 µm. Raw data (CEL Intensity files) were extracted from the scanned images using the Affymetrix GeneChip® Command Console (AGCC) version 4.1.2. CEL files were further processed with Affymetrix Expression Console software version 1.4.1 to calculate probe set signal intensities using Robust Multi-array Average (RMA) algorithms with default settings. Modulated molecular pathways were determined by using GSEA [69]. Individual differential gene expression of the selected candidates was evaluated through the Z score transformation. The dataset is publicly available in the NCBI Gene Expression Omnibus database (accession number GSE132638).

Analysis of gene expression using quantitative RT-PCR. RNA was extracted as described above and gene expression was assessed by qRT-PCR as described [60]. Gene expression was normalized to

GADPH expression. Primers and TaqMan® probes for *GAPDH*, *CDKN2C* and *SLC10A1* mRNA detection were obtained from ThermoFisher (TaqMan® Gene Expression Assays).

Protein expression. The expression of CDKN2C and *β*-tubulin was assessed by Western blot as described [5] using a monoclonal rabbit anti-CDKN2C antibody (anti-p18 INK4c, ab192239, Abcam), a rabbit polyclonal anti-*β*-tubulin antibody (GTX101279, Gentex) and a rabbit polyclonal anti-GAPDH (ab9485, Abcam), respectively. Peroxidase-AffiniPure Goat Anti-Rabbit IgG (H+L) (Jackson Research 111-035-144) was used as a secondary antibody. Protein expression was assessed using the ChemiDoc™ Imaging System (BioRad).

Analysis of nascent HBV RNA synthesis. Run-on assays were performed using Click-iT™ Nascent RNA Capture Kit from Thermofisher Scientific according to the manufacturer's instructions. HBV total and nascent RNA expression was assessed from HBV-infected Huh-106 cells overexpressing either *GFP* or *CDKN2C* by qRT-PCR four days after virus inoculation with 2 h of ethynyl uridine (EU) labeling. Actinomycin D (ActD, Sigma-Aldrich, Merck) was used as a negative control. Cells were pre-treated with ActD at 10 mg/mL for 20 min prior to EU labeling in presence of ActD. Specific primers and TaqMan® probes for total HBV RNAs (Pa03453406_s1) were purchased from Life Technologies. HBV RNA levels were normalized to GUSB expression using primers and TaqMan® probes from Life Technologies (Hs99999908_m1).

Analysis of CDKN2C expression in patients. For the analysis of *CDKN2C* mRNA expression in patients, *CDKN2C* mRNA expression was assessed in control healthy patients (n=6), HBV-infected patients with no detectable HBV DNA (n=32), HBV-infected patients with detectable HBV DNA (n=90) from GSE83148 [70]. Similarly, CDKN2C mRNA expression was assessed in HBV patients at different stages of virus infection including immune tolerant phase (n=22), immune clearance phase (n=50) and inactive carrier phase (n=11) from GSE65359. Alternatively, total RNA was extracted from liver tissue of 9 HBV-infected patients by using High Pure RNA Paraffin kit (Roche) according to the manufacturer's instruction, and gene expression analysis was performed by RNA-seq as previously reported [71]. To analyze the correlation between *CDKN2C* expression and the progression of liver disease in HBV-infected patients, *CDKN2C* mRNA expression was assessed in HBV-related liver fibrosis patients of different stages from GSE84044 [72] (n=37 score 0, n=33 score 1, n=34 score 2, n=15 score 3). Finally, *CDKN2C* expression in HBV-induced HCC patients was assessed from GSE65485 [73] (n=50 tumor tissue, n=5 non-tumor tissue) and from GSE14520 [74] (n=221 tumor tissue, n=199 non-tumor tissue). *CDKN2C* mRNA expression is shown as signal intensity values. For survival analysis, liver expression level of *CDKN2C* and survival data were derived from The Cancer Genome Atlas (TCGA, https://www.cancer.gov/about-nci/organization/ccg/research/structural-genomics/tcga) TCGA-LIHC database [75]. To analyze *CDKN2C* expression in liver tissue of patients with chronic liver disease, FPKM

values and clinical data were retrieved from TCGA. This data set includes samples from HCV-infected patients (34 tumor samples including 5 paired tumor/non-tumor samples), HBV-infected patients (76 tumor samples including 7 paired tumor/non-tumor samples), patients with alcoholic liver disease (ALD) (72 tumor samples including 8 paired tumor/non-tumor samples) and patients with non-alcoholic fatty liver disease (NAFLD) (11 tumor samples including 2 paired tumor/non-tumor samples).

Statistical information. For in vitro experiments, statistical analyses were performed using a two-tailed Mann-Whitney U test; $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***) were considered statistically significant. Significant p-values are indicated by asterisks in the individual figures and figure legends. The number of tested replicates is indicated in the figure legends (n). For microarray analyses, two-tailed unpaired Student's t-test was performed by comparing the values from three biological replicates per cell line. $p < 0.01$ was considered statistically significant. For clinical data, Mann-Whitney U test was used when comparing two groups (Fig. 9d-e). For multiple group comparison (Fig. 9b-c), Kruskal–Wallis H test was used. Correlation between *CDKN2C* expression and HBV viral load in patients was assessed using Spearman's rank correlation coefficient (Spearman's rho). Survival functions depending on *CDKN2C* expression were obtained using the Kaplan–Meier estimator. p-value was calculated using log-rank test for comparisons of Kaplan-Meier survival. $p < 0.01$ was considered statistically significant.

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Author contributions. ERV and TFB designed and supervised research. TFB initiated the study. CCC, GSC, DR, ERV and TFB designed the gain-of-function screen. LH, CCC, FP, GSC and DR performed and analyzed the screen. CE, LH and CCC performed the validation experiments. CE, LH, CCC, KM, JLup, MN, CSc, CSu, ERV and TFB analyzed the validation data. HES and ERV analyzed the microarray data. HES, FJ, NF, SYH, YH and ERV analyzed the clinical data. CSu performed the HBV Northern blots. CP and JLuc performed and analyzed the run-on data. PP and EF provided human hepatocytes. CE, LH, ERV and TFB wrote the manuscript. CE and LH contributed equally to this work as first authors. ERV and TFB contributed equally to this work as senior authors.

Competing interests. The authors have no competing interests to disclose.

Materials & Correspondence. Material and correspondence requests should be addressed to Prof. Thomas F. Baumert (thomas.baumert@unistra.fr) and Dr Eloi R. Verrier (e.verrier@unistra.fr).

Data availability. The dataset generated in this study, including the results from the gain-of-function primary screen, are available within Supplementary information. The microarray dataset is publicly available in the NCBI Gene Expression Omnibus database (accession number GSE132638). The rest of the data is available through the corresponding authors upon reasonable request.

SUPPLEMENTARY FIGURES

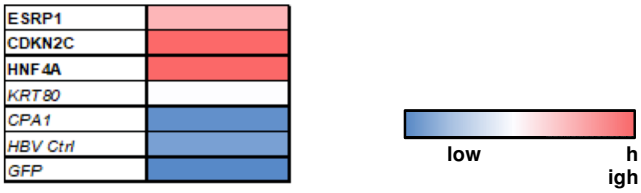

Figure S1. Heatmap of candidate validation by quantification of pgRNA (related to Figure 3a). Huh-106 cells were transduced with the indicated ORF and infected with HBV. HBV infection was assessed at 10 dpi by qRT-PCR quantification of pgRNA. Results are expressed as means relative pgRNA expression from 2 independent experiments (n=4).

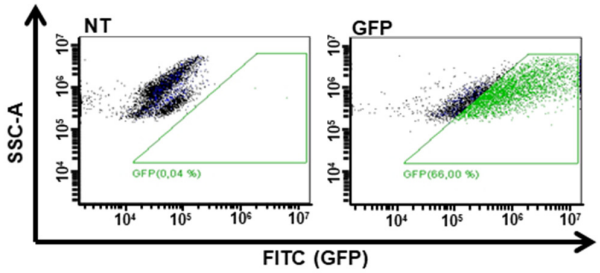

Figure S2. GFP control vector (related to Figure 5b). Expression of GFP in Huh-106 transduced with lentivirus for GFP overexpression (GFP) or non-transduced (NT). Quantification of GFP-expressing cell population by flow cytometric analysis in HBV-infected cells 10 dpi.

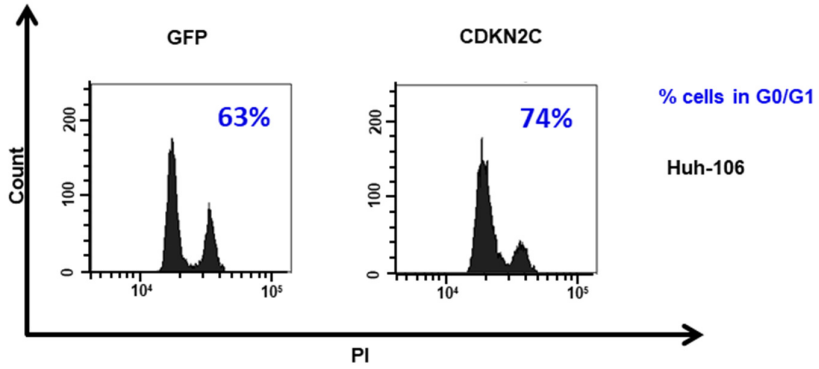

Figure S3. Effect of *CDKN2C* overexpression on cell cycle (related to Figure 7). Overexpression of GFP or CDKN2C in Huh-106 cells and cultivation in Williams Culture and 2% DMSO. Analysis of cell cycle using propidium iodide and flow cytometry after 3 days. One representative experiment out of 3 is shown (n=4).

