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

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# THÈSE présentée par :


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# Approches de génomique fonctionnelle pour la caractérisation de facteurs hépatiques impliqués dans l'infection par le virus de l'hépatite B

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”En vérité, le chemin importe peu, la volonté d’arriver suffit à tout.”

**Albert Camus -** Le Mythe de Sisyphe

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## TABLES DES MATIERES

## *LISTE DES ABRÉVIATIONS*

| | | |
|---|---|---|
| (-) | : | brin négatif ou replicatif |
| (+) | : | brin positif ou non réplicatif |
| AASLD | : | *American association for the study of liver diseases* |
| ADN | : | acide désoxyribonucléique |
| ADNccc | : | acide désoxyribonucléique circulaire clos de façon covalente |
| ADNdb | : | acide désoxyribonucléique double brin |
| ADNdbl | : | acide désoxyribonucléique double brin linéaire |
| ADN-rc | : | acide désoxyribonucléique relâché circulaire |
| AgHBc | : | antigène hépatite B core |
| AgHBe | : | antigène hépatite B e |
| AgHBs | : | antigène hépatite B de surface |
| AgHBx | : | antigène hépatite B x |
| ARN | : | acide ribonucléique |
| ARNdb | : | acide ribonucléique double brin |
| ARNm | : | acide ribonucléique messager |
| ARNpg | : | acide ribonucléique pré-génomique |
| ARNb | : | acide ribonucléique simple brin |
| C-ter | : | C-terminal |
| CAD | : | *carbamoyl-phosphate synthetase 2, aspartate transcarbamylase, and Dihydroorotase* |
| CDK | : | *cyclin dependent kinase* |
| CDK4/6 | : | *cyclin dependent kinase 4/6* |
| CDKN2C | : | *cyclin Dependent Kinase Inhibitor 2C* |
| cGAMP | : | *cyclic guanine monophosphate - alanine monophosphate* |
| cGAS | : | *2'3'-cyclic guanine monophosphate – alanine monophosphate synthase* |
| CHC | : | carcinome hépatocellulaire |
| CLIA | : | *chemiluminescent immunoassay* |
| CRISPR | : | *clustered regularly interspaced short palindromic repeats* |
| DAPI | : | 4',6-diamidino-2-phénylindole |
| db | : | double brin |
| DHBV | : | *duck hepatitis B virus* |
| DR | : | *direct repeated* |
| EASL | : | *european association for the study of the liver* |
| FACS | : | *fluorescence-activated cell sorting* |
| GFP | : | *green fluorescent protein* |
| GPC5 | : | glypican-5 |
| GTP | : | guanine triphosphate |
| HBSP | : | *HBV splicing-generated protein* |
| HLF | : | *hepatic leukemia factor* |
| HNF | : | *hepatocyte nuclear factor* |
| Hsp | : | *heat shock protein* |
| HSPG | : | protéoglycane à héparane sulfate |
| IF | : | immunofluorescence |
| IFITM3 | : | *interferon-induced transmembrane protein 3* |
| IFNα | : | interféron α |
| IFNα-Peg | : | interféron α pégylé |
| ISG | : | *interferon stimulated genes* |

| | | |
|---|---|---|
| KO | : | *knock-out* |
| Kb | : | kilo base |
| L | : | *large* |
| LGP2 | : | *laboratory of genetics and physiology 2* |
| Log2FC | : | l*og2 fold change* |
| LV | : | lentivirus |
| M | : | *medium* |
| MDA5 | : | *melanoma differentiation-associated protein 5* |
| MEC | : | milieu extracellulaire |
| MOI | : | *multiplicity of infection* |
| N-ter | : | N-terminal |
| NAFLD | : | *non-alcoholic fatty liver disease* |
| NF-κB | : | *nuclear factor κ B* |
| NGS | : | *next generation sequencing* |
| NK | : | *natural killer* |
| NKT | : | *natural killer T cell* |
| NLR | : | *nucleotide oligomerization domain-like receptor* |
| NLS | : | *nuclear localization signal* |
| NTCP | : | *sodium/taurocholate cotransporting polypeptide* |
| NUC | : | *nucleos(t)ide analogs* |
| OMS | : | Organisation Mondiale de la Santé |
| ORF | : | *open reading frame* |
| PAMP | : | p*athogen-associated molecular pattern* |
| PEG | : | polyéthylène glycol |
| PHH | : | *primary human hepatocyte* |
| Pol | : | polymérase |
| PTH | : | *primary tupaia hepatocyte* |
| PRR | : | *pattern recognition receptor* |
| qRT-PCR | : | *quantitative reverse transcription – polymerase chain reaction* |
| Rb | : | retinoblastoma |
| RIG-I | : | *RIG-like receptor* |
| RE | : | réticulum endoplasmique |
| RT | : | reverse transcriptase |
| S | : | *small* |
| sb | : | simple brin |
| siARN | : | *small intererence* acide ribonucléique |
| sgRNA | : | *single guide ribonucleic acid* |
| STING | : | *stimulator of interferon genes* |
| TBK1 | : | *TANK-binding kinase 1* |
| TDP2 | : | t*yrosyl-DNA phosphodiesterase 2* |
| TLR | : | *Toll-like receptor* |
| TP | : | *terminal protein* |
| uPa | : | *urokinase plasminogen activator* |
| VHA | : | virus hépatite A |
| VHB | : | virus hépatite B |
| VHC | : | virus hépatite C |
| VHD | : | virus hépatite D |
| VHE | : | virus hépatite E |
| VIH | : | virus immunodéficience humaine |
| WHBV | : | *woodchuck hepatitis B virus* |
| WHO | : | *World Health Organization* |

# INTRODUCTION

### 1/ Le foie : Structure et fonctions

De couleur brun/rouge et pesant près de 1,5 kg chez l'Homme, le foie est la glande abdominale la plus volumineuse du corps humain. Cet organe, appartenant au système digestif est fortement vascularisé et contient près de 13 % du volume sanguin du corps.

Le foie est principalement considéré comme une glande exocrine produisant la bile. Il possède également une propriété endocrine avec la sécrétion de différentes hormones telles que l'angiotensine impliquée dans la pression artérielle ou la thrombopoïetéine stimulant la production de plaquette.

Souvent assimilé à une plaque tournante du métabolisme, le foie est impliqué dans de nombreux processus physiologiques. Ceci inclut la régulation du volume sanguin, l'homéostasie du système immunitaire, le métabolisme et l'homéostasie des glucides, des protéines et des lipides dont la synthèse de la majorité des protéines du plasma. Il possède également une fonction exocrine de sécrétion des acides biliaires composant la bile, liquide biologique favorisant la digestion, à partir du cholestérol. Le foie assure également des activités de détoxification et d'épuration avec la dégradation des composés xénobiotiques tels que les médicaments et dans la régulation hormonale (Abdel-Misih et Bloomston, 2010 ; Kalra *et al.*, 2020).

Le foie est composé de plusieurs types cellulaires spécifiques à l'organe **(Figure 1) :**

- Les hépatocytes sont des cellules parenchymateuses spécialisées et constituent 65% des cellules du foie. Ils sont organisés en travées autour de capillaires permettant la circulation sanguine dans l'organe.
- Les cellules endothéliales ou sinusoïdales, bordent les vaisseaux sanguins et ont la particularité de ne pas reposer sur la membrane basale. Cette caractéristique spécifique favorise les échanges entre le sang et les hépatocytes.
- Les cellules de Küpffer représentent environ 10% des cellules du foie et sont des macrophages sédentaires de l'organe.
- Les cellules stellaires hépatiques ou cellules de Ito représentent 5% des cellules du foie. Ce type cellulaire est spécifiquement présent au niveau de l'espace entre les hépatocytes et les cellules sinusoïdales, appelé espace de Disse. Les cellules stellaires hépatiques ont été décrites comme ayant des propriétés physiopathologiques qui après activation, induisent un processus inflammatoire ou fibrotique (Higashi *et al.*, 2017).

Tous ces types cellulaires sont organisés autour de capillaires appelés sinusoïdes. Ils ont la propriété d'assurer la circulation sanguine dans l'ensemble du foie et ainsi les échanges entre le sang

et les hépatocytes via les fenestrations et l'espace de Disse. En parallèle du flux sanguin, les canalicules biliaires permettent la circulation de la bile sécrétée par les hépatocytes vers la vésicule biliaire et les intestins, et favorisant ainsi la digestion (Si-Tayeb *et al.*, 2010).

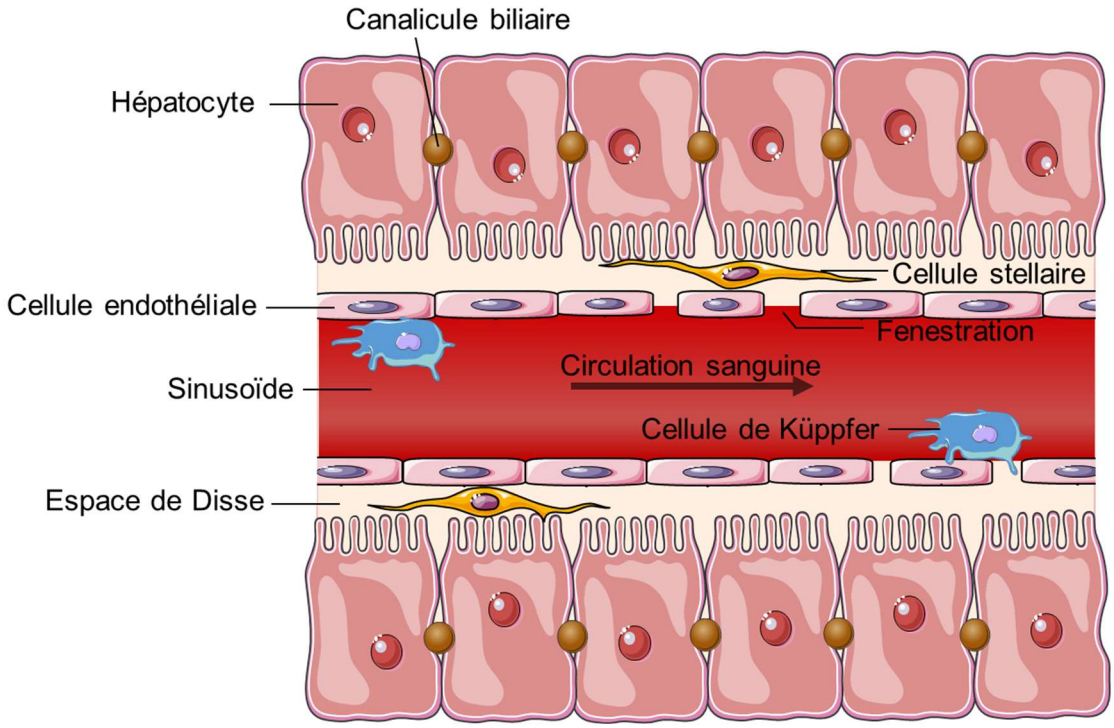


**Figure 1 : Représentation schématique de la structure d'une travée hépatique,** adaptée de (Prudencio *et al.*, 2006). Le sang sillonne le foie à travers des sinusoïdes qui sont recouverts d'un endothélium fragmenté de pores et fenestrations. Les cellules stellaires sont localisées dans l'espace de Disse, qui sépare le sinusoïde des hépatocytes et permet les échanges de métabolites entre le sang et les hépatocytes. Les canalicules biliaires permettent la circulation de la bile à travers le foie. Les cellules de Küpffer sont les macrophages sédentaires du foie qui migrent dans l'organe à travers les sinusoïdes.

### 2/ Les hépatites

L'affixe « hépato » provient du grec ancien « *hēpar* » pour décrire les pathologies du foie. Le terme « *hepatitis* » est dérivée de l'étymologie grec. La première description clinique de l'hépatite, la jaunisse, date de 400 ans avant Jésus-Christ est attribuée à Hippocrate :

" La **jaunisse** : si son corps est jaune, son visage jaune, ses yeux jaunes, si ses chairs deviennent flasques : c'est la **jaunisse.** "

- **Hippocrate** (460-370 environ avant J.C.)

L'hépatite désigne une atteinte inflammatoire du parenchyme du foie entraînant la destruction des hépatocytes. Elle peut provoquer une insuffisance hépatocellulaire plus ou moins importante et évoluer vers une forme grave dite fulminante, une cirrhose ou un cancer. En cas d'insuffisance hépatique terminale, seule la transplantation hépatique peut éviter la mort. Les hépatites sont majoritairement causées par des infections virales ou par la consommation abusive d'alcool, mais peuvent également être dues à certains médicaments ou toxines et à des maladies auto-immunes (Bernal et Wendon, 2013). Au vu des multiples fonctions vitales de cet organe, l'insuffisance hépatique est considérée comme un problème de santé majeur.

La physiopathologie des maladies hépatiques dépend de l'étiologie de l'insuffisance hépatique. Elles vont principalement générer une atteinte des voies métaboliques (lipides, graisses, protéines) et de détoxification, la perte des fonctions de stockage/libération des acides biliaires ou encore la transformation ou l'élimination des toxines ou bactéries. Toutes ces altérations sont généralement dues à une nécrose hépatocytaire ou à une importante apoptose des hépatocytes.

Les hépatites sont des maladies progressives pouvant évoluer vers une cirrhose ou encore un cancer du foie. Malheureusement, les traitements actuellement disponibles en cas de maladies hépatiques chroniques sont souvent de soutien, afin de prévenir ou traiter les symptômes de la maladie. Très souvent, seule la transplantation hépatique peut permettre la survie du patient (Chung *et al.*, 2012).

### 3/ Les hépatites virales

De nombreux virus peuvent être à l'origine d'une inflammation et de lésions hépatiques comme le cytomégalovirus (Sy *et al.*, 2013), le virus de l'herpès simplex (Flewett *et al.*, 1969) et le virus de la fièvre jaune (Oudart et Rey, 1970). Cependant, les hépatites virales sont très majoritairement provoquées par cinq virus hépatotropes nommés virus de l'hépatite A (HAV), B (HBV), C (HCV), D ou Delta (HDV) et E (HEV) (WHO, 2016).

Chaque année, les hépatites virales chroniques sont responsables de la mort de plus d'1,3 millions de personnes dans le monde, principalement due aux maladies chroniques du foie associées à l'infection virale telles que la cirrhose, l'insuffisance hépatique et au développement d'un cancer du foie (WHO, 2017).

L'épidémiologie et la pathogénèse des différents virus hépatiques ont été étudiées en détail. La caractérisation des structures et de l'organisation des génomes viraux ont permis le développement de thérapies et vaccins, ainsi que la prévention des maladies hépatiques associées comme la cirrhose et le carcinome hépatocellulaire (CHC) **(Tableau 1)**.

Une « Journée mondiale contre l'hépatite » a été créé par l'OMS (pour Organisation Mondiale de la Santé ou W*HO* pour *World Health Organization*) et a lieu le 28 juillet de chaque année. Cette date correspond à la date de naissance du Dr. Baruch Blumberg, qui découvrit le HBV. Cette journée a pour objectif de sensibiliser et d'informer les populations sur la prévention, la détection et les

traitements des hépatites. En 2016, l'Assemblée Mondiale de la Santé a adopté une stratégie internationale pour l'élimination des hépatites virales dans le monde d'ici 2030 (WHO, 2016). Cet objectif ambitieux nécessite encore de nombreuses avancées dans les domaines des hépatites virales chroniques et particulièrement sur le HCV et le HBV/HDV. En effet, en 2016, près de 6% de la population mondiale étaient chroniquement infectées par le HCV et/ou HBV dans le monde , soit près de 400 millions de personnes (WHO, 2016).

| Virus | Famille | Genre | Transmission | Génome | Chronicité | Vaccin | Thérapie curative |
|---|---|---|---|---|---|---|---|
| **HAV** | Picornaviridae | Hepatovirus | Orale | ARN-sb | - | Oui | Non |
| **HBV** | Hepadnaviridae | Orthohepadnavirus | Sang | ADN-dbp | + | Oui | Non |
| **HCV** | Flaviviridae | Hepacivirus | Sang | ARN-sb | +++ | Non | Oui |
| **HDV** | Hepadnaviridae | Deltavirus | Sang | ARN-sb | + | Oui | Non |
| **HEV** | *Hepeviridae* | Hepevirus | Oro-fécale | ARN-sb | - | A l'essai | Non |

**Tableau 1 : Classification, caractéristiques génomiques, modes de transmission et traitements des virus hépatotropes A, B, C, D et E.**

L'autorisation de mise sur le marché de plusieurs agents antiviraux à action direct (DAA) a permis la diminution de 30% du nombre de personnes chroniquement infectées par le HCV (WHO, 2017). Au contraire, aucun traitement actuel ne permet la guérison de l'infection chronique par le HBV. Malgré l'existence d'un vaccin prophylactique, le HBV reste une menace pour la santé mondiale et un frein à l'objectif d'éradication des hépatites virales dans le monde.

## L'INFECTION PAR LE VIRUS DE L'HEPATITE B

### 1/ Epidémiologie

Le HBV est la principale cause d'infection chronique du foie. En 2015, l'OMS estimait que plus de 257 millions de personnes étaient chroniquement infectées par ce virus à travers le monde (WHO, 2016). Cette même année, le nombre de morts estimé dues au HBV était chiffré à 887 000, le plus souvent des suites d'une cirrhose ou d'un CHC (Locarnini *et al.*, 2015 ; WHO, 2017)

A travers le monde, la prévalence des infections par le HBV est très hétérogène **(Figure 2).** Dans les zones de haute endémicité telles que l'Afrique Sub-saharienne et l'Asie du Sud-Est, plus de 8% de la population est porteuse chronique de l'infection par le HBV (Schweitzer *et al.*, 2015).

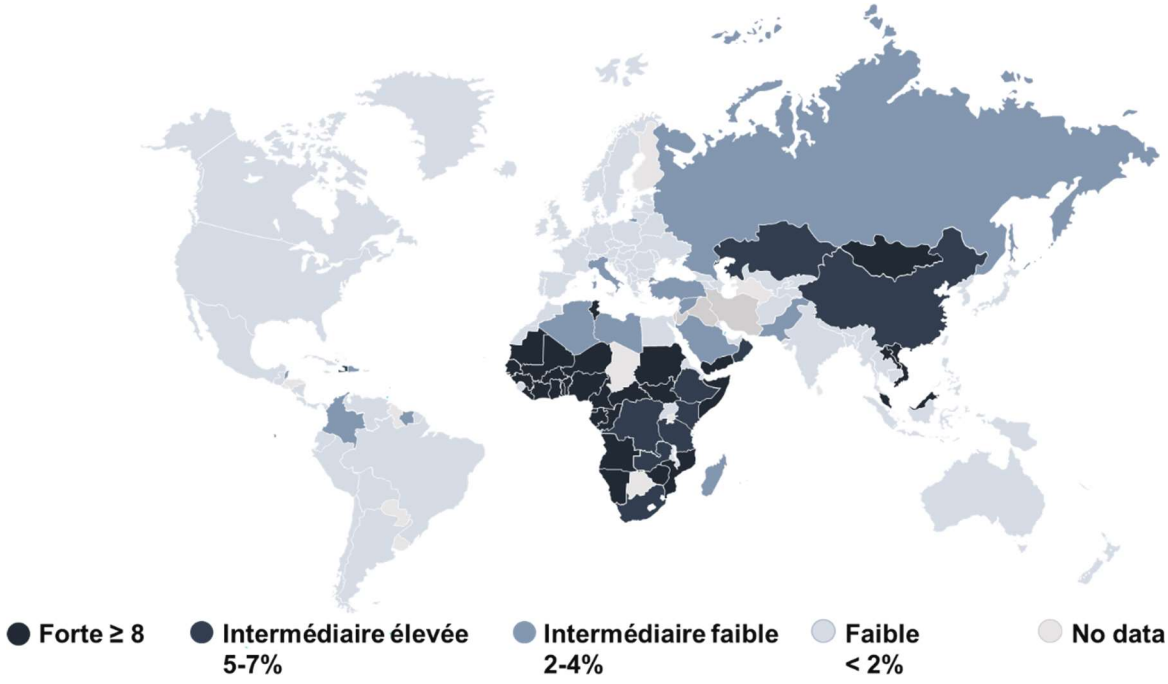


**Figure 2 : Carte de la prévalence de l'infection par le virus de l'hépatite B,** adaptée de (Schweitzer *et al.*, 2015).

### 2/ Transmission

Dans le cas du virus de l'hépatite B, il existe deux voies de transmission.

La voie horizontale correspond à la transmission par exposition à des fluides contaminés. La première description d'une épidémie d'hépatite transmise par le sang a été démontrée en 1885 chez 191 ouvriers de Breman en Allemagne. Ils présentaient des symptômes de jaunisse après une campagne de vaccination contre la variole, utilisant une préparation de lymphe humaine (Lurman, 1885). La contamination par exposition au sang peut donc survenir lors de transfusion sanguine ou transplantation d'organes, mais également lors d'utilisation de matériel médical contaminé ou dans le cadre des soins pour les personnels soignants (Trepo *et al.*, 2014). L'infection par voie sanguine a été démontrée comme très efficace et nécessitant moins de 20 particules virales pour infecter un foie entier (Candotti *et al.*, 2019). En parallèle, la voie sexuelle avec échange de fluides corporels est la voie de contamination principale (de Franchis *et al.*, 2003 ; Kidd-Ljunggren *et al.*, 2006). Le HBV est également détectable dans d'autres fluides corporels tels que la salive, les urines ou encore les larmes (Kidd-Ljunggren *et al.*, 2006). Selon l'OMS, le HBV peut se maintenir plus d'une semaine en dehors du corps humain sans perdre de sa virulence (WHO, 2017).

La voie de transmission verticale, ou périnatale, c'est-à-dire de la mère à l'enfant, est très fréquente dans les pays endémiques. Elle est la voie de transmission d'un tiers des contaminations dans les pays faiblement endémiques (WHO, 2017). La contamination se produit lors de l'accouchement par microtransfusions materno-fœtales au cours du travail ou par contact avec des sécrétions maternelles infectées. Lors des premiers mois après la naissance la transmission est possible par contact avec les fluides maternels contaminés (sang, salive, selles, urines ou lait maternel).

### 3/ Pathologies liées à l'infection par le HBV (Figure 3)

Le HBV est un virus non cytopathique et les pathologies associées à l'infection par le HBV proviennent majoritairement de la réponse immunitaire de l'hôte (Faure-Dupuy et Lucifora, 2016).

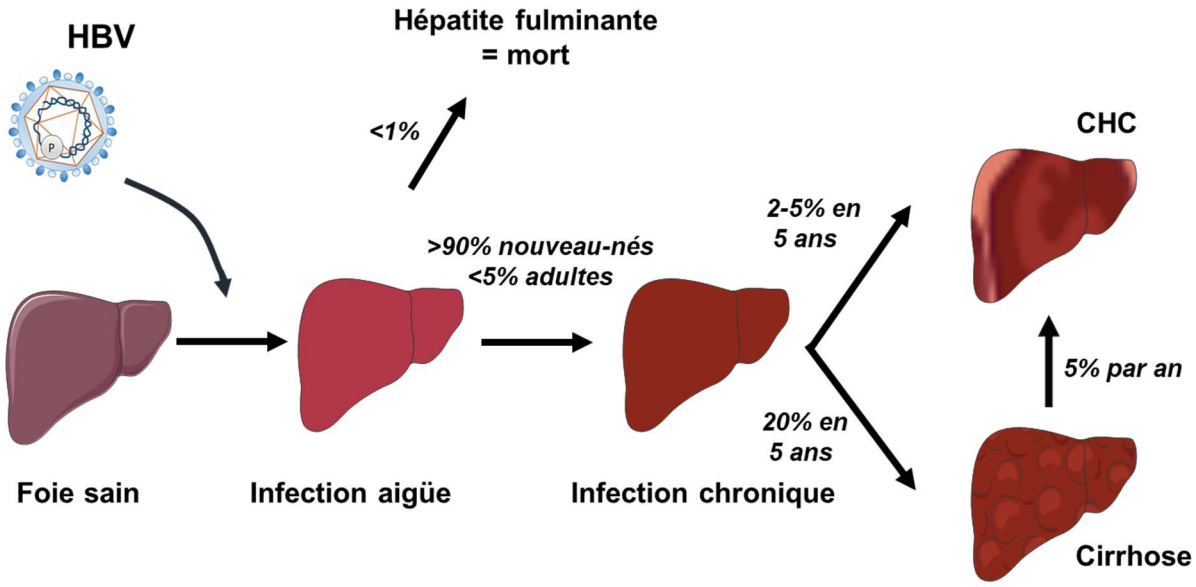


**Figure 3 : Schéma de la progression et des complications de l'infection chronique par le HBV.** Suite à la phase d'infection aigüe, moins de 5% des adultes développent une infection chronique contre plus de 90% pour les nouveau-nés. Après cinq ans d'infection chronique, environ 20% des cas évoluent en cirrhose puis dans environ 5% des cas par an en CHC. L'infection chronique peut également directement évoluer vers un CHC dans 2 à 5% des cas en cinq ans. Dans moins d'1% des cas, l'hépatite peut être fulminante et mortelle.

#### a) L'infection aigüe

L'infection aigüe est asymptomatique dans 75% des cas et donc non diagnostiquée. Dans les autres cas, les patients présentent une jaunisse, de la fatigue et des troubles digestifs dus à l'inflammation du foie. Durant cette phase, le taux d'alanine-amino-transférase (ALAT) - enzyme cytosolique des hépatocytes et marqueur des lésions hépatiques – est très élevé en raison de l'inflammation du foie.

La probabilité de progression de l'hépatite aiguë en hépatite chronique est fortement liée à l'âge au moment de l'infection. Alors que plus de 95 % des adultes infectés éliminent spontanément le virus en phase aigüe (Liang, 2009), les nourrissons infectés à la naissance ont 90% de chance de développer une infection chronique et les enfants de 1 à 5 ans ont 30% de chance (Edmunds *et al.*, 1993). La guérison de l'infection aigüe est liée au développement d'une immunité protectrice avec production d'anticorps dirigés contre les antigènes de surface du HBV - AgHBs (pour antigène de surface du HBV) - et ciblant les protéines de surface du virus (Fattovich, 2003).

b) L'infection chronique

L'infection est dite chronique lors d'une persistance virale, c'est-à-dire une détection de l'AgHBs, après six mois d'infection. Le passage en phase chronique est lié à l'inefficacité du système immunitaire à contrôler l'infection virale (Bertoletti et Ferrari, 2012). Dans 70 à 90% des formes chroniques, le patient ne développe pas de pathologie. Pour les autres, l'infection chronique par le HBV évolue en maladies progressives du foie telles que la fibrose, la cirrhose ou enfin le CHC.

c) Pathologies hépatiques viro-induites

La fibrose est la première étape de la dégénérescence hépatique. Elle correspond à une accumulation excessive de matrice extracellulaire (MEC), principalement due dans le cas des hépatites, à l'activation des cellules stellaires (Bataller et Brenner, 2005). Cet excès de MEC au niveau de l'espace de Disse induit la diminution des fenestrations des sinusoïdes et ainsi les échanges métaboliques entre le sang et les hépatocytes (Hernandez-Gea et Friedman, 2011). La fibrose peut être réversible dans le cas de dommages peu étendus et suite à l'élimination de la cause de la lésion hépatique (Ellis et Mann, 2012). Cette régression de la fibrose serait due à la dégradation de la MEC par des mécanismes physiologiques permettant le remodelage et la restauration de l'architecture de la matrice hépatique (Iredale *et al.*, 2013).

Après environ cinq ans d'infection chronique, 20% des patients voient évoluer leur fibrose en cirrhose, stade le plus avancé des maladies chroniques du foie et nécessitant à terme une transplantation hépatique (Tsochatzis *et al.*, 2014).

Selon le rapport du Global Disease Burden 2017, 40 % des CHC seraient dus au HBV (Collaborators., 2020). L'évolution de l'infection chronique du HBV vers le CHC est un processus multifactoriel (Buendia et Neuveut, 2015). Bien que l'intégration de l'ADN viral du HBV ne soit pas nécessaire pour le cycle réplicatif, certaines séquences virales défectives et réarrangées ont été observées dans le génome de la majorité des cellules de CHC liés au HBV (Tu *et al.*, 2018). L'intégration chromosomique est une des causes d'évolution de l'hépatite virale vers un cancer du foie et semble déjà avoir lieu dans la phase précoce de l'infection virale (Tu *et al.*, 2018). Par ailleurs, outre l'intégration du génome viral, l'âge, le genre masculin, la charge virale, la co-infection avec le HDV, HCV ou le virus de l'immunodéficience humaine (VIH), l'abus d'alcool, le diabète ou encore l'obésité sont d'autres facteurs augmentant le risque de développer un CHC chez les individus

chroniquement infectés par le HBV (Petruzziello, 2018). L'inflammation et l'accumulation de dommages génétiques, lors de la régénération massive du foie à la suite de la destruction des hépatocytes par le système immunitaire, sont des facteurs indirects de la carcinogénèse viro-induite (Bouchard et Navas-Martin, 2011). Par ailleurs, l'expression de la protéine virale X du HBV (HBx) module l'expression de différents mécanismes cellulaires tels que la transcription, la progression dans le cycle cellulaire et l'apoptose, processus ayant un rôle crucial dans le développement du CHC (Martin-Lluesma *et al.*, 2008 ; Wen *et al.*, 2008 ; Studach *et al.*, 2010). De manière intéressante, des il a été montré que la guérison de l'infection par le HBV n'éliminait pas totalement le risque de développement de CHC (Kanwal *et al.*, 2017).

d) L'infection chronique par le HDV et co-infection HBV/HDV

Le HDV ou virus delta (HDV) est un virus satellite du HBV qui détourne les antigènes de surface AgHBs du HBV lors de l'assemblage de ses particules infectieuses (Hughes *et al.*, 2011). L'hépatite D est la forme la plus sévère des hépatites chroniques viro-induites. Le HDV est le plus petit virus connu capable d'infecter les mammifères (Sureau et Negro, 2016).