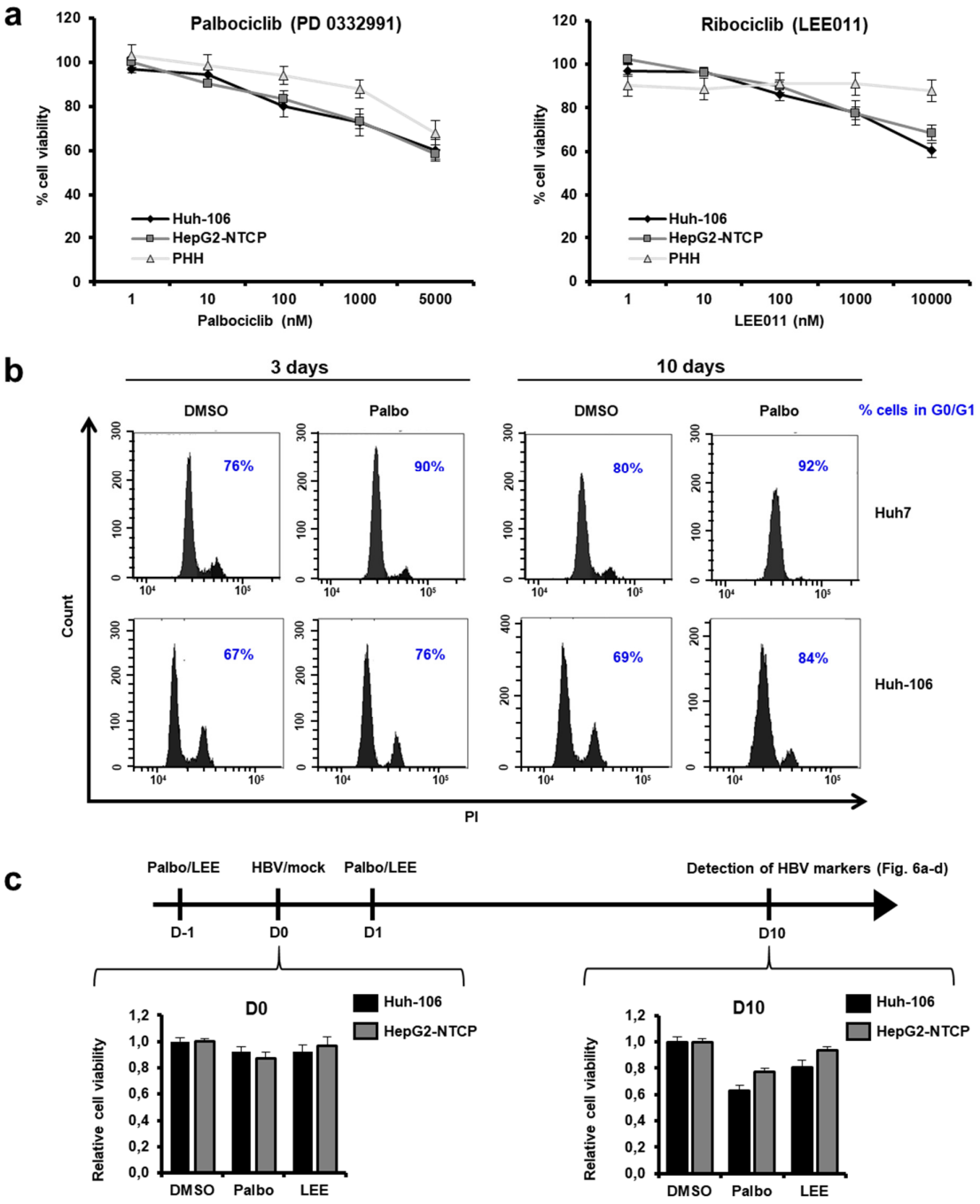

Figure S4. Effect of treatment with CDK4/6 inhibitors Palbociclib (Palbo) and LEE011 on cell viability and cell cycle (related to Figure 7). a Cell viability (prestoBlue) assay for Huh-106 cells, HepG2-NTCP cells and PHH from 3 different donors treated with different concentrations of Palbociclib (Palbo) or LEE011 for 3 days in 0,1% DMSO. Results are expressed as means +/- SEM % cell viability compared to 0,1% DMSO treated cells (set to 100%) from 3 independent experiments (n=10, n=12 for PHH). b Treatment of Huh7 cells and Huh-106 cells without (DMSO) or with 100 nM Palbociclib (Palbo) in Williams Culture and 2% DMSO. Analysis of cell cycle using propidium iodide and flow cytometry after 3 days and 10 days. One representative experiment is shown (n=4). c Cell viability (prestoBlue) assay for Huh-106 cells, HepG2-NTCP cells treated with 100 nM Palbociclib (Palbo) or 100 nM LEE011 (LEE) for 1 day (D0) or for 1 day before HBV infection and 9 days after removal of HBV inoculum (D10). Results are expressed as means +/- SEM % cell viability compared to DMSO-treated cells (DMSO – set 1) from 3 independent experiments (n=12) for D0 or as means +/- SEM rel. cell viability compared to untreated HBV-infected cells (DMSO - set to 1) from 3 independent experiments (n=9).

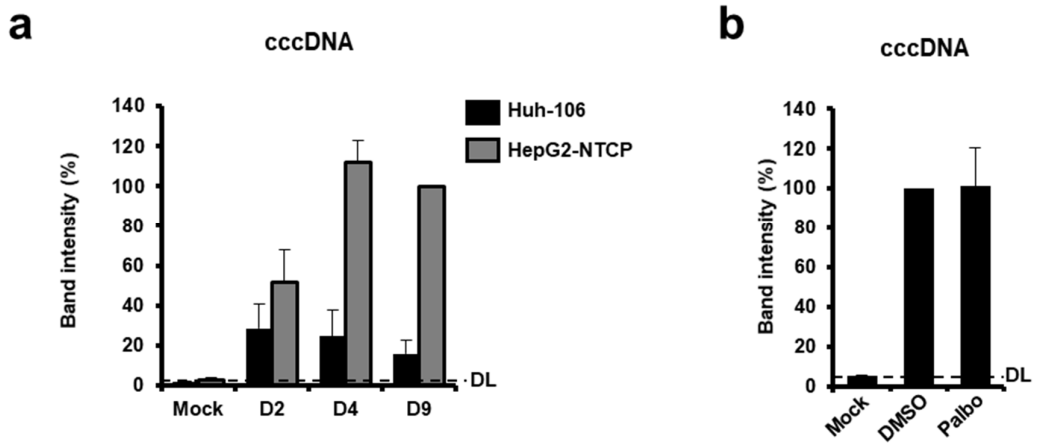

Figure S5. Quantification of Southern Blot cccDNA band using Image Lab Version 5.2.1 (related to (a) Figure 1e and (b) 7f). a Quantification of cccDNA bands in Mock or HBV infected Huh-106 and HepG2-NTCP cells 2 (D2), 4 (D4) or 9 (Mock, D9) days post HBV infection. Results are expressed as means +/- SEM % band intensity compared to HBV-infected HepG2-NTCP D9 (set to 100%) from 3 independent experiments. Dashed line indicates the detection limit (DL). Related to Figure 1e. b Quantification of cccDNA bands in Mock or HBV infected Huh-106 cells treated with DMSO or 100 nM Palbociclib 4dpi. Results are expressed as means +/- SEM % band intensity compared to DMSO (set to 100%) from 3 independent experiments. Dashed line indicates the detection limit (DL). Related to Figure 7f.

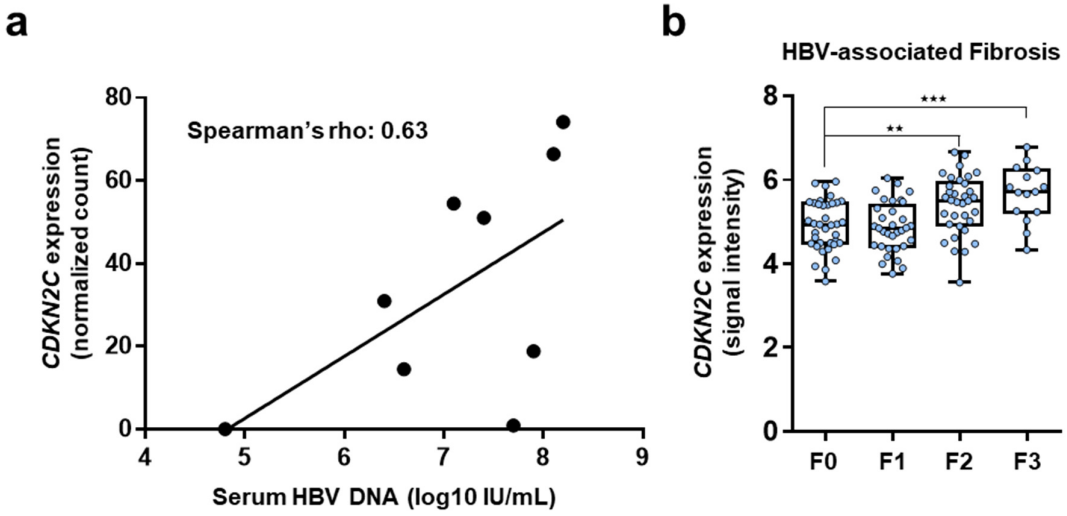

Figure S6. CDKN2C expression is associated with HBV infection and survival in patients (related to Figure 9). a Correlation between HBV DNA and *CDKN2C* expression in 9 HBV-infected patients. Serum HBV-DNA levels (log10 IU/mL) and liver tissue CDKN2C expressions showed a trend toward a positive correlation (Spearman's rho = 0.63, p = 0.076). **b** CDKN2C expression in fibrosis patients in different stages F0-F3. ★★ $p < 0.01$; ★★★ $p < 0.001$

SUPPLEMENTARY TABLE

Table S1. 90 candidates identified in the primary screen.

Gene Symbol	AVERAGE LFC HBV presort	Clone filter	Expression in the liver (HPA - TPM)
HIST1H4B	1,57	X	56.8 (FANTOM5)*
ASGR1	1,6	X	236,1
SDC1	1,59	X	144,7
TOB1	1,56	X	58,5
HLA-DRB3	1,9	X	49,5
USO1	1,93	X	40,1
CLEC1B	2,2	X	33,7
DEK	1,51	X	31,6
FGFR1OP	1,68	X	18,7
MAPK1IP1L	2,24	X	14
U2AF1	1,77	X	13,9
HCCS	2,37	X	13,6
ASMTL	1,55	X	12,9
TRIM24	1,69	X	11,9
MFSD1	1,5	X	11,5
NOTCH2	2,19	X	9,9
NGEF	2,25	X	9
TMEM38B	2,47	X	7,9
KIAA0232	2,69	X	7,8
LAMC1	1,64	X	7,1
HNF4A	1,52	X	6,9
ZNF326	1,52	X	6,9
PPP2R5D	2,49	X	6,7
KLHL15	1,51	X	6,6
VPS45	1,75	X	6,2
GRK5	1,63	X	6
CREB1	1,52	X	5,7
WWP2	1,9	X	5,6
ENTPD4	1,67	X	5,1
TCF3	1,59	X	4,6
PRKD2	2,06	X	3,7
ABHD8	1,58	X	3,2
CDKN2C	1,63	X	2,7
TOMM40L	1,57	X	2,6

TTLL3	2,23	X	2,6
SPATA24	1,68	X	2,5
ZNF37A	1,69	X	2,5
ZNF354A	2,07	X	1,7
RUFY2	2,24	X	1,2
ARPP21	2,18	X	1,1
KDF1	2	X	1,1
SEMA4A	1,67	X	1,1
LIPE	1,51	X	0,4
ESRP1	1,69	X	0,2
GPR123	2,04	X	0,1
GPR27	1,69	X	0,1
SLC13A2	2,45	X	0,1
ATP6V0A4	1,61	X	0
AVP	1,5	X	0
CLCA4	1,64	X	0
CREG2	1,53	X	0
DEFB121	1,75	X	0
FAM133A	1,59	X	0
LILRA1	1,61	X	0
OR2G3	1,77	X	0
OR51M1	1,72	X	0
OR5AP2	1,77	X	0
WBSCR28	1,69	X	0
ACVR1B	1,66		
ADCK2	1,54		
ADRBK1	1,87		
CCDC96	2,08		
CLK3	1,74		
CSF1R	1,93		
FGFR3	1,59		
FUK	2,32		
IRAK3	1,58		
JAK3	2,12		
LAG3	2,05		
MAP3K9	2,4		
MASTL	1,89		
NAPSA	1,6		
NEK8	2,01		
NME3	1,7		

PCSK9	1,56		
PDK1	2,08		
PIP5K1A	1,61		
PLCG2	2,13		
PSMB1	1,94		
PTGER1	2,09		
PTK2B	2,99		
RPL17	1,87		
SRC	1,81		
STK24	1,61		
STK35	1,87		
TEX264	1,5		
TLK2	1,59		
ULK4	1,74		
WDR1	1,73		
XRN2	1,78		
Controls			
KRT80	0,5		
CPA1	0,5		

* FANTOM5 data were used given the apparent problem with HPA RNAseq data for the gene in all tissues

DISCUSSION

In the framework of this thesis, *CDKN2C* was identified as proviral host factor implicated in HBV infection via its function as cell cycle regulator. The effect of cell cycle G1 arrest on HBV replication was confirmed by: (1) a significant increase/decrease in HBV infection after *CDKN2C* overexpression/knockout, respectively; (2) *CDKN2C* expression levels correlating to the susceptibility to HBV infection; and (3) a striking proviral effect of clinical CDK4/6 inhibitors. These results shed new light on the mutual impact between HBV infection and host cell cycle and highlight the importance of a broad knowledge about virus host interactions in view of developing improved model systems and novel HTA-based therapeutic strategies.