Cette co-infection ou surinfection est associée à une progression plus rapide de la maladie hépatique et présente un risque trois fois supérieur de développer un CHC, en comparaison à l'hépatite B chronique seule (Fattovich *et al.*, 2000). Une récente analyse suggère que 13 à 14 % des personnes chroniquement infectées par le HBV sont également infectées par le HDV, ce qui représente 50 à 60 millions de personnes dans le monde (Miao *et al.*, 2020). Il n'existe actuellement aucun traitement efficace pour l'infection chronique par le HDV mais le vaccin préventif contre le HBV permet également une protection efficace contre le HDV. Récemment, Perez-Vargas *et al.*, a mis en évidence la possibilité que le HDV puisse également s'associer à des glycoprotéines de surface d'autres virus que le HBV comme le HCV (Perez-Vargas *et al.*, 2019).

### 4/ Prévention et traitement de l'infection chronique par le HBV

a) Le traitement préventif

Le vaccin contre le HBV est disponible depuis les années 1980. A l'origine, il était obtenu à partir de plasma sanguin provenant de patients chroniquement infectés par le HBV et dont les AgHBs ont été purifiés (Maupas *et al.*, 1976). Actuellement, la majorité des vaccins administrés dans le monde sont produits par génie génétique et composés d'AgHBs recombinants (Michel et Tiollais, 2010). La vaccination contre le HBV est donc un traitement préventif de l'infection par le HBV. Depuis 2018 en France, la vaccination contre le HBV est rendue obligatoire pour tous les nourrissons (Décret n°2018-42). Selon l'EASL (pour *european association for the study of the liver)*, en plus d'être effective sur l'infection par le HBV et au vu du nombre élevé de CHC induit par le HBV, la stratégie de vaccination semblerait également être efficace pour la prévention des CHC (EASL, 2017).

Le vaccin induit une réponse immunitaire humorale (taux d'anticorps anti-HBs sanguin supérieur à 10mUI/ml) chez 85 à 90% des patients. Néanmoins, 10 à 15% de la population ne répondent pas ou mal à la vaccination et sont dits non-répondeurs. De plus, dans les pays en voie de développement - souvent les plus touchés par le HBV – le risque accru de transmission mère-enfant ainsi que la difficulté de l'acheminement des vaccins reste une vraie limitation à la campagne de vaccination mondiale proposée par l'OMS (WHO, 2016).

b) Les traitements disponibles

Les traitements actuels de lutte contre le HBV sont considérés comme des traitements fonctionnels. Leur rôle est de contrôler l'infection afin d'améliorer la qualité de vie des patients et d'augmenter la survie par prévention ou frein à l'évolution des maladies progressives (Zoulim *et al.*, 2016). Au niveau physiologique, cela se traduit par un taux d'AgHBs et d'ADN viral non détectable dans le sérum.

En routine, deux classes d'antiviraux sont proposées pour le traitement de l'infection chronique du foie, l'interféron α ou les analogues de nucléos(t)ides (NUC pour *nucleos(t)ide analogs) (Zoulim et al., 2016)*.

La première option est l'administration d'interféron α-pégylé (IFNα-peg) (Greenberg *et al.*, 1976). Cette cytokine a pour rôle principal de moduler l'immunité en stimulant la voie de l'IFN et ainsi l'expression de gènes antiviraux nommés ISG (pour *interferon stimulated genes*). D'autre part, le traitement à l'IFNα-peg peut également activer la différentiation et l'activation des cellules immunitaires telles que les lymphocytes NK ou NKT (pour *natural killer et natural killer T cell)* (Tong *et al.*, 2017). Le traitement à l'IFNα-peg a l'avantage de pouvoir être administré sur un court terme avec un taux de séroconversion de 10 à 40 % après 3 ans (Zoulim *et al.*, 2016). Malheureusement, pour des raisons encore mal connues, ce type de traitement n'a qu'un faible effet antiviral sur le HBV avec un maximum de 40% de patients répondeurs et une efficacité dépendante du génotype (Erhardt *et al.*, 2005). La mauvaise diffusion de l'IFNα-peg ou la résistance des hépatocytes au traitement pourraient être des explications plausibles de la limite d'efficacité de ce type de molécule.

La deuxième classe de traitement sont les NUC. Ces molécules ciblent directement l'activité transcriptase inverse de la polymérase virale. La Lamividine (LMV) et la Telbivudine (LdT) sont des analogues de première génération, alors que l'Adefovir (ADV), le Tenofovir (TDF) ou l'Entecavir (ETV) sont des molécules de deuxième et troisième génération. Ces nouvelles générations de NUC ont été développées pour mieux lutter contre la résistance au traitement. En effet, il a été observé une résistance à la suite de l'administration sur le long terme de Lamivudine. Cette résistance a été démontrée comme étant liée à des mutations du génome viral au niveau du motif YMDD de l'activité catalytique de la polymérase virale (Halegoua-De Marzio et Hann, 2014). Le traitement par les NUC permet une guérison fonctionnelle chez environ 80% des patients mais très rarement une guérison totale. Agissant directement sur une étape du cycle viral, le traitement avec les NUC peut induire des

phénotypes de résistance du virus au traitement et ainsi limiter leur activité antivirale (Zoulim et Durantel, 2015).

Afin d'améliorer la réponse sérologique au traitement, l'EASL et l'AASLD *(*pour a*merican association for the study of the liver disease*) proposent de combiner le traitement à l'INFα-peg avec les NUC (EASL, 2017 ; Terrault *et al.*, 2018). Néanmoins, la guérison totale du HBV avec ces traitements reste rare et la prise de ces médicaments tout au long de la vie est souvent nécessaire (Werle-Lapostolle *et al.*, 2004). Dans ce contexte, de nouvelles stratégies thérapeutiques sont donc indispensables pour résoudre l'infection chronique afin d'éradiquer le HBV à travers le monde.

## LE VIRUS DE L'HEPATITE B

### 1/ Découverte du HBV

La sérendipité est la conjonction du hasard heureux qui permet au chercheur de faire une découverte inattendue d'importance ou d'intérêt supérieur à l'objet de sa recherche initiale, et l'aptitude de ce même chercheur à saisir et à exploiter cette chance. C'est donc avec sérendipité que le Dr. Baruch Blumberg et son équipe découvrirent pour la première fois l'antigène « Australia », qu'ils identifieront plus tard comme l'agent responsable de l'hépatite B (Blumberg *et al.*, 1965). Le Dr. Blumberg a été récompensé par le Prix Nobel de Physiologie ou de Médecine en 1976 pour sa découverte du HBV et pour le développement d'un test de détection diagnostic.

Peu de temps après la description de l'antigène « Australia », les premières images par microscopie électronique ont permis de mettre en évidence la morphologie icosaédrique des particules virales (Dane *et al.*, 1970). Ce n'est que bien plus tard que la structure du génome viral ainsi que sa séquence complète ont été publiées (Summers *et al.*, 1975 ; Galibert *et al.*, 1982).

### 2/ Génotypes, sérotypes et distribution

Dès 1972, le HBV a été divisé en quatre grands sérotypes adr, adw, ayr et ayw et dix sous-sérotypes selon les variabilités antigéniques de l'AgHBs. Plus tard, le premier séquençage du génome complet du HBV a permis de mettre en évidence la variabilité génétique du virus. Des analyses phylogénétiques ont permis de classer le HBV en 8 génotypes (A-H), basés sur une diversité de séquence de 8% (Okamoto *et al.*, 1988 ; Norder *et al.*, 1994 ; Stuyver *et al.*, 2000 ; Arauz-Ruiz *et al.*, 2002). Deux autres souches ont ensuite été proposées comme issues de nouveaux génotypes (I-J) (Tatematsu *et al.*, 2009 ; Yu *et al.*, 2010a), bien que la notion de génotype pour la souche « J » soit encore controversée en raison de sa faible divergence par rapport aux autres souches. Les génotypes peuvent également être divisés en plus de 35 sous-génotypes avec une divergence de plus de 4% (Kramvis, 2014).

La distribution géographique varie en fonction des génotypes (Kay et Zoulim, 2007). Par exemple, les génotypes A et D sont retrouvés de façon ubiquitaire dans le monde, les génotypes B et C sont essentiellement trouvés en Asie, le génotype E en Afrique sub-saharienne et les génotypes F, G et H en Amérique (Velkov *et al.*, 2018). Le génotype I est spécifique de l'Asie et J du Japon.

Plusieurs études suggèrent un lien entre le génotype et la progression de la maladie hépatique, le développement du CHC, voire la réponse au traitement par IFNα-peg (revu dans (Sunbul, 2014)). Pour exemple, il a été décrit dans les pays d'Amérique du Sud que la co-infection HBV/HDV, et donc la forme sévère de l'infection chronique par le HBV, est fréquemment associée avec le génotype F (Crispim *et al.*, 2014).

### 3/ Classification du HBV

Selon la dernière taxonomie en vigueur, établie par l'ICTV (pour *international committee on taxonomy of viruses*), datant de 2018, le HBV appartient au domaine des *riboviria* car son génome code pour une ADN polymérase-ARN dépendante (https://talk.ictvonline.org/taxonomy/). Selon la Classification de Baltimore, le HBV est un virus de classe VII, correspondant aux virus à ADN bicaténaire nécessitant un intermédiaire d'ADNsb. L'ancienne taxonomie classe le HBV dans la famille des *Hepadnaviridae* et au groupe *Orthohepadnaviridae.* Les hepadnavirus (pour « *hepatotropic DNA virus »*) rassemblent des petits virus à symétrie icosaédrique, dont le génome est constitué d'ADN partiellement bicaténaire, qui possèdent une activité de rétro-transcription et qui causent des infections du foie. Jusqu'à récemment, les hepadnavirus étaient divisés en deux groupes selon la spécificité d'espèce hôte du virus : les *avihepadnaviridae* qui touchent les espèces aviaires et les *orthohepadnaviridae* qui infectent les mammifères. Parmi les avihepadnavirus, les virus de l'hépatite B du canard de Pékin (Mason *et al.*, 1980) ou du héron cendré (Sprengel *et al.*, 1988) ont la particularité de ne pas exprimer l'antigène X (van Hemert *et al.*, 2011).

Parmi les orthohepadnavirus, on retrouve le HBV infectant l'Homme et les chimpanzés, ainsi que des virus apparentés infectant les primates, avec des souches particulières à chaque espèce (Vaudin *et al.*, 1988 ; Lanford *et al.*, 1998 ; Warren *et al.*, 1999 ; Wieland, 2015). D'autres, plus éloignés, infectent la marmotte américaine (Tyler *et al.*, 1981), l'écureuil (Marion *et al.*, 1980) ou différentes espèces de chauve-souris (Drexler *et al.*, 2013 ; He *et al.*, 2013).

De récentes études auraient démontré l'existence d'hepadnavirus pouvant infecter les amphibiens (*herpetohepadnaviridae*) (Dill *et al.*, 2016) et les poissons (*metahepadnaviridae*) (Hahn *et al.*, 2015) (**Figure 4).**

Récemment, une nouvelle pseudo-famille de virus de poisson, nommée *nackedhepadnaviridae*, a été décrite comme ayant les caractéristiques clés des hepadnavirus - réplication avec étape de rétro-transcription, capside icosaédrique - mais ne possédant pas le gène de la protéine d'enveloppe S (Lauber *et al.*, 2017).

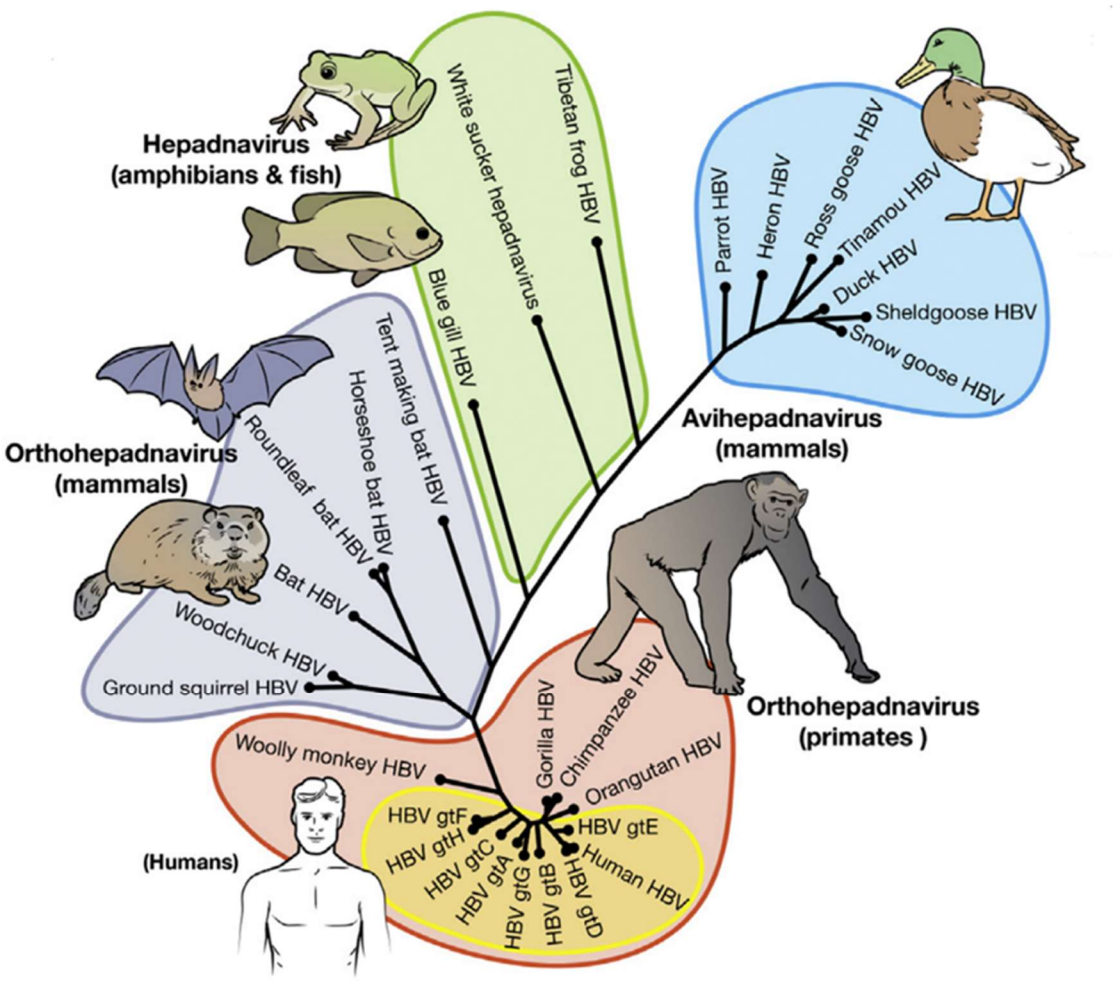


**Figure 4 : Arbre phylogénétique de la famille des hepadnavirus,** d'après (McNaughton *et al.*, 2019). La famille des hépadnavirus est divisée en deux groupes, les *orthohepadnaviridae* de mammifères et de primates et les *avihepadnaviridae* d'oiseaux. Un troisième groupe composé des virus d'amphibiens et de poissons a récemment été identifié.

L'hôte principal du HBV est l'homme (Dane *et al.*, 1970) mais ce virus est également capable d'infecter expérimentalement des primates non-humains tels que le chimpanzé et le gibbon ainsi qu'une petite musaraigne arboricole, le Toupaye de Belanger (*Tupaia Belangeri*) (Dane *et al.*, 1970 ; Dienes *et al.*, 1990 ; Walter *et al.*, 1996 ; Lanford *et al.*, 2000). Dans la nature, aucune infection naturelle n'a été démontrée pour ce mammifère et des souches spécifiques aux primates non-humains ont été décrites (Vaudin *et al.*, 1988 ; Lanford *et al.*, 1998 ; Warren *et al.*, 1999 ; Wieland, 2015).

Les récentes découvertes sur la phylogénie du HBV ont permis de faire évoluer les hypothèses sur l'origine du HBV. Lauber *et al.* émettent l'hypothèse d'une origine ancienne du HBV qui proviendrait de virus de poisson non-enveloppés (Lauber *et al.*, 2017). Au sein des mammifères, certaines analyses phylogénétiques suggèrent une évolution récente du HBV à partir d'un virus infectant le Toupaye de Belanger (Rasche *et al.*, 2019).

### 4/ Les cellules cibles du HBV

a) Les hépatocytes

Le HBV infecte exclusivement le foie, et en particulier un seul type cellulaire, les hépatocytes (Seeger et Mason, 2000). Ce sont des cellules différenciées ayant une demi-vie de plus de 6 mois. Elles ont également la caractéristique d'être quiescentes en conditions physiologiques. La combinaison d'une longue durée de vie et d'une faible croissance cellulaire est une des causes de la persistance virale après infection. De manière intéressante, il a été montré que la réplication du HBV était dépendante de la différentiation et de la division cellulaire de la cellule hôte (Ozer *et al.*, 1996).

Le tropisme cellulaire du HBV s'explique par les interactions entre le virus et des facteurs cellulaires spécifiques des hépatocytes. Ainsi, il a été démontré que les facteurs nucléaires hépatocytaires tels que HNF1α, HNF3β, HNF4α (pour *hepatocyte nuclear factor 1α, 3β, 4α*) ou HLF (pour *hepatic leukemia factor*) étaient primordiaux pour la réplication du HBV (Ishida *et al.*, 2000 ; Kim *et al.*, 2016). De plus, le transporteur des acides biliaires NTCP (pour *sodium-$Na^+$ Taurocholate Cotransporting Polypeptide*), quasi-exclusivement exprimé à la surface basolatérale des hépatocytes, est un récepteur indispensable à l'entrée virale HBV (Yan *et al.*, 2012 ; Ni *et al.*, 2014).

b) Les réservoirs extra-hépatiques du HBV

La réplication et la dissémination efficace du virus n'ont été démontrées que dans les hépatocytes. Pourtant, de l'ADN viral a pu être détecté chez des patients chroniquement infectés dans les tissus de pancréas, de rein, de rate, d'ovaire, de cœur et de la peau. Les cellules mononucléées du sang périphérique (PBMC) ou les cellules de la moelle osseuse peuvent également présenter des traces de HBV (Seeger *et al.*, 2000). Néanmoins, aucune réplication active n'a été décrite dans ces tissus.

### 5/ Les particules virales

Le HBV est l'un des plus petits virus actuellement connus. Le HBV a été observé pour la première fois par microscopie électronique dans les années 1970 et se présente sous différentes formes (Dane *et al.*, 1970 ; Huang *et al.*, 1972) **Figure 5 :**

- Les particules de Dane sont les formes infectieuses du virus et ont une taille de 42 nm. (Seeger *et al.*, 2000). Ces virions sont composés du génome du HBV sous une forme circulaire partiellement double brin associé à la polymérase virale, et entouré par une nucléocapside de 27 nm. La capside icosaédrique est enveloppée d'une bicouche lipidique provenant du bourgeonnement viral lors de la sortie de la cellule hôte. L'enveloppe porte les trois formes des glycoprotéines du virus à sa surface.
- Les sphères et les filaments sont les formes majoritairement sécrétées par les hépatocytes infectées ($10^4$ fois plus que les particules de Dane) (Blumberg, 1977). Les

filaments peuvent avoir une taille de plus de 100 nm et sont composés des formes S (pour *small*), M (pour *medium*) et L (pour *large*) des AgHBs, alors que les sphères ne font que 22 nm et ne sont composées que des formes S et M (Huang *et al.*, 1972 ; Heermann *et al.*, 1984). Il semblerait que ces formes virales leurrent l'immunité humorale de l'hôte en étant des cibles pour les anticorps neutralisants (Rydell *et al.*, 2017).

- Les particules vides sont composées des protéines de surface et d'une capside vide. La structure et le rôle de ces particules non infectieuses sont encore controversés.
- Les particules à ARN sont constituées d'une nucléocapside enveloppée contenant le génome viral sous forme d'ARN non rétrotranscrit. Cette forme particulière est encore peu décrite (Wang *et al.*, 2016).

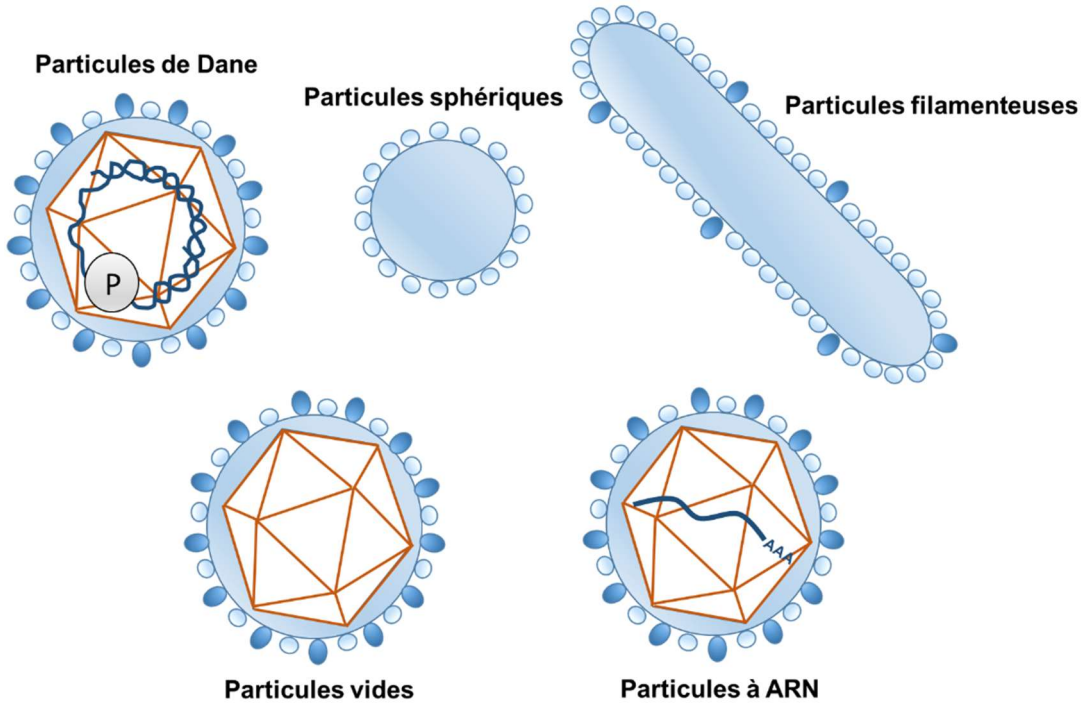


**Figure 5 : Représentation schématique des formes infectieuses et non infectieuses du HBV.** Les particules infectieuses, aussi appelées particules de Dane, sont enveloppées d'une bicouche lipidique portant les glycoprotéines virales de surface, qui entourent une nucléocapside contenant le génome viral. Les particules non infectieuses correspondent aux particules subvirales sphériques et filamenteuses, aux particules vides et aux particules contenant de l'ARN viral.

Les virions et les filaments ont été décrits comme partageant la même voie de sécrétion *via* les complexes ESCRT (pour *Endosomal Sorting Complex required Tranport)* et les corps multivésiculaires (Jiang *et al.*, 2015). Au contraire, les particules sphériques seraient sécrétées *via* d'autres voies de sécrétion cellulaire telle que la voie constitutive (via Golgi) (Patient *et al.*, 2009). Pour les autres formes virales, les voies impliquées dans la sécrétion restent méconnues (Hu et Liu, 2017).

### 1/ Organisation génétique

#### a) L'ADN relâché : ADN-rc

Dans les virions infectieux, le génome viral est présent sous forme d'ADN circulaire partiellement double brin de 3,2 kilo bases (kb) relâché appelé ADN-rc (Summers *et al.*, 1975). **Figure 6**

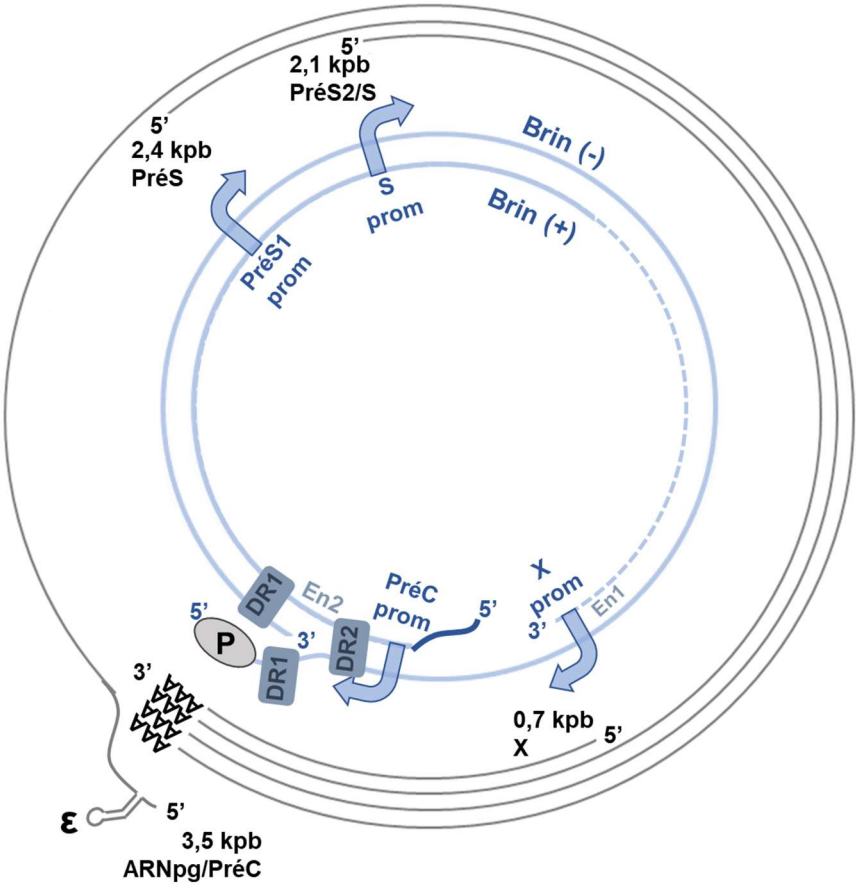


**Figure 6 : Organisation génétique du HBV,** d'après (McNaughton *et al.*, 2019). Le génome du HBV, représenté en bleu, est une structure circulaire partiellement double brin de 3,2 kb appelée ADN-rc. Le brin négatif (-) est lié à la polymérase viral P en 5'. Le brin (+) varie en taille et possède une séquence amorce d'ARN de 19 nucléotides représentée en bleu foncé. L'expression du génome est induite par quatre promoteurs nommés PréC prom, PréS1 prom, S prom, et X prom. Les séquences redondantes DR1/DR2 jouent un rôle dans la synthèse du génome viral lors de la réplication. En gris sont représentés les quatre ARN viraux synthétisés à partir du génome. L'ARNpg/préC possède une boucle ε en 5' impliquée dans l'encapsidation du génome lors de la réplication.

L'ADN-rc est composé d'un brin négatif (-), non clos, qui sert de matrice lors de la transcription des ARN viraux et qui est lié à la polymérase virale (Pol) en 5' (Gerlich et Robinson, 1980). Le brin positif (+) est également incomplet, de taille variable, et caractérisé par un court oligomère d'ARN en 5', ayant servi lors de la synthèse du brin (-) (Summers et Mason, 1982 ; Lien *et al.*, 1986). Ce brin de taille variable est composé d'environ 2/3 du génome et n'est pas transcrit lors de la réplication (Seeger et Mason, 2015). Une région cohésive d'environ 200 paires de base en 5' de chaque brin permet d'assurer la circularisation du génome lors de la réplication. En effet, des séquences redondantes DR1 et DR2, d'environ 11 nucléotides, sont indispensables à la synthèse de l'ADN viral (Nassal, 2015).

Le génome possède également des régions régulatrices de la transcription et de la réplication. Quatre promoteurs permettent l'initiation de la transcription et deux *enhancers* (En1 et En2) favorisent la transcription par liaison d'activateurs. L'ADN viral contient un unique signal de polyadénylation et un signal d'encapsidation ε (Nassal, 2015 ; Valaydon et Locarnini, 2017).

b) ADN circulaire clos de façon covalente : ADNccc

Lors de la réplication, l'ADN-rc nu pénètre dans le noyau de la cellule infectée sous la forme d'un épisome. Le génome viral subit des modifications structurales et est converti en une nouvelle forme superenroulée appelée ADNccc. La conversion de l'ADN-rc en ADNccc est un processus multifactoriel encore largement méconnu, qui impliquerait les mécanismes cellulaires de réparation de l'ADN dont des ADN polymérases, des ligases et des topoisomérases (Beck et Nassal, 2007). La forme superenroulée de l'ADNccc est due à son association avec des histones qui lui confèrent une structure en collier de perle et permettent la régulation de sa transcription (Bock *et al.*, 1994 ; Newbold *et al.*, 1995). D'autres facteurs cellulaires de transcription, des enzymes de modification des histones ainsi que des protéines virales telles que HBc, et indirectement HBx, interagissent également avec l'ADNccc et agissent donc dans la réplication du HBV (Lucifora et Protzer, 2016). **(Figure 7)**

Le nombre de copies ADNccc par hépatocyte infecté est estimé entre 0,1 à 10 (Werle-Lapostolle *et al.*, 2004). Chez les patients chroniquement infectés, l'ADNccc persiste dans le noyau sous la forme d'un minichromosome et le rebond de l'infection virale après arrêt du traitement antiviral indique que l'ADNccc reste en latence dans les hépatocytes infectés, et ceci pendant des années (Rehermann *et al.*, 1996 ; Bock *et al.*, 2001).