It is widely accepted that HBV preferentially infects and replicates in non-dividing cells (Aden *et al.*, 1979; Sureau *et al.*, 1986; Ozer *et al.*, 1996; Huang *et al.*, 2004). In addition, HBV may deregulate the cell cycle control to render the cellular environment more favorable for HBV infection (Chin *et al.*, 2010; Gearhart and Bouchard, 2011; Xia *et al.*, 2018). Recently, it was shown in engrafted PHHs in mice, that hepatocytes displaying high HBV replication levels have a lower proliferating capacity than cells negative for replication markers (Allweiss *et al.*, 2018). In this context, this study suggests that cell cycle arrest in the G1 phase renders cells more susceptible to HBV infection (see Eller, Heydmann *et al.* Figure 7 in the Results section). This is corroborated by increased HBV infection in cells overexpressing *CDKN2C*, and in cells treated with Palbociclib (PD-0332991), a clinical CDK4/6 inhibitor, both inducing cell cycle G1 arrest (Jeffrey, Tong and Pavletich, 2000; Fry *et al.*, 2004). The common mechanism of action of *CDKN2C* and Palbociclib with respect to HBV infection is confirmed by unchanged HBV replication in *CDKN2C*-expressing HepG2-NTCP cells irrespective of Palbociclib treatment. The herein identified effect of cell cycle arrest by *CDKN2C* expression or Palbociclib treatment on HBV infection can be further characterized by localizing its impact to a specific event in the HBV life cycle. Considering that Palbociclib treatment solely after removal of the HBV inoculum significantly and markedly increases HBV infection, G1 arrest may not promote viral entry, but rather a later event of the HBV life cycle. The step of the HBV life cycle affected by *CDKN2C*- or Palbociclib-induced G1 phase arrest can be further localized to an event occurring after cccDNA formation, as cccDNA levels remain unchanged independent of *CDKN2C* expression and Palbociclib treatment. Considering increased HBV RNA levels in the presence of *CDKN2C*, G1 phase arrest might have a positive effect on HBV RNA transcription or stability. The analysis of neosynthesized RNA in cells differentially expressing *CDKN2C* or in the presence and absence of Palbociclib using labelled uridine may give hints on whether HBV RNA formation is modulated. During co-evolution with their host cells, many viruses have found a way to subvert the cell cycle for the establishment of a cellular environment favorable to the viral life cycle (Fan, Sanyal and Bruzzone, 2018). In the case of oncogenic viruses, this often contributes to cell transformation and carcinogenesis (Chang *et al.*, 2017). There exist different

mechanisms by which viruses reshape the cell cycle, including the manipulation of cell cycle regulating proteins via functional inhibition or expression control (Fan, Sanyal and Bruzzone, 2018). Notably, *CDKN2C* expression has been shown to be modified by several viruses. For instance, the Tax oncoprotein of the human T-cell leukemia virus type 1 (HTLV-1) promotes the progression through S phase via different mechanisms, including the transcriptional repression of *CDKN2C*, the functional inhibition of other members of the INK4 family of CDK inhibitors (CKIs), and the activation of Cyclin D-CDK complexes in a CKI-independent manner (Suzuki and Kitao, 1996; Low *et al.*, 1997; Neuveut *et al.*, 1998). In contrast, the viral oncogenic E6 protein of the human papillomavirus (HPV) was shown to upregulate *CDKN2C* expression (X. Wang *et al.*, 2011). At first glance, it seems puzzling that both, repression and upregulation of *CDKN2C* expression, are thought to be implicated in cellular transformation and carcinogenesis by oncogenic viruses. In accordance with unleashing cell proliferation during carcinogenesis, repression of *CDKN2C* expression by HTLV-1 Tax leads to increased CDK activity and cell cycle progression. The other way around, upregulation of *CDKN2C* expression by HPV E6 increases CDK inhibition and induces cell cycle arrest via Rb protein. However, HPV E7 protein was shown to degrade the Rb protein, impairing the inhibitory function of CDKN2C (Gonzalez *et al.*, 2001). In this study, *CDKN2C* was shown to be overexpressed in HBV infected PHH. HBV-induced overexpression of *CDKN2C* may be seen in the light of a herein suggested proviral effect of G1 cell cycle arrest on HBV infection. While the induction of cell cycle arrest by HBV has already been shown, it remains controversial in which phase the arrest occurs, as divergent studies suggest G1 or G2 phase (Park *et al.*, 2000; Gearhart and Bouchard, 2010a; Xia *et al.*, 2018). For instance, a study in PHH suggests HBV to arrest infected cells in the G2/M phase and to replicate more favorably in this cell cycle phase (Xia *et al.*, 2018). However, the comparison of the proliferation ability of HepG2 and HepG2.2.15 with an integrated HBV genome indicated that HBV induces a G1 phase arrest (T. Wang *et al.*, 2011). Furthermore, HBx has been shown to induce G1 phase arrest in human hepatoma cells and to induce quiescent hepatocytes to exit G0 to remain in G1 phase, which might be beneficial for HBV replication (Park *et al.*, 2000; Gearhart and Bouchard, 2010a). Although HBx was further shown to block the G1/S transition of the hepatocyte cell-cycle in HBx transgenic mice, the effect of HBx on the cell cycle remains controversial, as HBx has also been shown to stimulate cell cycle progression and entry into S phase, which would correlate with its role in carcinogenesis (Benn and Schneider, 1995; Wu *et al.*, 2006). In summary, this study supports the hypothesis that HBV preferentially infects non-dividing cells, potentially arrested in the G1 phase of the cell cycle and induces overexpression of *CDKN2C*, a proviral host factor, to produce a cellular environment that promotes HBV replication. Whether *CDKN2C* expression in HBV infected hepatocytes contributes to hepatocarcinogenesis remains to be investigated.

The identification of HBV host interactions is of great interest because it is closely linked to the development of improved infectious model systems for HBV research. Indeed, the ignorance of essential

proviral host factors and the lack of suitable model systems have mutually hampered advances in the respectively other field. The fact that the discovery of host factors can trigger innovation of model systems has been manifested in the case of NTCP. Its identification as HBV/HDV receptor has paved the way for major progresses in the fields of infectious model systems and therapeutic strategies (Yan *et al.*, 2012; Ni *et al.*, 2014). It has been established that there exist model systems which can be infected with HBV, though under unphysiological conditions including a very high MOI and the presence of PEG and DMSO in the media (Verrier *et al.*, 2016b). Although progress is made, the tools for robust and physiological HBV infection *in cellulo* are not yet achieved. For the development of improved models, it is important to comprehend the limitations of currently available systems. Comparing the ability of HepG2-NTCP and Huh7-NTCP cell lines to support HBV and HDV infection, it has been recognized that, despite permitting HDV infection, Huh7-NTCP cells are very poorly susceptible to HBV infection (Yan *et al.*, 2012; Ni *et al.*, 2014). However, the mechanisms underlying the restriction of HBV infection in Huh7-NTCP cells have not been investigated (Ni *et al.*, 2014). Here, the differences between HepG2-NTCP and Huh-106 (a Huh7-derived cell line constitutively expressing hNTCP) cells with respect to HBV infection have been further characterized by evaluating virion binding, cccDNA formation, pgRNA levels and concentrations of secreted viral antigens during the course of infection. In accordance with a previous study, cccDNA levels in infected HepG2-NTCP cells primarily increased within the first two days of infection and pgRNA and HBeAg levels started increasing markedly after four days of infection (Qi *et al.*, 2016). Despite comparable virion binding and cccDNA formation at a similar pace in both cell lines, absolute cccDNA formation was strongly reduced in Huh-106 cells. Therefore, the restriction of HBV infection in Huh-106 cells was pinned down to a step between viral entry and cccDNA formation, indicating that host functions modulating early steps of the viral life cycle are differentially expressed. However, the question remains, whether proviral host factors promoting HBV infection are missing in Huh-106 cells, or restrictive host factors are overexpressed in this cell line. In an elegant study aiming at clarifying whether restriction of HBV infection in NTCP-overexpressing cells is caused by the lack of a dependency factor or the activity of a restriction factor, human NTCP (hNTCP)-overexpressing mouse and human cell lines were fused with replication-competent HepG2 cells (Lempp *et al.*, 2016). Heterokaryotic cells supported HBV infection, suggesting that the studied non-susceptible cell lines require supplementation with a proviral host factor and are not limited by a restriction factor (Lempp *et al.*, 2016). To address the same question concerning Huh-106 cells, a similar approach may be applied by fusion of Huh-106 cells with HepG2 cells and subsequent HBV infection. Unexpectedly, the screen described here did not reveal a host factor involved in an early step of the life cycle, as demonstrated by unchanged cccDNA levels in HBV infected Huh-106 cells irrespective of *CDKN2C*, *HNF4A*, or *ESRP1* overexpression. Revealing limitations of the screening strategy applied here, this may be interpreted in two different ways. Either one or more host restriction factor(s) may impede cccDNA formation in Huh-106 cells, or the missing proviral host function may not be mediated by a single factor but rather by a complex of which several parts are lacking in Huh-

106 cells. While the screen failed to identify host functions involved in the establishment of infection, it revealed that overexpression of *CDKN2C*, *HNF4A*, or *ESRP1* promotes HBV replication in a step post cccDNA formation. This indicates that later phases of the viral life cycle like cccDNA-mediated transcription and translation of viral proteins may not be efficient in naïve Huh-106 cells neither. A useful tool for a better understanding of mechanisms underlying the susceptibility of cell culture models to HBV infection is comparative gene expression profiling. Notably, comparison of gene expression profiles between restricted HepaRG cells and their susceptible differentiated counterparts was used to confirm NTCP as receptor for HBV/HDV infection (Ni *et al.*, 2014). Here, gene expression profiles of permissive HepG2-NTCP and restricted Huh-106 cells were compared. Importantly, the overexpression of *CDKN2C* in HepG2-NTCP cells as compared to Huh-106 cells correlates with the susceptibility to HBV infection and further corroborates the role of *CDKN2C* as proviral host factor for HBV infection. Overall, Huh-106 cells were shown to be restricted to HBV infection at different steps of the life cycle and this restriction can be attenuated by the supplementation with a lacking proviral host factor. Towards more robustly infectious cell culture systems, several complementary proviral host factors could be overexpressed in Huh-106 or HepG2-NTCP cells in order to render these cells more susceptible to HBV infection. For this, the identification of further proviral host factors can only be beneficial.

Here, *CDKN2C* was identified as host factor for HBV infection in Huh-106 cells, contributing to a better global understanding of HBV host interactions. However, many other host functions involved in the HBV life cycle still remain obscure. This study should, therefore, also be considered as a proof of concept for the successful identification of host functions modulating HBV infection using functional genomics. As discussed above, the presence of (a) restriction factor(s) in Huh-106 cells may contribute to the poor susceptibility to HBV infection. In this case, an inverse screening strategy using the CRISPR/Cas9 system in a loss-of-function approach could be applied (Shalem *et al.*, 2014). Such a screen could include delivery of a sgRNA library via lentiviral vectors into Cas9-overexpressing Huh-106 (Huh-106-Cas9) cells, followed by HBV infection, sorting of HBsAg positive cells, DNA extraction and deconvolution as performed in this study. Alternative approaches for the identification of further host factors may exploit other cell lines. Interestingly, cells from different tissue and species origins were shown to support HDV but not HBV infection, when reconstituted with hNTCP (Yan *et al.*, 2013; Li *et al.*, 2014; Lempp *et al.*, 2016). These include the mouse hepatoma cell lines Hepa1-6 and Hep56.1D, the human cervical cancer cell line HeLa, and primary mouse hepatocyte (Yan *et al.*, 2013; Li *et al.*, 2014). A genome-wide gain-of-function screen for the identification of proviral host factors could be envisioned in a hNTCP-overexpressing mouse hepatoma cell line. Importantly, there is evidence that HBV infection in hNTCP-Hepa1-6 and hNTCP-Hep56.1D is restricted by the lack of a proviral host factor (Lempp *et al.*, 2016). The identification of host factors enabling HBV infection in hNTCP-complemented mouse hepatoma cell lines could be of particular interest considering the absence of immunocompetent small-animal models supporting HBV infection. In line with HBV transgenic mice

supporting HBV replication and secretion, the block of HBV infection in hNTCP-Hepa1-6 and hNTCP-Hep56.1D was shown to occur upstream of cccDNA formation (Guidotti *et al.*, 1995; Lempp *et al.*, 2016). This indicates that one or more host dependency factor(s) implicated in the HBV life cycle between entry and cccDNA-mediated transcription could be identified in a functional genomics screen using these cell lines. Being the template for transcription of all viral RNAs, cccDNA is a key intermediate in the HBV life cycle and is also considered responsible for viral persistence (Rehermann *et al.*, 1996). However, currently available antivirals do not target cccDNA but life cycle events downstream of cccDNA formation. Novel therapeutic strategies allowing the prevention of cccDNA formation, its inhibition, or its degradation may therefore be required to achieve viral cure. Host factors involved in these processes may represent suitable targets for the development of HTAs. Although host functions are known to mediate rcDNA to cccDNA conversion, and the host DNA damage response is thought to be involved, the key players and mechanisms remain unclear (Schreiner and Nassal, 2017). A major goal of current virologic HBV research is therefore the identification of host dependency factors modulating cccDNA formation and preceding steps. In a screen pursuing this goal, a suitable readout would be intracellular cccDNA levels. This requires specific and sensitive detection of cccDNA, which is tricky due to low cccDNA copy numbers in infected cells, rarely rising above one copy per infected hepatocyte in chronically infected patients (Werle–Lapostolle *et al.*, 2004b). Because cccDNA detection via Southern blot is not adapted for high-throughput methods, and the specific detection of cccDNA by qPCR has been hampered by missing specificity and detection of excess input HBV DNA, new qPCR protocols are being developed (X. Li *et al.*, 2017; Qu *et al.*, 2018). Alternatively, surrogate models can be used that are based on higher cccDNA levels in ducks infected with DHBV and woodchucks infected with, which can reach copy numbers of over 50 copies per cell (Zhu *et al.*, 2001; Zhang *et al.*, 2003; Schreiner and Nassal, 2017). Such models are often based on the stable integration of DHBV or HBV into hepatoma derived cell lines and a tetracycline (Tet)-regulated promoter which allows initiation of virus replication and cccDNA formation upon Tet withdrawal (Cai *et al.*, 2012; Königer *et al.*, 2014; Long *et al.*, 2017). As host factors involved in cccDNA formation have already been identified using stable DHBV TetOFF cell lines, further screens in such models could be envisioned (Königer *et al.*, 2014; Long *et al.*, 2017). For instance, knockdown or knockout of genes belonging to the DNA repair machinery might be an interesting approach (Schreiner and Nassal, 2017). To facilitate high-throughput studies using stable DHBV TetOFF systems, cccDNA-dependent production of HBeAg can be easily detected via ELISA (Cai *et al.*, 2016; Schreiner and Nassal, 2017). To sum things up, this study identified *CDKN2C* as a previously unknown host factor involved in HBV infection using a functional genomics screen, highlighting the possibility to exploit technological progress for the identification of virus host interactions, which remain poorly understood.

Taken together, this study identifies *CDKN2C* as a novel HBV host factor, acting through inhibition of CDK4/6 and prevention of cell cycle G1 progression. This sheds new light on the relationship

between HBV infection and the host cell cycle. It moreover encourages further effort for the identification of virus host interactions using innovative technologies, as this will pave the way for the development of physiological infectious model systems for drug discovery.

RÉSUMÉ DE LA THÈSE

Un criblage gain-de-fonction identifie CDKN2C comme facteur d'hôte impliqué dans le cycle viral du virus de l'hépatite B

INTRODUCTION

L'infection chronique par le virus de l'hépatite B (VHB) est l'une des principales causes de maladie hépatique dans le monde. Ces maladies incluent le carcinome hépatocellulaire, sixième cancer le plus fréquent et deuxième cancer le plus meurtrier au monde (Lamontagne, Bagga and Bouchard, 2016). Malgré l'existence d'un vaccin préventif très efficace, environ 250 millions de patients sont chroniquement infectés par ce virus (Schweitzer *et al.*, 2015). Les traitements actuels, basés sur l'interféron-α et des analogues nucléos(t)idiques (*e.g.* entecavir, tenofovir, lamivudine) permettent le contrôle de la réplication virale et réduisent la progression de la maladie hépatique mais ne permettent pas l'élimination du virus. En effet, le virus persiste dans le noyau de la cellule infectée sous forme d'épisome ce qui constitue un réservoir qui est réactivé dès l'arrêt des traitements. De nouvelles stratégies thérapeutiques permettant l'éradication du virus sont impatiemment attendues par le corps médical.

Le VHB est un petit virus à ADN appartenant à la famille des *Hepadnaviridae*. Il possède un spectre d'hôte restreint et infecte les hépatocytes humains. Les particules virales infectieuses, nommées particules de *Dane*, sont composées d'une enveloppe lipidique intégrant la protéine d'enveloppe virale HBs et d'une nucléocapside composée de la protéine HBc contenant le génome du virus lié à la polymérase virale. Le VHB est un virus de petite taille (3,2 kpb) dont le génome formé d'un ADN circulaire relaxé n'est que partiellement double brin (*relaxed circular DNA - rcDNA*). Il contient quatre cadres de lecture ouverts chevauchants (Galibert *et al.*, 1979). En plus des protéines structurales HBs (enveloppe) et HBc (nucléocapside), le génome viral code une polymérase virale et la protéine HBx qui régule l'expression des gènes viraux. Le VHB s'attache à sa cellule cible suite à l'interaction entre les protéines d'enveloppe du virus et les protéoglycanes à sulfate d'héparane (HSPGs) présents à la surface de l'hépatocyte, dont Glypican 5 (GPC5) (Verrier *et al.*, 2016a). Il pénètre ensuite dans la cellule suite

à son interaction avec son récepteur, le *sodium taurocholate co-transporting polypeptide* (NTCP/ *SLC10A1*). Dans le noyau de la cellule hôte, le génome du VHB sous forme rcDNA est complété et converti en un ADN super-enroulé, (*covalently closed circular DNA*- cccDNA). Ce mécanisme fait intervenir plusieurs facteurs de l'hôte mais reste encore mal compris. Le cccDNA est considéré comme un facteur clé du cycle viral et sert de matrice pour la transcription de tous les transcrits viraux dont l'ARN pré-génomique (pgRNA) (Nassal, 2015). Le pgRNA est ensuite rétro-transcrit en rcDNA qui sera encapsidé lié à la polymérase afin de permettre la production de nouveaux virions. Le cccDNA quant à lui, persiste dans le noyau de la cellule hôte. L'éradication du cccDNA des hépatocytes infectés nécessaire à la résolution définitive de l'infection demeure un défi à relever afin de guérir les patients (Lucifora and Protzer, 2016).

Les facteurs clés du cycle viral, et les interactions virus-hôte restent peu connus du fait de l'absence de modèles *in vitro* et *in vivo* permettant de reproduire de manière robuste l'infection chronique par le VHB. Les lignées cellulaires HepG2 et Huh7, dérivées de carcinomes hépatoblastome/hépatocellulaires humains, constituent des modèles cellulaires pertinents, mais ces lignées ne sont pas naturellement permissives à l'infection par le VHB. La découverte récente du récepteur cellulaire NTCP comme récepteur pour le VHB, a rendu possible le développement de modèles cellulaires infectieux à partir de la lignée cellulaire HepG2 et ainsi l'étude du cycle viral complet (Yan *et al.*, 2012; Ni *et al.*, 2014). Dès la découverte de ce récepteur, des lignées HepG2 et Huh7 surexprimant le récepteur NTCP ont étés établis. Alors que la surexpression de NTCP dans des cellules HepG2 rend ces cellules susceptibles à l'infection par le VHB, les cellules Huh7 restent très peu permissives **(Eller, Heydmann *et al.* Figure 1a)**. Ceci est en faveur d'un blocage du cycle viral dans les cellules Huh7 surexprimant le récepteur NTCP pouvant être dû à l'absence d'un ou plusieurs facteurs de l'hôte impliqués dans l'établissement de l'infection ou la réplication du virus. Le but de mon projet de thèse a été d'identifier et de caractériser de nouveaux facteurs d'hôte du VHB à partir de ces observations.