L'ADNccc est primordial pour la réplication virale car il est la matrice pour la synthèse de tous les transcrits viraux dont l'ARNpg, précurseur de l'ADN viral néosynthétisé (Nassal, 2015).

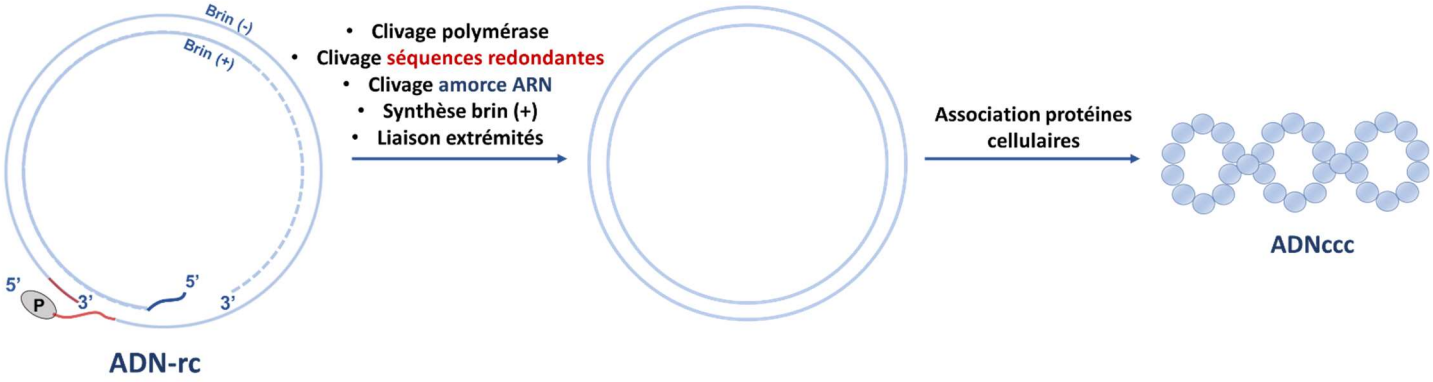


**Figure 7 : Représentation schématique de la conversion de l'ADN-rc en ADNccc,** d'après (Lucifora *et al.*, 2016). La formation de l'ADNccc à partir de l'ADN-rc nécessite le clivage de la polymérase en 5' du brin (-), de l'amorce ARN en 5' du brin (+) et des séquences redondantes DR1/2. La liaison des extrémités implique des mécanismes de réparation de l'ADN, de modifications de structure, de synthèse et de liaison. Le surenroulement d'ADN viral implique des protéines cellulaires telles que les histones.

c) ARN pré-génomique : ARNpg

L'ARNpg est synthétisé par transcription de l'ADNccc par la polymérase cellulaire II et est la matrice pour la traduction de la protéine core (AgHBc) et de la polymérase virale Pol (Rall *et al.*, 1983 ; Sells *et al.*, 1988). Lors de la réplication, l'ARNpg s'associe avec des protéines AgHBc et Pol afin de former une nucléocapside immature (Seeger *et al.*, 2000). Cette étape est dépendante de la liaison de Pol à la séquence d'encapsidation ε présent en 5' de l'ARNpg, qui régule également la transcription inverse de l'ARNpg en ADN afin de former une nucléocapside mature (Bartenschlager *et al.*, 1990 ; Knaus et Nassal, 1993).

d) ADN double brin linéaire : ADNdbl

Chez les patients chroniquement infectés, environ 10% des nucléocapsides matures libérées contiennent une forme double brin linéaire du génome viral (ADNdbl) (Tu *et al.*, 2017). Lors de la réplication virale et en particulier lors de la rétrotranscription de l'ARNpg en ADN, la polymérase virale conserve une séquence d'ARN de 18 nucléotides en 5' de la matrice, qui servira d'amorce à la synthèse du brin (+). La circularisation de l'ADN viral est induite par la fixation de l'amorce au niveau de la séquence DR2 du brin (-) néosynthétisé. Au contraire, une fixation de l'amorce sur la séquence DR1 induit la conservation de la forme linéaire de l'ADNdb (Staprans *et al.*, 1991 ; Yang *et al.*, 1996). Comme la forme circulaire, la forme linéaire du génome viral peut être libérée dans le milieu extracellulaire ou recyclée dans le noyau (Zhang *et al.*, 2003). Néanmoins, le recyclage des nucléocapsides contenant de l'ADNdbl peut engendrer la formation d'ADNccc défectueux par recombinaison non homologue (NHEJ). De plus, la forme double ADNdbl du HBV est également le substrat principal de l'intégration de l'ADN viral dans le génome de la cellule hôte (Staprans *et al.*, 1991 ; Yang *et al.*, 1996).

e) Intégration de l'ADN viral

L'intégration de l'ADN du HBV dans le génome des cellules infectées est décrite depuis de nombreuses années (Yang et Summers, 1999 ; Mason *et al.*, 2016 ; Tu *et al.*, 2018). Contrairement aux rétrovirus, l'intégration du HBV n'est pas nécessaire à la réplication virale (Tu *et al.*, 2017) et se produirait dans les modèles animaux dans environ une cellule sur 100 en phase précoce de l'infection virale (Caballero *et al.*, 2018 ; Tu *et al.*, 2018). L'intégration implique la forme ADNdbl du génome du HBV et le mécanisme de réparation de l'ADN en jonction d'extrémités non homologues alternative (A-NHEJ) (Yang *et al.*, 1999 ; Zhao *et al.*, 2016).

Dans les tissus non tumoraux, l'intégration de l'ADN du HBV semble être aléatoire et dispersée dans tout le génome cellulaire (Sung *et al.*, 2012). Cependant, des études ont montré que l'intégration au niveau de certains sites chromosomiques favorisent l'expansion clonale des hépatocytes décrivant ainsi un mécanisme possible de carcinogénèse. Pour exemple, plusieurs études ont montré une intégration récurrente d'ADN viral au sein des oncogènes codant pour TERT (pour transcriptase inverse de la télomérase) ou MLL4 (pour *mixed-lineage leukemia 4*) (Paterlini-Brechot *et al.*, 2003 ; Saigo *et al.*, 2008 ; Nault *et al.*, 2013 ; Nault *et al.*, 2014).

**2/ Transcription du génome**

a) Cadre de lecture

L'ADN du HBV présente une organisation de lecture complexe avec le chevauchement de 4 cadres ouverts de lecture, ORF (pour *Open Reading Frame*), répartis sur le brin (-) et permettant la synthèse des sept protéines du HBV. **Figure 6**

- ORF P code pour la polymérase virale et chevauche tous les autres ORF
- ORF C (préC/C) code pour la protéine structurale core (AgHBc) et l'antigène soluble E (AgHBe)
- ORF S (PreS1/PreS2/S) code pour les trois protéines de surface (AgHBs)
- ORF X est le plus petit ORF et code pour la protéine soluble X (HBxAg)

La superposition des ORF se traduit par le fait que 2/3 des nucléotides du génome codent pour plus d'un élément fonctionnel.

b) Transcrits viraux

Lors de la réplication, le génome viral est transcrit par la machinerie cellulaire en cinq ARN viraux dont quatre codent pour des protéines virales (Cattaneo *et al.*, 1983 ; Sells *et al.*, 1988) :

- ARN PréC de 3,5 kb pour le précurseur de l'AgHBe
- ARN PréS1 L de 2,4 kb pour la synthèse de la protéine de surface L
- ARN PréS2/S de 2,1 kb pour la synthèse des protéines de surface M et S

- ARN X de 0,7 kb pour la synthèse de HBx.
- ARNpg de 3.5 kp pour la synthèse de la protéine core (AgHBc) et de la polymérase (Pol)

Il est à noter que l'ARN PréC et l'ARNpg ont une taille supérieure à la taille initiale du génome (3,2 kb pour le génome et 3,5 kb pour les transcrits). Le site de polyadénylation est en effet ignoré par la polymérase lors de son premier passage, entrainant la production de transcrits plus longs.

c) Epissage alternatif

Tous les ARNm subissent une maturation avec ajout d'une coiffe et d'une queue polyadénylée. En plus des ARN décrits précédemment, de nombreux autres transcrits sont synthétisés à la suite de l'épissage alternatif de l'ARNpg (Suzuki *et al.*, 1989 ; Wu *et al.*, 1991). Le variant majoritairement épissé à partir de l'ARNpg est nommé SP1RNA et est délété d'1/3 du génome (Δ2447/489). Il peut représenter jusqu'à 30% des ARNpg totaux chez un patient chroniquement infecté. Certains de ces ARN épissés peuvent être assemblés, rétro-transcrits et libérés sous la forme de particules virales défectives (Terre *et al.*, 1991). De manière encore controversée, la production et le ratio de ces particules virales défectives auraient un lien avec la progression des maladies hépatiques (Chen *et al.*, 2015), dans l'échec au traitement basé sur l'interféron (Bayliss *et al.*, 2013), ainsi que dans l'échappement à la réponse immunitaire (Duriez *et al.*, 2017). Les différents ARN viraux sont traduits en sept protéines virales.

### 3/ Traduction du génome

a) Les protéines de surface : AgHBs

Le cadre de lecture S, composé des gènes S, PréS1 et PréS2, code pour la synthèse de trois protéines de surface appelées AgHBs S, M, et L. Lors de la réplication, l'ARN viral PréS1 sert de matrice pour la traduction de L et l'ARN PréS2/S pour M et S (Heermann *et al.*, 1984). La présence d'un unique codon stop dans les transcrits PréS1 et PréS2/S induit la synthèse de protéines partageant la même extrémité C-terminale (C-ter) composée du domaine S. M et L sont composées de S et des extensions PréS2 ou PréS2/PréS1 respectivement au niveau N-terminal (N-ter) (Seeger *et al.*, 2000) **Figure 8.**

Les protéines de surface sont synthétisées à la membrane du réticulum endoplasmique (RE) et subissent des maturations post-traductionnelles au niveau de l'appareil de Golgi (Chua *et al.*, 2005). Ainsi, le domaine commun S et la partie N-ter de M possèdent un site de N-glycosylation et la protéine L peut être myristoylée en N-ter (Urban *et al.*, 2014).

Une fois mature, les protéines de surface forment des homo- ou hétérodimères qui bourgeonnent au niveau de la lumière du RE. Elles sont alors transportées dans l'appareil de Golgi puis secrétées au niveau de la membrane cellulaire. Lors du bourgeonnement, les particules de Dane seront composées des protéines de surface L, M et S selon un ratio 1 :1 :4 enfermant une

nucléocapside mature (Heermann *et al.*, 1984). Les domaines PréS1 et PréS2 ont été décrits comme ayant un rôle dans la liaison avec la nucléocapside lors de l'enveloppement (Seitz *et al.*, 2007). En parallèle, les protéines de surface M et S peuvent former des particules immatures.

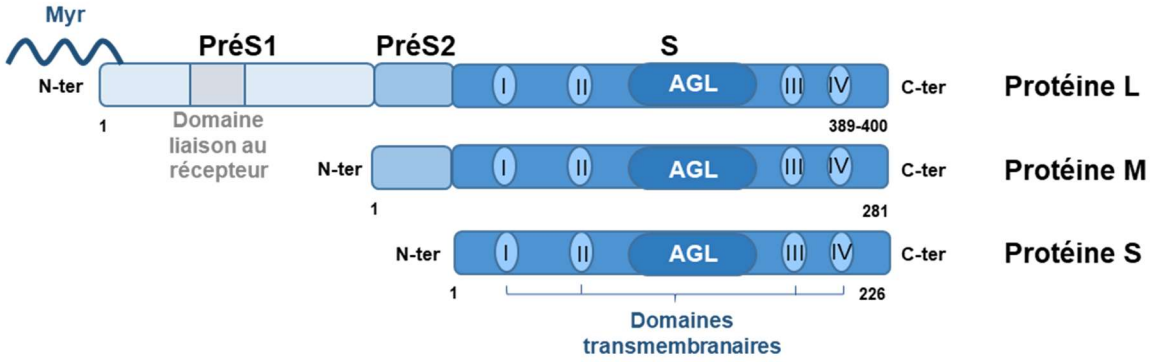


**Figure 8 : Représentation schématique de la structure des protéines de surface AgHBs du HBV,** inspirée de (Urban *et al.*, 2014). Les protéines de surface S, M et L partagent la même séquence S en C-ter. S est composé de 4 domaines transmembranaires I, II, III et IV. La boucle antigénique AGL et la séquence myristoylée en N-ter sont impliqués dans l'entrée virale.

Les protéines de surface ont un rôle clé dans l'infectiosité des particules virales (Urban *et al.*, 2014). En effet, le domaine PréS1 de la protéine L possède une extrémité myristoylée, indispensable à l'attachement du HBV à la surface des hépatocytes et jouant un rôle dans la spécificité de l'hôte (Bruss *et al.*, 1996 ; Engelke *et al.*, 2006 ; Meier *et al.*, 2013). La boucle antigénique AGL (pour *antigenic loop*) formée au niveau des domaines transmembranaires II et III de S est également un déterminant dans l'entrée virale et est impliquée dans la liaison avec les HSPG (pour protéoglycanes à héparane sulfate) à la surface des hépatocytes (Sureau et Salisse, 2013).

Lors de la découverte du HBV, l'antigène « Australia » décrit par Blumberg *et al.* correspondait à l'antigène HBs (Blumberg *et al.*, 1965). En effet, étant exposées sur la face externe du virus, les protéines de surface sont fortement immunogènes (Fagan et Williams, 1986 ; Waters *et al.*, 1986). Elles induisent la production d'anticorps neutralisants par le système immunitaire de l'hôte et ont été utilisées lors de la conception du vaccin anti-HBV.

b) <u>La protéine core : AgHBc</u>

L'antigène AgHBc est codé par l'ORF PréC et est synthétisé à partir de l'ARNpg. Cette protéine de 21 KDa est l'unité de structure de la capside virale (Cohen et Richmond, 1982 ; Zlotnick *et al.*, 2015). Après sa synthèse, la protéine HBc est rapidement dimérisée. La capside du HBV est composée d'un assemblage d'hexamères de dimères jusqu'à obtenir une structure icosaédrique de 22 nm (Birnbaum et Nassal, 1990). La capside est ainsi composée de 120 dimères de AgHBc. L'extrémité N-ter de la protéine correspond au domaine de dimérisation et d'assemblage de la capside (Birnbaum *et al.*, 1990).