RESULTATS

Criblage pour l'identification de facteurs de l'hôte nécessaires à l'infection par le VHB

Afin d'identifier les facteurs de l'hôte impliqués dans le cycle viral du VHB, un criblage à haut débit de type « gain de fonction » a été réalisé en collaboration avec le *Broad Institute à Boston* (David E. Root, Federica Piccioni) à l'aide d'une banque de lentivirus codant plus de 16000 ORFs humains (X. Yang *et al.*, 2011). Le but de ce criblage était d'identifier des facteurs d'hôte rendant les cellules Huh-106 permissives pour le VHB. Pour ce criblage, nous avons utilisé des cellules Huh7 surexprimant le récepteur NTCP (lignée Huh-106) établie au laboratoire (Verrier *et al.*, 2016a) Cette lignée est très faiblement permissive à l'infection par le VHB **(Eller, Heydmann *et al.* Figure 1)**. Les cellules Huh-106 transduites avec la banque de lentivirus ont été infectées par le VHB 3 jours plus tard. Dix jours après l'infection, les cellules ont été triées en fonction de leur phénotype (infectées/non-infectées), par cytométrie de flux en utilisant un anticorps dirigé contre l'antigène d'enveloppe du VHB (HBsAg). A l'aide de technique de séquençage de nouvelle génération (*next generation sequencing* - NGS) l'accumulation d'ORF dans les populations de cellules infectées et non-infectées a été quantifié par nos collaborateurs. L'enrichissement de certains ORF dans les cellules infectées a été déterminé en calculant la valeur *log2 fold change* (LFC) entre les populations *HBV pre-sort* et *HBV sorted*. Les candidats les plus enrichis dans les cellules infectées ($LFC \geq 1.5$) ont été sélectionnés comme facteurs d'hôte potentiels **(Eller, Heydmann *et al.* Figure 2)**. Pour valider le rôle des candidats sélectionnés dans le cycle viral du VHB, des cellules Huh-106 ont été transduites avec des lentivirus pour surexpression individuelle des ORF des 35 candidats sélectionnés. Dix jours après l'infection avec le VHB, le taux d'infection a été analysé en dosant les antigènes du VHB secrétés (HBe et HBs) dans le milieu de culture par *chemoluminescence immunoassay* (CLIA) **(Eller, Heydmann *et al.* Figure 3a)**. L'identification de gène codant l'*hepatocyte nuclear factor 4a* (HNF4A), un facteur de transcription déjà connu pour être impliqué dans la réplication du VHB (Raney *et al.*, 1997; Quasdorff *et al.*, 2008), valide l'approche expérimentale. Au cours du criblage, deux candidats potentiels ont été identifiés, l'un codant le *cyclin dependent kinase inhibitor 2C* (CDKN2C*)* et l'autre l'*epithelial splicing regulatory protein 1* (ESRP1). Pour valider davantage le rôle de ces deux candidats, des lentivirus pour surexpression des ORFs ont été produits. Ensuite, des cellules Huh-106 ont été transduites avec des lentivirus pour surexpression

individuelle des ORF des candidats et des contrôles. Dix jours après l'infection avec le VHB, le taux d'infection a été analysé en quantifiant l'ARN pré-génomique du VHB (pgRNA) par qRT-PCR. En effet, la surexpression de *CDKN2C* et *ESRP1* augmente significativement l'expression d'ARNpg par rapport aux contrôles (1) cellules non-transduites (HBV), (2) cellules transduites avec un vecteur lentiviral vide (ctrl), et (3) cellules transduites avec un vecteur codant pour un gène control (KRT80) **(Eller, Heydmann *et al.* Figure 4a)**. Ces données indiquent que *CDKN2C* et *ESRP1* sont des facteurs de l'hôte impliqués dans l'infection du VHB. ESRP1 n'étant que peu exprimé dans le foie sain, la focale a été centré sur le candidat CDKN2C, un régulateur du cycle cellulaire qui contrôle la progression de la phase G1 en interagissant avec les *cyclin dependent kinases* 4 et 6 (CDK4/6).

Validation de CDKN2C comme facteur de l'hôte pour l'infection par le VHB

Pour valider la fonction de *CDKN2C* dans un modèle alternatif, des cellules HepG2-NTCP fortement permissives à l'infection par le VHB ont été utilisées pour examiner l'effet du *silencing* de *CDKN2C* à l'aide de siRNA sur l'infection du VHB. Une baisse d'infection par le VHB a été observée dans ces cellules traitées avec des siRNAs ciblant *CDKN2C* ou *SLC10A1* (NTCP) avant l'infection, confirmant le rôle de *CDKN2C* comme facteur d'hôte pro-viral **(Eller, Heydmann *et al.* Figure 4b, c)**. Pour exclure des effets non-spécifiques des siRNAs, l'effet du knockout (KO) de *CDKN2C* à l'aide du système CRISPR/Cas9 sur l'infection du VHB a été étudié. Ainsi, le rôle de *CDKN2C* comme facteur d'hôte a été confirmé par une baisse d'infection par le VHB dans ces cellules HepG2-NTCP KO-CDKN2C **(Eller, Heydmann *et al.* Figure 4d, e)**. Comme décrit précédemment, l'absence ou la faible expression d'un ou plusieurs facteurs d'hôte proviral dans les cellules Huh-106 est responsable de la faible susceptibilité de ces cellules à l'infection par le VHB. Nous avons pu mettre en évidence que la surexpression de *CDKN2C* permet d'augmenter significativement l'infection virale, suggérant que ce facteur nécessaire au cycle viral est certainement peu exprimé dans les Huh-106. Cette hypothèse a été confirmée, car nous avons mis en évidence une faible expression de *CDKN2C* dans les Huh-106 en comparaison aux cellules HepG2-NTCP, qui sont des cellules permissives au VHB **(Eller, Heydmann *et al.* Figure 3d, e)**. Ces données indiquent que la faible expression de *CDKN2C* dans les cellules Huh-106 contribue à la faible permissivité de ces cellules à l'infection par le VHB. Pour aller plus loin dans

la compréhension du rôle de *CDKN2C* dans l'infection par le VHB, l'étape du cycle viral impactée par *CDKN2C* a été étudié. La détection de HBsAg par IF (immunofluorescence) et sa quantification par cytométrie de flux ont confirmé un taux d'infection élevé dans les cellules surexprimant *CDKN2C* (**Eller, Heydmann *et al.* Figure 5a, b**). Afin de comprendre si CDKN2C module le cycle viral avant ou après la formation du cccDNA, les intermédiaires génomiques (cccDNA et ARN viraux) ont été détectés par *Southern blot* et *Northern blot*. La surexpression de *CDKN2C* dans les cellules Huh-106 avant l'infection par VHB n'a pas d'effet sur le niveau de cccDNA **Eller, Heydmann *et al.* Figure 5d)** indiquant que *CDKN2C* est impliqué dans une étape se situant après la formation du cccDNA. La détection des ARN viraux par *Northern blot* montre des niveaux d'ARN du VHB plus haut dans les cellules surexprimant *CDKN2C* comparé aux cellules contrôle surexprimant le GFP (**Eller, Heydmann *et al.* Figure 5e**). Ceci indique un rôle de *CDKN2C* dans le cycle viral du VHB au cours d'une étape après la formation du cccDNA et avant la traduction des antigènes viraux. Afin de mieux caractériser la fonction de CDKN2C pour l'infection par le VHB, des études fonctionnelles avec deux inhibiteurs cliniques de CDK4/6, palbociclib (Fry *et al.*, 2004) et ribociclib (Kim *et al.*, 2013), ont été réalisées. Les taux d'infection ont été quantifiés dans des cellules Huh-106 traitées avec les inhibiteurs avant et après l'infection avec le VHB (**Eller, Heydmann *et al.* Figure 6a**). La visualisation de HBsAg intracellulaire a révélé une forte augmentation du niveau d'infection après traitement avec palbociclib ou ribociclib (**Eller, Heydmann *et al.* Figure 6b**). Cet effet a été confirmé par la quantification de l'ARNpg et le HBsAg, avec des taux d'infection plus élevés dans des cellules traitées avec des inhibiteurs de CDK4/6. Collectivement, ces données identifient *CDKN2C* comme nouveau facteur de l'hôte, agissant à travers l'inhibition de CDK4/6 et provoquant un arrêt du cycle cellulaire en phase G1 (**Eller, Heydmann *et al.* Figure 7**).

DISCUSSION

L'infection chronique par le VHB est l'une des principales causes du carcinome hépatocellulaire et représente un problème majeur de santé publique (El-Serag, 2012). Le génome et la structure du VHB sont bien décrits, mais la plupart d'interactions moléculaires entre virus et facteurs hépatiques sont encore méconnues, en partie à cause du manque de modèles d'étude *in vitro* satisfaisants (Verrier *et al.*,

2016b). L'identification de facteurs de l'hôte impliqués dans l'infection du VHB est de ce fait cruciale pour la découverte de nouvelles cibles thérapeutiques antivirales. De plus, la découverte de nouveaux facteurs de l'hôte est nécessaire pour le développement de nouveaux modèles cellulaires qui permettront d'étudier l'intégralité du cycle viral. Dans cette étude, nous avons mis en évidence le rôle de *CDKN2C* dans la réplication du VHB. La protéine CDKN2C est un régulateur du cycle cellulaire qui freine la progression en phase S par l'inhibition des CDK4/6. Cela mène à une accumulation de cellules en phase G1 permettant au VHB de mieux répliquer. Le même effet a été observé dans des cellules traitées avec un inhibiteur des CDK4/6, le palbociclib. Cette molécule est déjà utilisée en clinique pour traiter le cancer du sein (Vijayaraghavan and Moulder, 2018) et est actuellement évaluée pour le traitement d'autres cancers, dont le cancer primitif du foie (Bollard *et al.*, 2017). La compréhension de l'effet activateur de l'inhibition des CDK4/6 sur la réplication du VHB pourrait constituer un obstacle au développement clinique de palbociclib pour le traitement du CHC.

En résumé, dans le cadre de cette thèse, CDKN2C a été identifié comme facteur d'hôte pro-virale impliqué dans la réplication du VHB à travers sa fonction de régulateur du cycle cellulaire. L'effet de l'arrêt du cycle cellulaire dans la phase G1 sur l'infection du VHB a été confirmé par : (1) une augmentation/baisse significative d'infection du VHB après la surexpression/le knockout de CDKN2C, respectivement ; (2) des niveaux d'expression de CDKN2C correspondant à la susceptibilité à l'infection du VHB ; et (3) un effet pro-viral éclatant d'inhibiteurs cliniques de CDK4/6.

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ANNEX

Review article:

Eller, C. *et al.* (2018) 'The functional role of sodium taurocholate cotransporting polypeptide NTCP in the life cycle of hepatitis B, C and D viruses', *Cellular and Molecular Life Sciences*, 75(21), pp. 3895–3905. doi: 10.1007/s00018-018-2892-y.

The functional role of sodium taurocholate co-transporting polypeptide NTCP in the life cycle of hepatitis B, C and D viruses

Carla Eller[1,2], Laura Heydmann[1,2], Che C. Colpitts[3], Eloi R. Verrier[1,2], Catherine Schuster[1,2], Thomas F. Baumert[1,2,4]*

[1]Inserm, U1110, Institut de Recherche sur les Maladies Virales et Hépatiques, 67000 Strasbourg, France; [2]Université de Strasbourg, 67000 Strasbourg, France; [3]Division of Infection and Immunity, University College London, London, United Kingdom; [4]Institut Hospitalo-Universitaire, Pôle Hépato-digestif, Nouvel Hôpital Civil, 67000 Strasbourg, France.

***Corresponding author**: Prof. Thomas F. Baumert, MD; Inserm U1110, Institut de Recherche sur les Maladies Virales et Hépatiques, Université de Strasbourg, 3 Rue Koeberlé, 67000 Strasbourg, France; Phone: +33 3 68 85 37 03, Fax: +33 3 68 85 37 24, e-mail: thomas.baumert@unistra.fr

ORCID of the authors: 0000-0001-7835-9468, 0000-0002-2273-2267, 0000-0003-2474-1834, 0000-0002-9022-5611, 0000-0001-7281-4511, 0000-0002-8864-2168

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Abstract

Chronic hepatitis B, D and C virus (HBV, HDV and HCV) infections are a major cause of liver disease and cancer worldwide. Despite employing distinct replication strategies, the three viruses are exclusively hepatotropic and therefore depend on hepatocyte-specific host factors. The sodium taurocholate co transporting polypeptide (NTCP), a transmembrane protein highly expressed in human hepatocytes that mediates the transport of bile acids, plays a key role in HBV and HDV entry into hepatocytes. Recently, NTCP has been shown to modulate also HCV infection of hepatocytes by regulating innate antiviral immune responses in the liver. Here we review the current knowledge of the functional role and the molecular and cellular biology of NTCP in the life cycle of the three major hepatotropic viruses, highlight the impact of NTCP as an antiviral target and discuss future avenues of research.

Keywords: Liver cell biology, bile acid transport, host factor, anti-viral therapy, hepatocytes.

Introduction

Every year, viral hepatitis is estimated to cause around 1.3 million deaths worldwide, mainly through chronic liver disease and hepatocellular carcinoma (HCC). Approximately 95% of these deaths are caused by hepatitis B and C viruses (HBV, HCV) [1]. Despite the availability of an effective vaccine for HBV, 250 million people are chronically infected by the virus worldwide [2]. An estimated 5% of HBV patients are co-infected with hepatitis D virus (HDV), a satellite virus hijacking HBV envelope proteins to assemble its infectious viral particles. HDV co-infection worsens the outcome of HBV infection and treatment of HBV-HDV co-infected patients is less effective [3, 4]. Moreover, around 70 million people are living with chronic HCV infection and, despite the existence of effective curative strategies, the incidence of HCV is still increasing [3].

Remarkable progress has recently been made for treatment of HCV infection. The development and approval of direct acting antivirals (DAAs) specifically targeting viral proteins now allows for HCV cure, but these therapies remain inaccessible for the majority of HCV patients [5]. For chronic HBV infection, two therapeutic approaches are used to suppress viral replication: pegylated interferon and nucleos(t)ide analogues (NUCs). While these treatments allow control of HBV infection, viral eradication is rare and, in most cases, lifelong therapy is required [6]. For patients with chronic HBV/HDV co-infection, the current treatment options are limited to interferon-alpha (IFNα) and its pegylated derivative. Furthermore, although current antivirals decrease the risk of HCC, they are not sufficient to eliminate the risk [7, 8]. In order to effectively combat these hepatotropic viruses, it is necessary to improve existing therapies and uncover new strategies for prevention and treatment of viral hepatitis.

Alternative strategies against chronic HBV and HCV infection include host-targeting agents (HTA), which modify the host cell function to inhibit viral replication. HTAs have been shown to be

promising candidates for the prevention and treatment of infections by various pathogens, including HBV and HCV [9–11]. This approach requires a profound understanding of the viral life cycle and the virus-host interactions involved. Indeed, the identification of the human sodium taurocholate co-transporting polypeptide (NTCP) as a functional receptor for HBV/HDV infection [12, 13] opened perspectives for new antiviral strategies. Several entry inhibitors for treatment of HBV infection targeting NTCP are now in development [14–19]. Furthermore, this crucial discovery has allowed the development of novel infectious model systems that will enable an improved understanding of the complete HBV/HDV viral life cycle [20]. However, the regulatory role of NTCP in HCV host cell infection, and its potential immunomodulatory activities in hepatocytes, should not be overlooked. The aim of this review is to summarize what is known about the interactions of NTCP with three major hepatitis viruses during infection, to describe the molecular mechanisms, and to highlight possible applications in research and therapy.

Sodium taurocholate co-transporting polypeptide, a bile acid transporter

The circulation of bile and bile components between human intestine enterocytes and liver parenchymal cells is known as the enterohepatic circulation (EHC) [21]. In the liver, bile acids are mainly involved in cholesterol metabolism and elimination of toxic compounds [22]. Interestingly, bile acids have also been shown to inhibit interferon (IFN) signaling pathways, resulting in reduced expression of IFN-stimulated genes (ISG) [23, 24]. In hepatocytes, bile acid homeostasis is maintained by the interplay between uptake, synthesis and secretion of bile acids. The major hepatic uptake transporter for conjugated bile acids in humans is sodium taurocholate co transporting polypeptide (NTCP) [25]. NTCP is predominantly expressed at the hepatic basolateral membrane and is involved in the recycling of bile acids from portal blood to hepatocytes in a sodium-dependent manner [21]. NTCP is a member of the solute carrier family SLC10 and is encoded by *SLC10A1* [26, 27]. *SLC10A1* mRNA is translated into a 349 amino acid glycosylated phosphoprotein with seven or nine transmembrane domains [21, 28–31]. While the exact function of some SLC10 family members remains unknown, all of them are thought to be sodium-dependent transporters [21]. Interestingly, bile acid transport through NTCP can be blocked by small molecules already in clinical use, such as cyclosporine A (CsA, an immunosuppressive drug used in transplantation) or ezetimibe (used for hypercholesterolemia) [16, 32].

Hepatic bile acid metabolism is tightly regulated, including at the transcriptional level (see Figure 1) [33]. Upon bile acid activation, the nuclear factor Farnesoid X Receptor (FXR) indirectly downregulates several target genes through transcriptional induction of the small heterodimer partner (SHP) [34, 35], including the first and rate-limiting enzyme in bile acid biosynthesis cholesterol 7α-hydroxylase (CYP7A1) [36, 37]. FXR also directly activates the expression of the bile salt export pump (BSEP, ABCB11), which is expressed at the apical membrane and secretes conjugated bile acids into the bile canaliculus in an ATP-dependent manner [38, 39]. FXR does not directly interact with the promoter of human *SLC10A1* but induces the expression of different factors to indirectly repress *slc10a1*

expression in rat and mouse, although mechanisms of transcriptional regulation of human NTCP remain unknown [40–42]. In hepatic inflammation, the cytokines tumor necrosis factor alpha (TNF-α), interleukin (IL)-1β, and IL-6 downregulate mRNA levels of *SLC10A1* and reduce the transporter protein expression [43–45]. The downregulation of NTCP expression in the human liver has been implicated in several cholestasis pathologies. The reduction of NTCP expression could explain impaired hepatic bile acid uptake, resulting in cholestatis and jaundice. Several studies have shown a downregulation of bile salt transporters in primary biliary cirrhosis [46, 47]. Interestingly, a recent study showed a suppression of NTCP expression via cyclin D1 in hepatocellular carcinoma (HCC) [48]. These data may explain the low expression level of NTCP in HCC-derived cell lines, such as Huh-7 and clones or HepG2.

The localization and membrane expression of NTCP is controlled by post-translational mechanisms [49]. For example, cyclic adenosine monophosphate (cAMP) plays a role in stimulating the dephosphorylation and membrane translocation of NTCP (see Figure 1) [50–52]. Sequencing analysis of NTCP revealed the existence of several ethnic-dependent single nucleotide polymorphisms (SNPs) which may alter NTCP activities [53]. For example, mutation S267F, found in 7.5% of allele frequencies in Chinese Americans, is associated with an almost complete loss of bile acid uptake function. However, no pathologies have been described resulting from these NTCP polymorphisms and their clinical roles remain controversial [54]. Besides its major role in the bile acid uptake system, Yan et al. described the crucial role of NTCP on HBV and HDV entry [12]. For the time being, NTCP remains the only described HBV and HDV entry receptor.

NTCP is a host factor for HBV/HDV infection

Hepatitis B virus is the prototypic member of the *Hepadnaviridae* family of small enveloped hepatotropic DNA viruses. Its envelope consists of three different forms of the HBV surface protein (HBsAg) – the small (S), middle (M) and large (L) proteins. Importantly, the preS1-domain of L envelope protein is known to bind hepatocyte cell surface and is required for HBV and HDV entry [55]. The HBV capsid is comprised of HBV core protein (HBcAg) and carries a partially double-stranded relaxed circular DNA (rcDNA) genome of 3.2 kilobases. Upon infection of hepatocytes, genomic rcDNA is converted into a covalently closed circular DNA (cccDNA), a minichromosome-like structure that persists in the nucleus as central transcription template for all viral RNAs [56]. The presence of cccDNA in the nucleus is thought to be responsible for viral rebound after withdrawal of NUC therapy that targets reverse transcription, a late step in the HBV life cycle. Therefore, removal of cccDNA from HBV-infected hepatocytes will be essential to achieve the goal of HBV cure [57].

HDV is a defective hepatotropic virus which depends on HBV surface proteins for assembly of infectious virions and viral entry [58]. The HDV genome is a negative single-stranded circular RNA of nearly 1700 nucleotides containing one functional open reading frame, which encodes the hepatitis delta protein (HDAg) expressed in small and large form. Replication of HDV RNA and transcription of HDAg mRNA in the nucleus depends on host cell polymerases, including DNA-dependent RNA

polymerase II. Both forms of the delta protein are then produced and reimported in the nucleus where they bind to genomic RNA to form the ribonucleoprotein (RNP), which is then exported into the cytoplasm and is associated with HBV envelope proteins to form a mature HDV virion [59]. Thus, HDV enters hepatocytes using the same pathways as HBV, and depends on the same host factors for host cell binding and entry. HDV is therefore a useful surrogate model for HBV entry.