Outre son importance dans la structure de la particule virale, de nombreuses études décrivent également un rôle de HBc dans d'autres étapes du cycle viral (Diab *et al.*, 2018). Ainsi, la région C-ter est impliquée dans la liaison avec l'ARNpg lors de l'encapsidation et contient un signal de translocation nucléaire (Li *et al.*, 2010). HBc joue également un rôle de protéine chaperonne et de régulateur de la transcription de l'ADNccc par modification épigénétique (Bock *et al.*, 2001).

c) La protéine HBe : AgHBe

La protéine HBe est synthétisée à partir de l'ARN PréC et à la suite du clivage protéolytique de son précurseur PréC (Takahashi *et al.*, 1983). Cette forme tronquée de PréC est sécrétée dans le milieu extracellulaire et est appelée AgHBe. Cet antigène soluble de 17 KDa ne semble pas nécessaire à la réplication virale et n'intervient pas dans la structure du virus. Des mutations défectives pour la production d'AgHBe ont été retrouvés chez de nombreux patients. Il a été suggéré que ces souches mutantes pourraient être corrélées à des hépatites fulminantes. Ainsi, l'AgHBe serait impliqué dans le contrôle de la sévérité de l'infection (Scaglioni *et al.*, 1997).

L'AgHBe, sécrété dans le milieu extracellulaire, est également immunogène. La production d'anticorps anti-AgHBe indique en général une diminution de la réplication virale et ces anticorps sont actuellement utilisés en diagnostic comme marqueur sérologique (Fagan *et al.*, 1986).

d) La polymérase virale : Pol

La polymérase virale Pol est codée par l'ORF P et traduite à partir de l'ARNpg pour obtenir une protéine multifonctionnelle de 91 KDa.

Cette enzyme a pour rôle de synthétiser l'ADN viral à partir de l'ARNpg, tout en dégradant la matrice dans la nucléocapside néo-formée. Elle est composée de 4 domaines ayant chacun une fonction spécifique (Radziwill *et al.*, 1990) **Figure 9 :**

- Le domaine N-ter, appelé TP (pour *terminal protein*) est spécifique aux hepadnavirus et joue un rôle dans la synthèse du brin (-) par un mécanisme de protéine-amorce via un résidu tyrosine en position 63 (Lanford *et al.*, 1999).
- Un domaine espaceur favorisant la flexibilité de la protéine. Cette séquence est superposée avec la région PréS1 sur le génome et est la région la plus variable du génome.
- Le domaine de l'activité catalytique de rétrotranscriptase ou RT possédant une séquence hyperconservée YMDD. Cette région est responsable de l'activité ADN polymérase-ARN dépendante pour la synthèse du brin (-) à partir de l'ARNpg et ADN polymérase-ADN dépendante pour la synthèse du brin (+) à partir du brin (-).
- Le domaine C-ter comprend l'activité ribonucléase H de l'enzyme qui catalyse la digestion de la matrice ARNpg après la synthèse du brin (-).

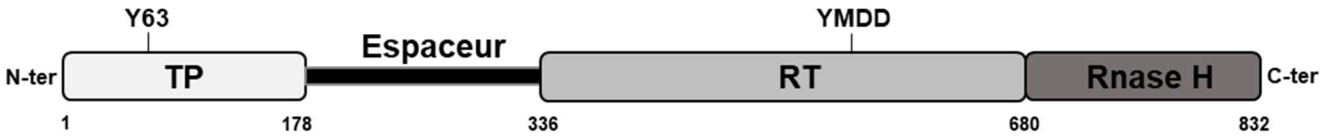


**Figure 9 : Représentation schématique de la polymérase virale du HBV,** inspirée de (Nassal, 2008). Le domaine TP initie la synthèse d'ADN via un mécanisme amorce-protéine. Le domaine RT permet l'activité ADN polymérase-ARN dépendant et ADN polymérase-ADN dépendant. Le domaine RNase H permet l'activité ribonucléase H. L'espaceur permet la flexibilité de l'enzyme.

Le magnésium ainsi que certaines protéines chaperonnes sont indispensables à l'activité de la polymérase et sont encapsidés lors de l'assemblage (Seeger *et al.*, 2000 ; Beck *et al.*, 2007).

Pol ne possède pas d'activité de relecture. Cette caractéristique implique la possibilité d'insérer des mutations lors de la réplication du génome (Park *et al.*, 2003) et l'évolution du génome avec sélection de souches soumises à la pression de sélection. La polymérase, et en particulier son activité de rétrotranscription, est la cible des traitements antiviraux actuellement utilisés basés sur l'utilisation de NUC chez les patients chroniquement infectés (Zoulim *et al.*, 2016).

e) <u>La protéine X : HBx</u>

La protéine X est codée par l'ORF X et synthétisée à partir du plus petit transcrit du génome viral, l'ARN X (Guo *et al.*, 1991). HBx est produit par tous les hepadnavirus excepté le genre des Avihepadnavirus où elle n'est pas fonctionnelle (van Hemert *et al.*, 2011). Le nom X de cette protéine de 17,5 KDa provient du fait qu'elle ne présente aucune homologie avec d'autres protéines connues, ne permettant pas de supposer son rôle dans le cycle viral (Bouchard et Schneider, 2004). Depuis, de nombreuses études ont décrit HBx comme étant un facteur favorisant la réplication virale via des mécanismes directs ou indirects impliquant la machinerie cellulaire (Lucifora *et al.*, 2011 ; Slagle et Bouchard, 2018). Hbx est caractérisé par une localisation nucléaire, cytoplasmique mais également mitochondriale et agit sur la réplication à différentes étapes du cycle viral. Par exemple, HBx est impliqué dans l'activation de la transcription virale par un mécanisme épigénétique d'inhibition de la désacétylation des histones liés à l'ADNccc (Tang *et al.*, 2005 ; Belloni *et al.*, 2009) ou dans l'amélioration de l'activité de la polymérase via la voie de signalisation du calcium au niveau de la mitochondrie (Bouchard *et al.*, 2001). Au niveau cytoplasmique, HBx a également un rôle dans la régulation du protéasome cellulaire en inhibant la dégradation des nucléocapsides lors de l'assemblage et en inhibant l'activité antivirale du protéasome (Zhang *et al.*, 2010). Au cours des dernières années, de nombreux partenaires cellulaires tels que DDB1 (pour *damage specific DNA binding protein 1)* CREB (pour *C-AMP response element-binding protein)* ont été décrits, suggérant une implication de HBx dans le détournement de la machinerie cellulaire, notamment l'inhibition du cycle cellulaire en régulant l'expression et de l'activité des kinases dépendantes des cyclines comme

CDK4 (Gearhart et Bouchard, 2010). Enfin, HBx jouerait un rôle de co-facteur dans le mécanisme d'oncogenèse. Différentes études ont montré le rôle de HBx dans la carcinogénèse par des modifications épigénétiques contribuant à l'activation des oncogènes et l'inhibition des gènes suppresseurs de tumeurs (Tian *et al.*, 2013 ; Hamamoto *et al.*, 2018 ; Gao *et al.*, 2020). Ainsi, l'activité d'HBx semble être une des actions directes majeures du virus sur la carcinogénèse en parallèle de l'intégration du génome viral.

f) La protéine HBSP

La protéine HBSP provient de la traduction de l'ARN épissé SP1RNA produit lors de la maturation de l'ARNpg. L'épissage alternatif de l'ARNpg induit une modification du cadre de lecture et donc une région C-ter différente de la protéine. Ainsi, HBSP est composé des 46 premiers acides aminés de Pol et de 47 acides aminés originaux (Soussan *et al.*, 2000). Cette protéine a été retrouvée dans le tissu hépatique de nombreux patients atteints d'infection chronique. La fonction exacte de cette protéine dans l'évolution de la pathogénicité reste incertaine. Néanmoins, différentes études *in vitro* ont montré que HBSP aurait un rôle dans la viabilité cellulaire, la prolifération et plus récemment dans la voie de signalisation de TNFα (Pol *et al.*, 2015 ; Duriez *et al.*, 2017).

## LE CYCLE VIRAL DU HBV ET LES INTERACTIONS VIRUS-HOTE

### 1/ Cycle viral

Les différentes étapes du cycle viral du HBV sont assez bien décrites. Néanmoins, les interactions entre le virus et les facteurs cellulaires de l'hôte sont encore peu connues et les étapes clés telles que le transport de la nucléocapside jusqu'au noyau ou encore la cinétique de formation de l'ADNccc restent floues ou controversées. Pourtant, le cycle viral du HBV est étroitement lié aux mécanismes cellulaires et aux facteurs de l'hôte. Nous présenterons ici une description non exhaustive des interactions HBV-hôte actuellement connues.

Une représentation schématique du cycle viral du HBV est proposée en **Figure 10.**

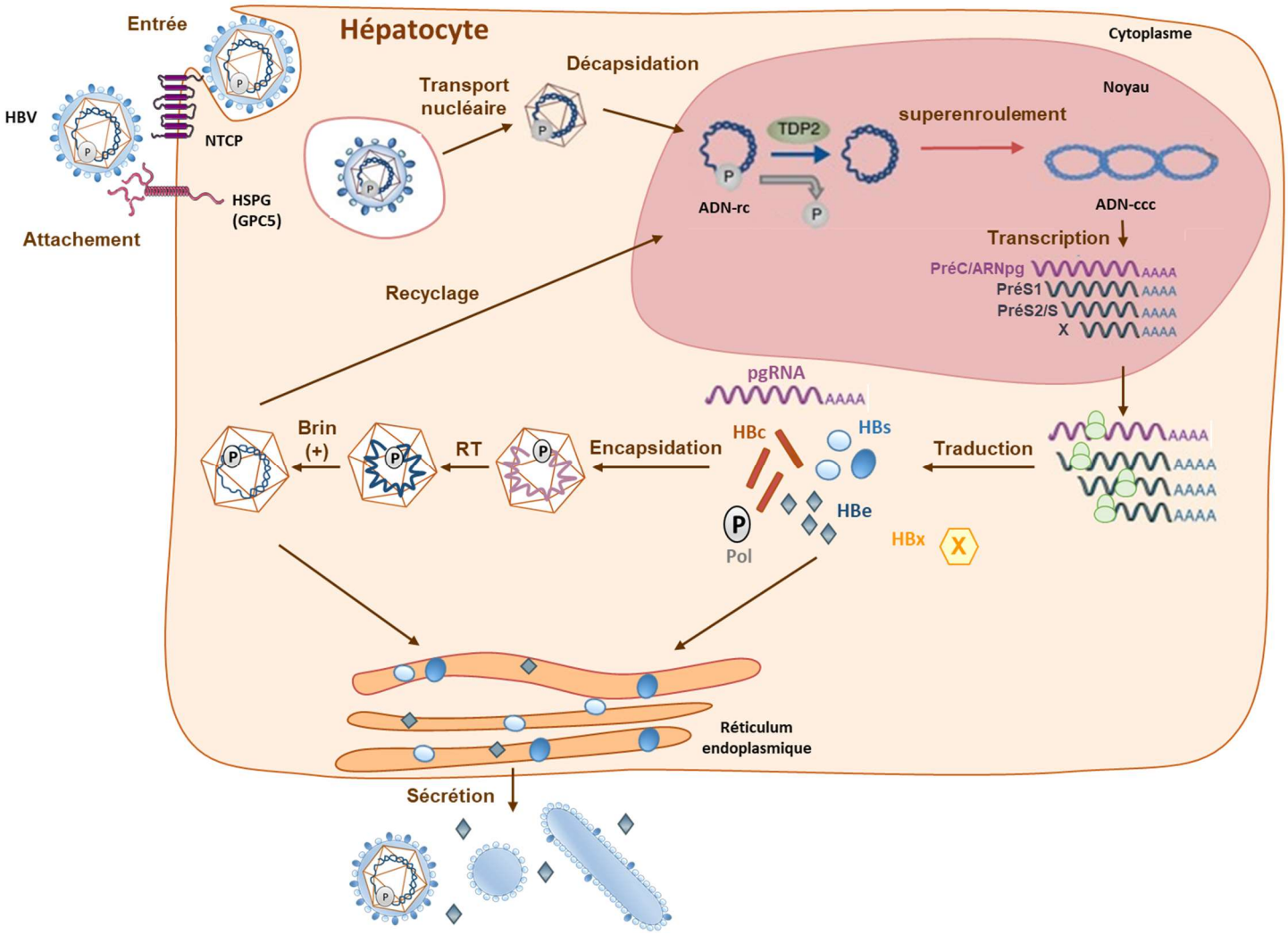


**Figure 10 : Représentation schématique du cycle viral du HBV** d'après (Baumert *et al.*, 2015)**.** L'attachement de la particule virale à GPC5 et la liaison au récepteur NTCP induit l'endocytose du HBV. La nucléocapside est transportée dans le noyau à travers des pores nucléaires. L'ADN-rc est converti en ADNccc par un processus multi-étape. Les ARN viraux sont transcrits à partir de l'ADNccc et exportés dans le cytoplasme. L'ARNpg est empaqueté dans une néo-nucléocapside avec la polymérase virale où il est rétrotranscrit en ADN. Les nucléocapsides virales matures peuvent être recyclées dans le noyau ou sécrétées, après acquisition de son enveloppe, dans le milieu extracellulaire.

a) <u>Attachement et entrée</u>

Le cycle viral du HBV débute par l'attachement des particules virales infectieuses à la surface de l'hépatocyte. Lors de cette étape, la boucle antigénique AGL du domaine S de l'AgHBs s'attache via des liaisons de faible affinité aux HSPG (Sureau *et al.*, 2013). Cette étape entraine un enrichissement des particules virales infectieuses à la surface des cellules hôtes (Schulze *et al.*, 2007).

Ce n'est qu'en 2012 que Yan *et al.* ont identifié le polypeptide de transport des acides biliaires NTCP comme étant un récepteur d'entrée du HBV (Yan *et al.*, 2012). Il est impliqué dans l'entrée des virions via des liaisons de haute affinité avec le domaine myristoylé PréS1 de l'AgHBs L (Yan *et al.*, 2012 ; Ni *et al.*, 2014). Ce transporteur est presque exclusivement exprimé à la surface basolatérale

des hépatocytes et a pour rôle physiologique de maintenir l'homéostasie des sels biliaires dans le foie (Doring *et al.*, 2012). Lors de mes travaux de thèse, j'ai participé à la rédaction de deux revues décrivant le rôle fonctionnel de NTCP dans l'infection virale par le HBV (Eller *et al.*, 2018 ; Verrier, 2018). Une revue est présentée en **annexe 1**.