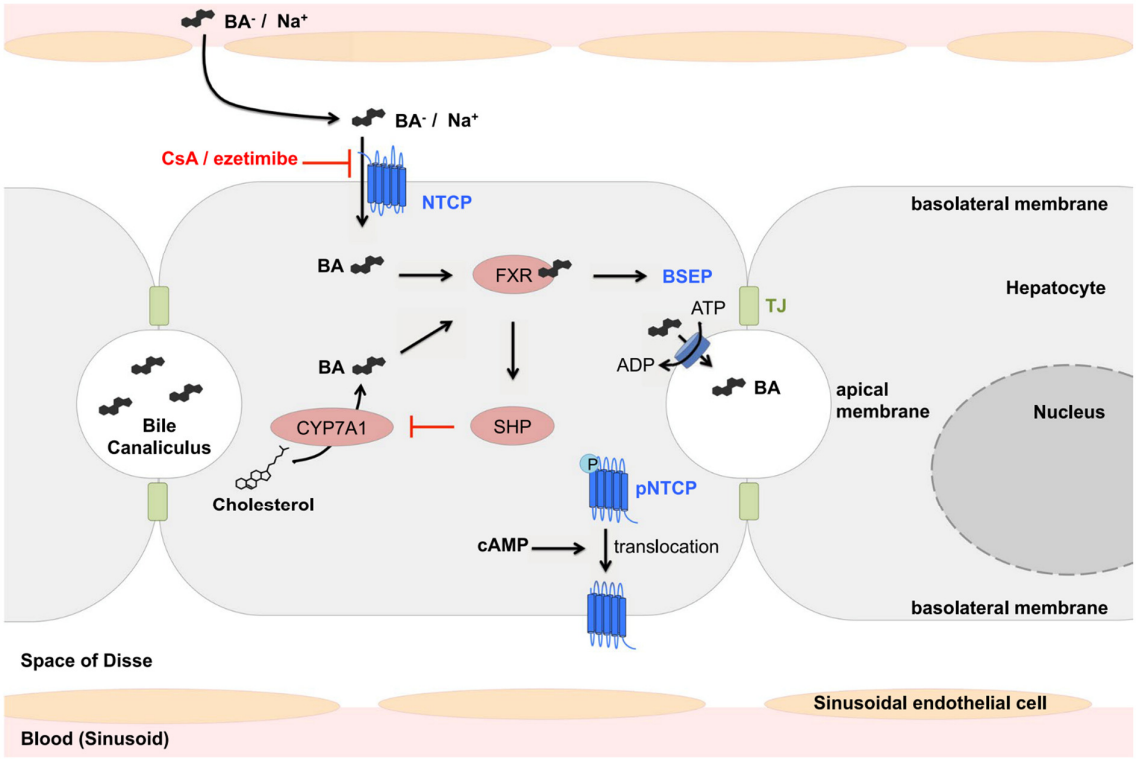

Fig. 1 Model of the functional role of NTCP in hepatic bile acid transport and metabolism. Transport of bile acids from portal blood into hepatocytes via NTCP depends on a sodium gradient and is inhibited by CsA or ezetimibe. Secretion into the bile canaliculus via bile salt export pump (BSEP) in an ATP-dependent manner and synthesis from cholesterol are regulated by bile acid-mediated activation of FXR. cAMP mediates dephosphorylation and membrane translocation of NTCP. NTCP: Sodium taurocholate co transporting polypeptide; BSEP: bile salt export pump; FXR: Farnesoid X Receptor; SHP: small heterodimer partner; CYP7A1: cholesterol 7α-hydroxylase; BA: bile acid; TJ: tight junction; CsA: cyclosporin A; cAMP: cyclic adenosine monophosphate.

The first step of viral infection is virion binding to attachment factors and receptors at the host cell surface. This specific interaction between viral surface proteins and host entry receptors often determines the tissue tropism and host range of the virus [60]. HBV and its satellite virus HDV share HBV envelope proteins and are known to exclusively infect human, chimpanzee and tree shrew (*Tupaia belangerii*) hepatocytes, suggesting the involvement of species- and liver-specific cell surface factors in the common entry process of these viruses [20]. Two elements of the HBV envelope proteins are necessary for interaction with these factors. One determinant of infectivity resides in the surface-exposed cysteine-rich antigenic loop (AGL), a polypeptide located in the S domain of all three envelope proteins [61, 62]. The second known infectivity determinant is a receptor binding site in the N-terminal

pre-S1 domain of the L-HBsAg [55]. This domain is post-translationally modified by addition of myristic acid [63], and this myristoylation is essential for virion infectivity [64, 65]. A synthetic myristoylated peptide comprising the N-terminal amino acids 2 to 78 of the pre S1 domain prevents HBV infection [66].

As for many viruses [67, 68], HBV/HDV infection requires the initial attachment to the glycosaminoglycan (GAG) side chains of heparan sulfate proteoglycans (HSPGs) [69]. Both the antigenic loop of all HBV envelope proteins and the preS1-region of HBsAg-L are involved in this interaction [69, 70]. Indeed, glypican-5 (GPC5), a member of the glypican family of HSPGs, acts as an entry factor for HBV and HDV (see Figure 2) [71]. After this initial step of HBV/HDV attachment to HSPGs, the virions bind to a high-affinity receptor via the preS1-domain [72], allowing uptake into hepatocytes. Despite the discovery of several preS1 interacting proteins without biological activity in HBV infectivity [73–78], the identity of the HBV/HDV entry receptor remained unclear until 2012, when Yan *et al.* identified NTCP as a functional receptor for HBV and HDV infection. Using a labeled preS1 peptide as a bait in *Tupaia* hepatocytes, a mass spectrometry purification of preS1-bound proteins, and validation in human hepatocytes, they showed that NTCP specifically interacts with the HBV receptor-binding domain preS1, allowing viral entry [12]. Zhong *et al.* showed that *Tupaia* NTCP mediates entry of woolly monkey HBV, indicating that NTCP orthologs act as a common cellular receptor for known primate hepadnaviruses [79]. Differential gene expression patterns between non-susceptible undifferentiated and susceptible differentiated HepaRG cells validated the role of NTCP as a specific receptor for HBV and HDV [13]. Moreover, silencing of NTCP in primary *Tupaia* hepatocytes (PTH) or differentiated HepaRG cells inhibited HBV and HDV infection [12, 13]. Exogenous expression of NTCP directly renders non-susceptible hepatoma cell lines susceptible to HBV and HDV infection, while entry inhibitors derived from the preS1 peptide efficiently inhibit this infection [12]. In addition, the S267F mutant of NTCP, conferring a loss of bile acid uptake function is significantly associated with resistance to chronic hepatitis B and decreased risk of cirrhosis and liver cancer development, supporting the role of NTCP as cellular receptor for HBV in human infection [80–82]. However, S267F homozygote patients can still be infected by HBV, suggesting the existence of alternative receptors allowing viral entry in the absence of functional NTCP [83].

Interestingly, expression of human (but not mouse) NTCP in non-susceptible hepatocarcinoma cells confers limited susceptibility to infection. For robust infection, addition of dimethyl sulfoxide (DMSO) to culture medium is essential [13]. The fact that human hepatoma cell lines HepG2 and Huh7 are not susceptible to HBV and HDV infection without exogenous expression of NTCP is consistent with reports that NTCP expression is reduced in human hepatocellular carcinoma cells [48, 84]. NTCP expression rapidly decreases over time following isolation of cultured PTHs, which supports observations that primary human hepatocytes (PHH) remain susceptible to HBV infection *in vitro* only for a few days after isolation [12, 85]. Considering the predominant expression of NTCP in the liver, this receptor is likely to contribute to the hepatotropism of both viruses [12]. In addition, NTCP protein

sequences vary among mammalian species, which might contribute to the narrow species tropism of HBV and HDV infection. For example, monkey NTCP does not support HBV and HDV infection despite a high protein sequence homology to human NTCP. Replacing amino acids 157–165 of nonfunctional monkey NTCP with the human counterpart conferred susceptibility to both HDV and HBV infection [12]. The fact that hepatocytes from cynomolgus and rhesus macaques and pigs become fully susceptible to HBV upon hNTCP expression indicates that NTCP is the key host factor limiting HBV infection in these species [86].

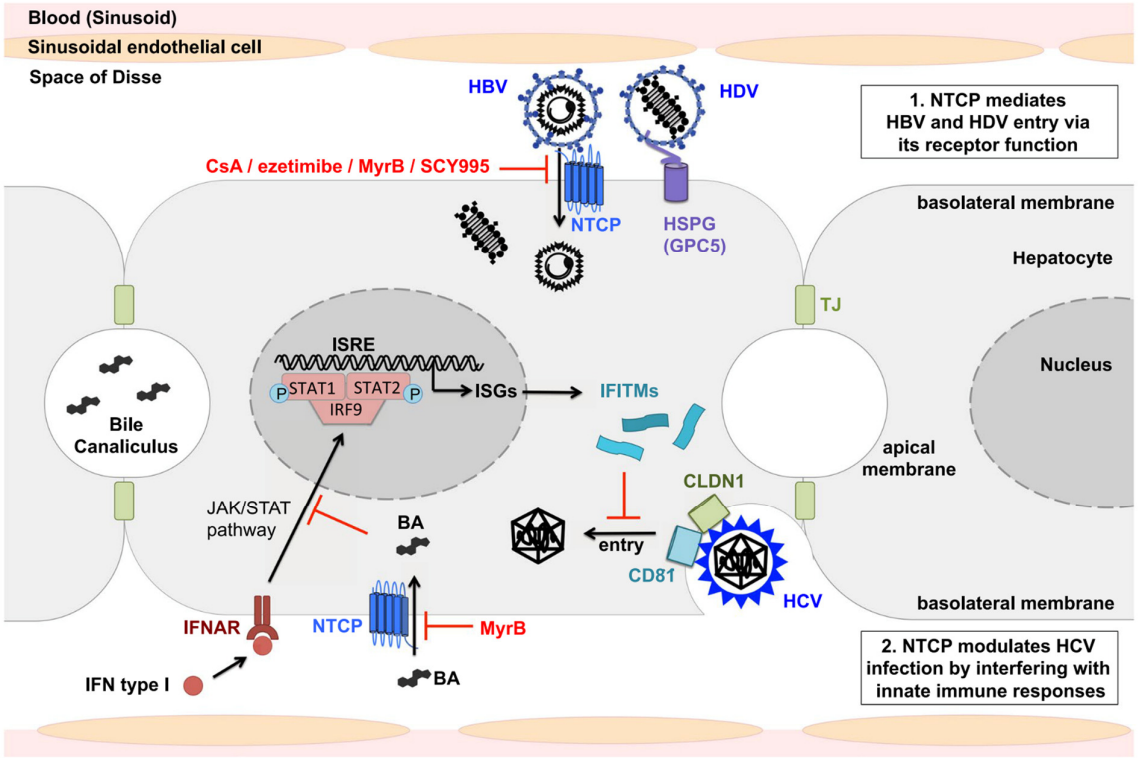

Fig. 2 Model of interactions between NTCP and the entry of HBV, HDV, and HCV in hepatocytes. After initial attachment to HSPG including GPC5, HBV and HDV virions bind to the receptor NTCP through the preS1-domain of the large envelope protein. NTCP inhibitors CsA and ezetimibe block viral entry like preS1 derived MyrB and CsA-derived SCY995. NTCP modulates HCV infection by interfering with innate immune responses. Bile acids interfere with the IFN signaling pathway and thereby favor HCV entry. Inhibition of NTCP-mediated bile acid import into hepatocytes promotes inhibition of HCV entry through the upregulation of ISGs including IFITMs. HBV: hepatitis B virus; HCV: hepatitis C virus; HDV: hepatitis D virus; HSPG: heparan sulfate proteoglycan; GPC5: glypican-5; NTCP: Sodium taurocholate co-transporting polypeptide; MyrB: myrcludex B; CsA: cyclosporin A; SCY995: synthesized CsA derivative 995; IFN: interferon; IFNAR: IFN-α/β receptor; JAK: Janus kinase; STAT: signal transducer and activator of transcription; IRF9: Interferon regulatory factor 9; ISRE: IFN-sensitive response element; ISG: IFN-stimulated gene; IFITM: IFN-induced transmembrane protein; CLDN1: Claudin 1; CD81: cluster of differentiation 81; BA: bile acid; TJ: tight junction

As a key host factor enabling HBV and HDV infection *in vitro*, the discovery of NTCP has been crucial for the development of novel animal models supporting virus infection. Indeed, only Chimpanzee and Tupaia can experimentally support HBV and HDV infections [87]. The state-of-the-art mouse model for the study of HBV/HDV life cycles consists of liver-engrafted humanized chimeric uPa/SCID or FRG

mice, which support virus entry and replication, but lack an efficient immune system limiting the study of virus-host interactions [87]. The recent development of human NTCP-expressing transgenic mice opened perspectives for the development of novel immune-competent animal models for the investigation of HDV infection and HDV-induced pathogenesis *in vivo* [88]. As HBV infection is limited in mouse cells expressing hNTCP, probably because of the lack of a key host factor [89], it should be noted that hNTCP-transgenic mice are not susceptible to HBV infection. Recently, an elegant study demonstrated that vector-mediated expression of hNTCP in the hepatocytes of rhesus macaques conferred susceptibility to HBV infection, providing a robust and relevant model for the study of HBV infection, including its interaction with adaptive immunity and the understanding of viral clearance [90].

Overall, NTCP was identified as the long-sought preS1-specific HBV receptor contributing to HBV liver tropism and species specificity [13]. Targeting the interactions between the HBV preS1-domain and its receptor NTCP required for HBV/HDV entry is a promising strategy to block viral entry for both viruses.

NTCP as a therapeutic target for HBV/HDV infection

Even before the identification of NTCP as HBV/HDV receptor, entry inhibitors derived from the HBV preS1 were shown to efficiently inhibit HBV infection *in vitro* and *in vivo* [91, 92]. One of these compounds, the myristoylated preS1-derived peptide (also called Myrcludex B or MyrB), efficiently prevents HBV dissemination in vivo and hinders amplification of the cccDNA pool in infected human hepatocytes [14]. MyrB is the first HBV/HDV entry inhibitor targeting NTCP to reach clinical trials [93], where it was shown to have a good safety profile with a mild and reversible elevation of serum bile acid salts [93, 94]. Phase IIa clinical studies revealed a marked antiviral effect of MyrB, as measured by HDV RNA, HBV DNA and improvement of biochemical disease activity (ALT), when used in combination with IFN therapy, although there was no significant decrease in HBsAg levels. In monotherapy, however, MyrB did not show significant antiviral activity [94]. Further studies are necessary to confirm these results obtained in small patient cohorts [95].

Importantly, the identification of NTCP as the first HBV/HDV entry receptor has accelerated the discovery and development of several new potential entry inhibitors. Binding of myristoylated preS1-derived peptide to NTCP was shown to interfere with the physiological bile acid transport function of NTCP, indicating that NTCP-inhibiting drugs might be able to block HBV infection [96]. In a study evaluating FDA approved therapeutics with documented inhibitory effect on NTCP cellular function against HDV entry, three of these molecules (irbesartan, ezetimibe, and ritonavir) inhibited HDV infection *in vitro* [97]. The inhibitory effect of ezetimibe on HBV infection had already been described previously without understanding its interactions with NTCP [98]. In 2014, Watashi *et al.* evaluated the effect of compounds on the early phase of the HBV life cycle to identify cyclosporine A as an HBV entry inhibitor targeting NTCP [15]. In the same year, Nkongolo *et al.* characterized the effect of cyclosporine A, a cholestasis-inducing drug inhibiting NTCP bile acid transport [32, 97, 98],

against HBV/HDV infection and found that inhibition of entry resulted from interference with the NTCP receptor [16]. The screening of FDA/EMA-approved drugs or small molecules for interaction with NTCP allowed the identification of several additional potential HBV/HDV entry inhibitors targeting NTCP [18, 19]. All of these NTCP-targeting HBV/HDV entry inhibitors concomitantly inhibit the transporter function of NTCP and impair bile acid uptake into hepatocytes, increasing the risk of adverse effects. NTCP deficient mice and a patient with NTCP deficiency were shown to exhibit an elevated level of serum bile acids and to develop related pathologies including growth retardation and hypercholanemia [101, 102].

Two different strategies to selectively inhibit HBV entry without impairing bile acid uptake have been suggested recently. Shimura et al. showed that cyclosporine A derivatives SCY450 and SCY995 inhibit HBV/HDV entry without interfering with the NTCP transporter activity (see Figure 2) [17]. Tsukuda *et al.* identified an oligomeric flavonoid, proanthocyanidin (PAC) and its analogs, as a new class of entry inhibitors, which directly target the preS1-domain of the HBV large envelope protein and thereby prevent its attachment to NTCP. By directly targeting HBV particles, PAC impaired HBV infectivity without affecting the NTCP mediated bile acid transport activity [103]. Further studies are required to determine if these novel inhibitory strategies will show efficacy in vivo and in clinical studies in co-treatment with NUC therapy.

NTCP is a host factor for HCV infection

Hepatitis C virus is an enveloped single-stranded positive-sense RNA virus in the *Flaviviridae* family. The host cell derived lipid envelope contains the two viral envelope glycoproteins, E1 and E2 [104]. Within the envelope, an icosahedral capsid contains the RNA genome of 9.6 kilobases. Like HBV and HDV, attachment of HCV to hepatocytes is mediated by HPSGs on the host cell surface [105–107]. Following attachment, the envelope glycoprotein E2 mediates interactions with a series of specific cellular entry factors, including CD81 and claudin-1 (see Figure 2) [108–111]. HCV is internalized via endocytosis in a clathrin- and dynamin-dependent process [112]. Following fusion with early endosomal membranes, the HCV genome is released into the cytosol, where it is translated into a polyprotein cleaved by viral and host proteases. The HCV genome is replicated directly into RNA without passing through a DNA intermediate [113]. Therefore, HCV entry and replication steps are very distinct from those described for HBV/HDV. Nonetheless, the mutual hepatotropism of these three viruses mediated by tissue specific factors suggests a possible overlap in usage of common hepatocyte specific host factors like NTCP.

Following establishment of the pivotal role of NTCP for HBV and HDV entry into hepatocytes, a recent study implicated the transporter in HCV infection (see Figure 2). Exogenous overexpression or silencing of NTCP increased or decreased HCV infection *in vitro*, respectively [114]. Unlike HBV, however, no direct interaction between HCV envelope proteins and NTCP was identified. Instead, the bile acid transporter function of NTCP was found to be important for HCV entry [114]. Bile acids are

known to modulate cellular antiviral responses by inhibiting interferon (IFN) type I signaling and thereby decreasing the expression of IFN stimulated genes (ISGs) [23, 24]. NTCP was shown to regulate HCV infection by inducing the bile acid mediated repression of ISG expression in hepatocytes, including IFITM1, IFITM2 and IFITM3 [114]. These transmembrane proteins are known to restrict the entry of several viruses, including HCV [115]. IFITM1 blocks the interaction between HCV and its receptors [116], whereas IFITM2 and IFITM3 inhibit entry at a post endocytosis step by blocking the release of virions into the cytoplasm [117]. NTCP facilitates HCV infection by modulating innate antiviral responses via its bile acid transport function. As bile acids have been shown to enhance HCV replication [118], it is likely that NTCP expression and activity modulates HCV infection through a multimodal mechanism of action. Interestingly, MyrB mediated inhibition of NTCP blocks the import of bile acids, which in turn stimulates the expression of ISGs, inhibiting HCV entry and infection [114]. However, it still needs to be determined whether the inhibition of NTCP-mediated bile acid entry affects the HBV life cycle through similar mechanisms as described for HCV. The potential of NTCP-targeting antivirals to enhance innate antiviral responses and to engage the host immune system to clear infection may be a useful property for the treatment of all hepatotropic viruses, including HBV, HCV and HDV.

Conclusions

The discovery of NTCP as the first HBV/HDV receptor was a milestone in the study of the life cycle of these viruses. This landmark discovery enabled significant progress in understanding HBV/HDV entry and virus-host interactions. Moreover, based on this discovery, novel infectious model systems based on transduced cell lines stably expressing NTCP have been developed which allow detailed study of the early steps of the viral life cycle. By allowing the study of authentic infection in cell lines, these model systems will help to understand the formation and degradation of HBV cccDNA, which is a key target to achieve the ultimate goal of HBV cure. Robust human NTCP expressing animal model systems will enable the in vivo validation of virus-host interactions and antiviral therapies. Moreover, NTCP has been established as an antiviral target, and several molecules targeting NTCP are in clinical development with the goal to improve current therapies in the future. The recent discovery of NTCP as a host-dependency factor in HCV infection underscores its essential role in virus-hepatocyte interactions.

Author contributions

CE, LH, CCC, ERV, CS, TFB wrote the manuscript.

Conflicts of interest

The authors have no conflicting interests to disclose.

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Université de Strasbourg

Carla ELLER

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

Un criblage gain-de-fonction identifie *CDKN2C* comme facteur d'hôte impliqué dans le cycle viral du virus de l'hépatite B

Résumé

L'hépatite B est causée par le virus de l'hépatite B (VHB) qui est une cause majeure du carcinome hépatocellulaire, deuxième cancer le plus meurtrier au monde. Le VHB infecte des hépatocytes humains, et, dû à la petite taille de son génome, dépend de nombreux facteurs de l'hôte, qui contribuent au tropisme d'espèce et à sa spécificité tissulaire. Cependant, au niveau moléculaire les interactions virus-hôtes nécessaires au cycle viral restent mal connues, à cause de l'absence de modèles cellulaires robustes pour l'étude de l'infection par le VHB. Un criblage innovant de génomique fonctionnel a révélé le rôle de CDKN2C comme facteur d'hôte proviral promouvant la réplication du VHB lors d'une étape du cycle viral postérieure à la formation de l'ADN super-enroulé, ceci, par sa fonction de régulateur du cycle cellulaire. Les travaux réalisés offrent une meilleure compréhension des interactions virus-hôte et des limites des systèmes de culture cellulaire actuellement disponibles, et contribuera au développement de systèmes modèles infectieux plus performantes et à l'élaboration de stratégies thérapeutiques novatrices pour lutter contre l'hépatite B chronique.

Mots-clés : Virus de l'hépatite B, systèmes modèles, interactions virus-hôte, CDKN2C

Résumé en anglais

Hepatitis B is caused by the hepatitis B virus (HBV) and is a major cause of progressive liver disease including cirrhosis and hepatocellular carcinoma (HCC), the second leading cause of cancer death worldwide. HBV infects human hepatocytes, and, because of the tiny size of its genome, depends on multiple host functions, contributing to species and tissue tropism. However, fundamental virus-host interactions remain obscure, owing to the lack of robust infectious models for HBV research. An innovative functional genomics screen revealed the role of CDKN2C as proviral host factor promoting HBV replication in a step of the life cycle after the formation of covalently closed circular (ccc) DNA via its function as cell cycle regulator. This provides a better understanding of virus-host interactions and limitations of currently available cell culture systems, and will contribute to the development of physiological infectious model systems and novel therapeutic strategies for viral cure.

Key words: Hepatitis B virus, model systems, virus-host interactions, CDKN2C