Durant mes travaux de thèse, notre équipe a identifié le HSPG Glypican-5 (GPC5) comme étant un facteur impliqué dans l'entrée du HBV et du HDV dans les hépatocytes (Verrier *et al.*, 2016a)

b) Fusion et transport intracellulaire

L'internalisation de la particule virale à la suite de la liaison avec NTCP est induite par le récepteur à l'EGF (pour *epidermal growth factor*), EGFR (pour *epidermal growth factor receptor*) (Iwamoto *et al.*, 2019). Très tôt, un mécanisme d'endocytose indépendant du pH a été proposé par Hagelstein *et al.* (Hagelstein *et al.*, 1997). Récemment, une étude a clairement démontré le rôle clé de l'endocytose dépendante de la clathrine dans l'internalisation du virus (Herrscher *et al.*, 2020). Ainsi, l'entrée puis la libération de la nucléocapside dans le cytoplasme impliquerait les mécanismes dépendants de l'effecteur AP2 (pour *adaptator protein 2*) (Huang *et al.*, 2012 ; Herrscher *et al.*, 2020). La dynamique des vésicules d'endocytose est régulée par les protéines Rab et leurs effecteurs. Il a ainsi été démontré que les protéines Rab5/7 jouent un rôle dans le transport intracellulaire des vésicules précoces d'endocytose vers le noyau (Macovei *et al.*, 2013).

Les étapes de l'import nucléaire du génome viral restent peu décrites. Une des hypothèses serait qu'une modification structurelle de la nucléocapside dans l'endosome engendrerait sa libération dans le cytoplasme et son transport à la périphérie nucléaire grâce aux microtubules (Rabe *et al.*, 2006 ; Stoeckl *et al.*, 2006). Les séquences signales NLS (pour *nuclear localization signal*) de la protéine core seraient alors reconnues par les facteurs de transport nucléaires importines α et β qui permettraient la fixation de la capside mature à la membrane nucléaire et sa translocation via les pores tel que la nucléosporine 153 (Kann *et al.*, 1999 ; Schmitz *et al.*, 2010). Une fois à la membrane, le génome viral serait libéré dans le noyau après décapsidation.

c) Formation de l'ADNccc

Une fois libéré dans le noyau, l'ADN-rc est convertit en ADNccc via des processus encore largement méconnus qui impliquent différents facteurs de la machinerie cellulaire, notamment différentes enzymes impliquées dans la réparation de l'ADN.

Ainsi, lors de la formation de l'ADNccc, la polymérase virale est libérée en 5' du brin (-). Koniger *et al,* ont démontré que l'enzyme TDP2 (pour *tyrosyl-DNA phosphodiesterase 2*) était impliquée dans le processus de déprotéination de l'ADN (Koniger *et al.*, 2014). Une étude récente a également démontré que l'endonucléase FEN1 (pour *flap structure-specific endonuclease 1*) était impliquée dans le clivage de l'amorce en 5' du brin (+) (Kitamura *et al.*, 2018). Afin de former un minichromosome circulaire clos, les séquences manquantes du génome doivent être synthétisées et les brins liés. Les ADN polymérases translésionnelles Pol κ, Pol λ ou Pol η sont impliquées dans la synthèse des brins

manquants (Qi *et al.*, 2016). Puis, Long *et al.* ont montré que les ADN ligases 1 et 3 étaient impliquées dans la liaison des brins lors de la formation de l'ADNccc chez les Hepadnavirus (Long *et al.*, 2017). Enfin, le changement de topologie de l'ADN pendant ces processus serait dû aux ADN topoisomérases 1 et 2 (Sheraz *et al.*, 2019). La cinétique de ces processus est encore peu décrite et des formes intermédiaires de la formation de l'ADNccc restent encore à caractériser (Luo *et al.*, 2017), notamment l'ADN-rc-(cM) (pour ADN-rc-closed minus-strand) contenant le brin (-) clos de façon covalente et le brin (+) non clos.

L'ADN viral circularisé est associé à des histones, formant une structure superenroulée épisomale dite « minichromosome », en forme de collier de perles (Bock *et al.*, 1994 ; Newbold *et al.*, 1995). Les histones H3 et H4 ont notamment un rôle dans la régulation de la transcription de l'ADNccc (Pollicino *et al.*, 2006).

d) Expression des gènes viraux et encapsidation

L'activité transcriptionnelle de l'ADNccc est également régulée par de multiples facteurs cellulaires (Lucifora *et al.*, 2016). Ainsi, le promoteur PréS1 contient des sites de liaison pour les facteurs hépatocytaires HNF3 (Raney *et al.*, 1995). La transcription à partir des promoteurs PréS2/S et PréC/C est induit par le facteur de transcription SP1, RXRα (pour *retinoid X receptor α*), PPARα (pour *peroxisome proliferator-activated receptor α*) ou HNF4α (Raney *et al.*, 1992 ; Tang et McLachlan, 2001). La transcription est également favorisée par CEBP (pour *CCAAT-enhancer-binding protein*) ou ATF (pour *activating transcription factor*). Des enzymes de modifications épigénétiques sont également impliquées dans l'activité transcriptionnelle de l'ADNccc comme HDAC1 (pour *histone desacetylase 1*), SIRT1 et SIRT3 (pour *sirtuin 1/3)*, PRMT1 et PRMT5 pour *protein arginine methyltransferase 1/5 (Belloni et al., 2009)* et des facteurs nucléaires, HNF4α ou HLF (Ishida *et al.*, 2000 ; Kim *et al.*, 2016).

Les ARN viraux sont synthétisés à la suite du détournement de la polymérase II cellulaire (Beck *et al.*, 2007). En parallèle, la protéine virale HBx induit la dégradation du complexe Smc5/6 connu pour bloquer la transcription du génome viral (Decorsiere *et al.*, 2016 ; Murphy *et al.*, 2016 ; Niu *et al.*, 2017). Les transcrits subissent une maturation pour devenir des ARNm avec ajout d'une coiffe, d'une queue poly-adénine et potentiellement des réarrangements par épissage alternatif. Li *et al.* ont montré que les ARNm viraux, ainsi que l'ARNpg étaient exportés dans le cytoplasme via la protéine cellulaire TIP et le facteur nucléaire d'export 1 (TAP-NXF1) (Li *et al.*, 2010).

Une fois dans le cytoplasme, les ARNm viraux sont traduits par la machinerie ribosomale cellulaire pour synthétiser les sept protéines du HBV. Afin de permettre la formation de néo-nucléocapside, Yao *et al.* ont montré que la liaison de RBM24 (pour *RNA-binding protein 24)* en 5' et 3' de l'ARNpg inhibe la synthèse de HBc et favorise la liaison de Pol sur la séquence **ε** (Yao *et al.*, 2018). Cette liaison initie l'empaquetage de l'ARNpg pour former une nucléocapside immature. L'activité de transcription inverse de Pol dans la néo-nucléocapside est régulée par des protéines

chaperonnes cellulaires telles que Hsp90, Hsp60 ou Hsp40 (pour *Heat shock protein*) qui sont encapsidées avec l'ARNpg (Locarnini, 2005 ; Beck *et al.*, 2007).

e) Synthèse de l'ADN viral

La transcription inverse est initiée par la liaison du domaine TP de la polymérase avec son résidu Y63 et la synthèse d'une amorce de 4 bases par Pol elle-même (Beck *et al.*, 2007). Le complexe amorce/Pol est alors transféré vers la région DR1 en 3' de l'ARNpg afin de débuter la synthèse du brin (-). La transcription inverse est simultanément suivie par la dégradation de l'ARNpg via l'activité RNase H de Pol, jusqu'au 18 derniers nucléotides. Cette séquence d'ARN restante sert d'amorce pour la synthèse du brin (+).

De manière générale, l'ADN-rc est alors formé à la suite du transfert du complexe Pol/ARN vers la région DR2 du brin (-) néosynthétisé et la synthèse du brin (+). Au contraire, si l'amorce est transloquée vers la séquence DR1, le génome ne sera pas circularisé et restera sous la forme de l'ADN dbl (Staprans *et al.*, 1991 ; Yang *et al.*, 1996).

La maturation de la capside nécessite la phosphorylation de l'AgHBc via des kinases cellulaires tels que CDK2 (pour *cyclin-dependent kinase 2*) ou PLK1 (pour *Polo like-kinase 1*) (Ludgate *et al.*, 2012 ; Diab *et al.*, 2017).

f) Sécrétion ou recyclage

La nucléocapside néoformée mature peut être soit recyclée pour l'amplification intracellulaire d'ADNccc (Tuttleman *et al.*, 1986) ou assemblée avec les protéines de surface dans le RE afin de former des particules virales infectieuses qui seront exportées hors de la cellule (Dandri et Locarnini, 2012). Les mécanismes de bourgeonnement et de libération des virions sont initiés par le transport des nucléocapsides à la surface des corps multivésiculaires cellulaires *via* NEDD4 (pour *neural precursor cell expressed, developmentally down regulated 4*) et AP1G2 (pour *adaptator related protein complex 1 subunit gamma 2*). Enfin, les protéines du complexe ESCRT induisent le bourgeonnement des nucléocapsides et l'enveloppement avec les AgHBs via le complexe de sortie endosomale (Lambert *et al.*, 2007). La fusion des corps multivésiculaires avec la membrane plasmique permet la libération des virions dans le milieu extracellulaire (Watanabe *et al.*, 2007).

**2/ Le cycle cellulaire et la réplication virale**

L'état général de la cellule et son stade dans le cycle cellulaire jouent un rôle dans le cycle viral et la réplication du HBV. Très tôt dans les investigations sur les mécanismes d'infection et de carcinogénèse, il a été décrit que la production d'AgHBs était augmentée dans les cellules quiescentes dérivées de tumeur et ayant intégré le génome viral (Aden *et al.*, 1979). Ces résultats ont été confirmés quelques années plus tard *in vitro* (Sureau *et al.*, 1986). Il est intéressant de souligner que la plupart des modèles d'étude cellulaire *in vitro* de l'infection par le HBV nécessite l'utilisation du DMSO. En plus de permettre la différentiation des hépatocytes, il a été montré que le DMSO induit

un ralentissement de la prolifération cellulaire (de Abreu Costa *et al.*, 2017). En parallèle, le virus lui-même a la propriété de modifier le cycle cellulaire, ce qui favorise sa réplication. De récentes études ont montré que le HBV modifie le cycle cellulaire et en particulier en phase G1 (Wang *et al.*, 2011a ; Xia *et al.*, 2018). Le cycle cellulaire est régulé par les kinases dépendantes des cyclines (CDK). Comme présenté précédemment, la protéines CDK2 induit la phosphorylation de l'AgHBc (Ludgate *et al.*, 2012). CDK2 a également un rôle indirect sur le cycle viral du HBV en phosphorylant et en inactivant le facteur de restriction SAMHD1 - déjà décrit comme inhibant la réplication du VIH (Hu *et al.*, 2018). De plus, un inhibiteur de CDK9 a été montré comme inhibant la propagation du HBV en diminuant l'ADNccc (Tanaka *et al.*, 2016).

Néanmoins, l'impact des phases du cycle cellulaire sur la réplication virale reste toujours controversé. En effet, différentes études indiquent que la protéine HBx induit l'activation de CDK2 et stimule la progression du cycle cellulaire et favorise ainsi la réplication virale et la carcinogénèse (Benn et Schneider, 1995 ; Gearhart *et al.*, 2010).

## REPONSE CELLULAIRE INNEE DE L'HOTE A L'INFECTION VIRALE

Le système immunitaire met en jeu des mécanismes biologiques complexes et coordonnées de reconnaissance et de défense du soi face au non-soi. La réponse immunitaire correspond à l'activation du système immunitaire face à une infection, dont l'infection virale.

L'immunité innée est la première ligne de défense de l'hôte face au HBV et correspond à une réponse non spécifique. Lors de l'infection par le virus, la cellule va reconnaitre les protéines ou les génomes viraux aussi appelés PAMP (pour *Pathogen-associated molecular* patterns) via les PRR (pour *Pattern Recognition Receptors*). La détection des antigènes viraux initie l'expression de gènes antiviraux pro-inflammatoires permettant l'inhibition de l'infection et de sa propagation.

### 1/ Les PRR

Les PRR sont des senseurs reconnaissants notamment des motifs viraux, bactériens et parasitaires présents dans les différents compartiments cellulaires - surface des cellules, cytoplasme et endosomes – afin de répondre rapidement à l'infection d'un large spectre d'agents pathogènes. Leur activation, via la détection de glycoprotéines ou d'acides nucléiques, induit l'expression d'IFN et d'ISG. Dans le paragraphe suivant, nous allons nous concentrer sur la description des PRR qui reconnaissent les acides nucléiques intracellulaires.

Les PRR intracellulaires sont composés des TLR, des RLR (pour *Retinoic acid-inducible gene I-Like Receptor*), les NLRs (pour *Nucleotide oligomerization domain-Like Receptor*) et les senseurs à ADN cytosoliques (Thompson *et al.*, 2011).

a) Les TLR

La découverte des TLR chez la drosophile et leur rôle anti-fongique a permis à Jules Hoffman et ses collaborateurs d'obtenir un prix Nobel de Médecine en 2011 (Lemaitre *et al.*, 1996). Les TLR sont des protéines transmembranaires de type I composées d'un domaine extracellulaire permettant la reconnaissance des PAMP et d'un domaine intracellulaire jouant un rôle dans l'activation de cascades de signalisation. Chez l'Homme, 10 TLR ont été décrits et sont caractérisés par leur localisation. Les TLR 3, 7, 8 et 9, sont présents dans les membranes endosomales et sont impliqués dans la reconnaissance des acides nucléiques viraux (Crozat et Beutler, 2004 ; Heil *et al.*, 2004 ; Vercammen *et al.*, 2008).

Ainsi, TLR3 reconnaît de l'ADN et de l'ARN double brin, TLR7 et TLR8 sont activés par de l'ARN simple brin et TLR9 détecte les motifs CpG non méthylés de l'ADN double brin (Thompson *et al.*, 2011). La reconnaissance des différentes formes des génomes viraux par les TLR active des voies de signalisation via l'adaptateur TRIF (pour *TIR-domain-containing adapter-Inducing interferon-β*) ou MyD88 (pour *Myeloid Differentiation primary response protein 88*). Ces cascades de signalisation impliquent les IKRF (pour *Interferon Regulatory Factors*) et NF-κB (pour *Nuclear Factor κ B*) et induisent l'expression d'IFN et de cytokines pro-inflammatoires (Kawai et Akira, 2007) **Figure 11.**

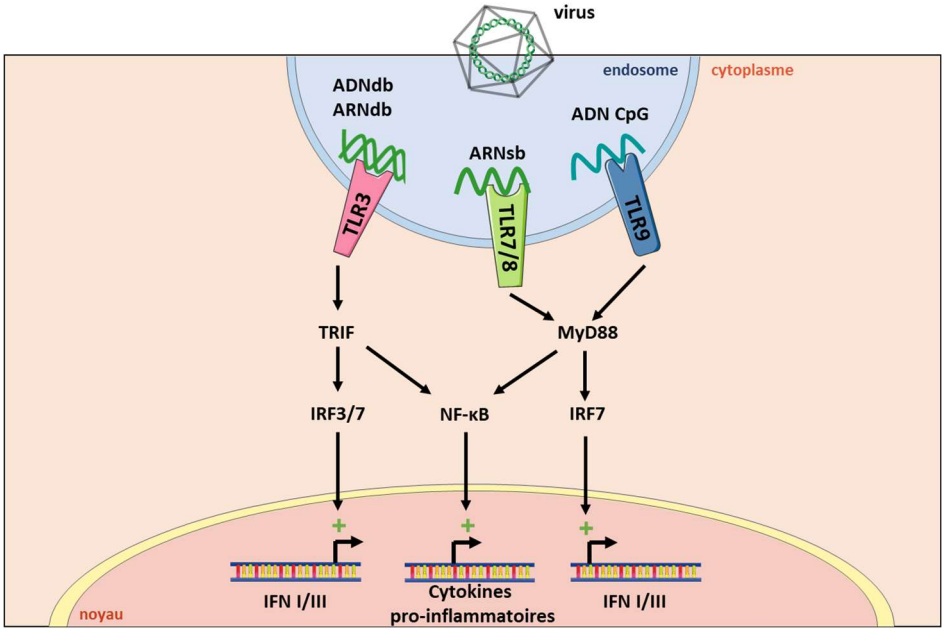


**Figure 11 : Représentation schématique des voies d'induction de l'IFN par les TLR des endosomes.** Les acides nucléiques viraux sont détectés par les TLR dans les endosomes. TLR3 reconnaît l'ADN/ARN (ARNdb/ARNb) double brin, TLR7/8 l'ARN simple brin (ARNb) et TLR9 l'ADN aux motifs CpG non methylés (ADN CpG). La détection active les cascades de signalisation via les adaptateurs TRIF et MyD88 et les facteurs de transcription IRF3/7 et NF-κB qui induisent l'expression des gènes de l'IFN et des cytokines pro-inflammatoires.

b) Les senseurs cytosoliques

Les ARN viraux peuvent également être détectés dans le cytoplasme via les voies RLR, NLR et les senseurs cytosoliques (Yoneyama *et al.*, 2004 ; Kanneganti *et al.*, 2007) **Figure 12.**

Les senseurs RLR sont des hélicases cytoplasmiques exprimées dans tous les types cellulaires. Ils comportent trois membres connus, RIG-I (pour *RIG-like Receptor*), MDA5 (pour *melanoma differentiation-associated protein 5,* ou IFIM1) et LGP2 (pour *Laboratory of Genetics and Physiology 2*) (Thompson *et al.*, 2011). Après détection de l'ARN viral, l'adaptateur MAVS (pour *Mitochondrial Antiviral-Signaling Protein*) active les facteurs de transcription IRF3/7 et NF-κB et induit l'expression des IFN et des cytokines pro-inflammatoires.

Les NLR sont des adaptateurs cytosoliques possédant différentes activités antivirales (Kanneganti *et al.*, 2007). La protéine NOD2 a été décrite comme activant la réponse IFN à la suite de la détection d'ARNb viral et l'activation d'IRF3 (Sabbah *et al.*, 2009).

Les ADN viraux sont également détectés par des senseurs cytosoliques. En effet, la présence d'ADN viral dans le cytoplasme active la réponse immunitaire (Paludan et Bowie, 2013). Différents senseurs de l'ADNdb ont été identifiés tels que AIM2 (pour *Absent in Melanoma 2),* DAI (pour *DNA-dependent activator of IKRF*), ou cGAS (*pour 2'3'-cyclic GMP-AMP synthase*) (Paludan *et al.*, 2013). AIM2 a été décrit comme pouvant induire une inflammation chez les patients infectés par le HBV (Zhen *et al.*, 2014). Le senseur DAI a lui été identifié comme pouvant inhiber la réplication du HBV via l'activation de NF-κB (Chen *et al.*, 2012). Enfin, le senseur cGAS a récemment été décrit comme ayant une activité antivirale contre un grand nombre de virus à ADN et à ARN (Sun *et al.*, 2013 ; Schoggins *et al.*, 2014). La détection des ADN viraux cytosoliques par ces senseurs induit l'expression d'IFN via la voie de signalisation STING/TBK1 (pour *Stimulator of Interferon Genes* et *TANK-Binding Kinase 1*) (Xiao et Fitzgerald, 2013) **Figure 12.**

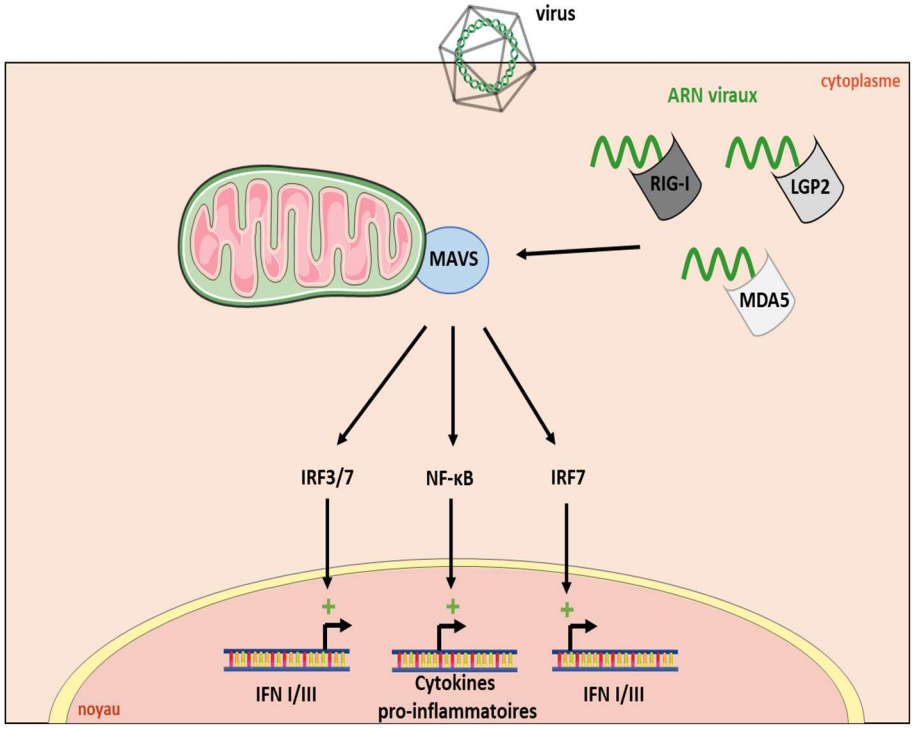


**Figure 12 : Représentation schématique des voies d'induction de l'IFN par les hélicases cytosoliques.**

RIG-I, MDA5 et LGP2 détectent les formes d'ARN viral (ARN double brin ou 5'-triphosphaste) et induisent l'expression d'IFN et de cytokines pro-inflammatoires via l'adaptateur MAVS et des effecteurs secondaires IRF3/7 et NF-κB

c) Cas particulier : la voie de signalisation cGAS-STING

cGAS a la capacité de se fixer à l'ADN - de façon non spécifique – au niveau de la liaison sucre-phosphate et forme un oligomère (Zhang *et al.*, 2014). Une séquence d'ADN de 15 paires de base est suffisante pour activer cGAS. La voie de signalisation cGAS/STING peut également être initiée par une structure hybride ADN/ARN ou ADNsb comme dans le cas de l'infection par le VIH-1 (Mankan *et al.*, 2014). cGAS a été identifié comme ayant une homologie de structure avec un autre senseur à ARNdb, OAS1 (Kranzusch *et al.*, 2013)

cGAS est une enzyme cytoplasmique qui catalyse la formation de cGAMP (pour *cyclic GMP-AMP)* à partir de l'ATP (pour adénine triphosphate) et du GTP (pour guanine triphosphate) (Sun *et al.*, 2013 ; Wu *et al.*, 2013). Le produit enzymatique cGAMP agit comme messager secondaire dans la voie de signalisation. Sa liaison directe à STING induit un changement conformationnel de la protéine et l'induction d'IFN via l'activation de TBK1 (Xiao *et al.*, 2013) **Figure 13**. cGAMP peut également être transmis aux cellules voisines via les jonctions gap et ainsi induire une réponse antivirale dans les cellules non infectées (Ablasser *et al.*, 2013). De plus, il a été décrit que cGAMP peut être incorporé dans des néo-nucléoparticules virales et ainsi permettre une réponse IFN rapide lors de l'infection de nouvelles cellules (Gentili *et al.*, 2015).

cGAS a été décrite comme ayant une activité antivirale contre un large spectre de virus (Schoggins *et al.*, 2014). Un des mécanismes d'échappement des virus à la détection par les senseurs est d'éviter la libération d'ADN viral dans le cytoplasme et de « cacher » leur génome dans la nucléocapside. C'est le cas du VIH qui rétro-transcrit son génome viral en ADN complémentaire au sein de la capside virale et qui le libère directement dans le noyau afin d'éviter la détection par les senseurs cytosoliques (Rasaiyaah *et al.*, 2013). Néanmoins, lors de la réplication virale, des acides nucléiques viraux sont tout de même libérés dans le cytoplasme et activent finalement la voie cGAS/STING (Herzner *et al.*, 2015).

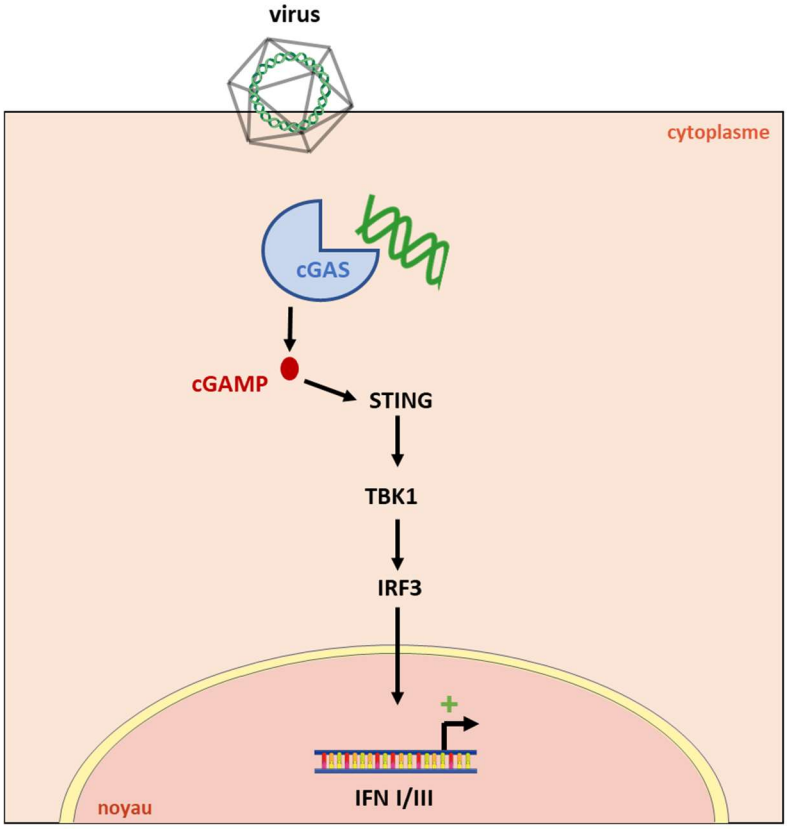


**Figure 13 : Représentation schématique des voies d'induction de l'IFN par les senseurs cytosoliques.** cGAS détecte les formes d'ADN viral (ADN double brin ou 5'-triphosphaste) et induit l'expression d'IFN *via* la voie de signalisation STING/TBK1.

### 2/ Les interférons et ISG

À la suite de la détection des PAMP par les senseurs cellulaires, les voies de signalisation de l'immunité innée cellulaire sont activées, dont la réponse IFN.

Les IFN sont des glycoprotéines de la famille des cytokines qui permettent à une cellule de devenir résistante aux pathogènes. Cette protéine fût découverte en 1957 par Isaacs et Lindenmann qui ont également mis en évidence l'activité autocrine et paracrine de ces cytokines (Isaacs et Lindenmann, 1957). Les IFN sont divisés chez l'Homme en trois types selon leurs caractéristiques et leurs récepteurs (Pestka *et al.*, 2004). Les IFN de type I, aussi nommés IFN-α, β, δ, ε, κ, ω et τ, sont directement induits par les infections virales. L'IFN de type II, ou IFN-γ, régule l'activation des cellules de l'immunité tels que les NK ou les macrophages. Enfin, les IFN de type III n'ont été que très récemment découverts et regroupent l'IFN-λ1, -λ2 et -λ3 (aussi nommées respectivement IL-28A, IL-28B et IL-29) (Sheppard *et al.*, 2003 ; Vilcek, 2003). Ces IFN sont spécifiquement présents dans les cellules épithéliales et permettent une réponse immunitaire directe, de façon similaire aux IFN de type I.

Ces cytokines agissent de manière autocrine et paracrine en se fixant sur leurs récepteurs respectifs à la surface cellulaire, ce qui active des cascades de signalisation intracellulaire induisant la transcription d'effecteurs antiviraux secondaires nommés ISG (pour *Interferon Stimulated Genes*) (Ivashkiv et Donlin, 2014). Les IFN de type I et III induisent environ 300 à 400 ISG, permettant une

réponse immunitaire ciblant toutes les étapes de l'infection. Parmi ces ISG, nous retrouvons des PRR tels que cGAS, augmentant la vigilance des cellules face au PAMP (Ma *et al.*, 2015). En plus de jouer un rôle dans la réponse antivirale pour la cellule infectée, les IFN activent les voies de signalisation des cellules du système immunitaire innée et adaptative (cellules T, macrophages, cellules dendritiques ...) (Gonzalez-Navajas *et al.*, 2012).

Face à la réponse antivirale aux IFN, les virus présentent des mécanismes de contournement des défenses de la cellule hôte (Randall et Goodbourn, 2008). Dans le cas du HBV, les interactions entre le virus et l'immunité innée cellulaire sont encore très floues. Il est cependant clair que face à ce virus, la réponse immunitaire innée et l'expression des IFN est faible, voire inexistante (Mutz *et al.*, 2018 ; Suslov *et al.*, 2018).

### 3/ Interactions entre HBV et immunité innée

Les interactions entre le HBV et la réponse immunitaire innée sont très controversées. En 2004, une étude génomique réalisée chez des chimpanzés infectés par le HBV a montré que l'infection virale n'induisait l'expression d'aucun gène de la réponse immunitaire innée (Wieland *et al.*, 2004). Dès lors, le HBV est décrit comme un virus silencieux. Cette hypothèse soulève de nombreux questionnements sur les mécanismes impliqués et reste aujourd'hui discutée. En 2009 dans une revue basée sur de multiples études *in vitro et in vivo,* Durantel et Zoulim suggèrent que le HBV est tout de même détecté par les PRR et induit une réponse IFN (Durantel et Zoulim, 2009). Néanmoins, ils suggèrent également que le HBV est capable d'échapper à la réponse innée en inhibant les voies antivirales, expliquant l'aspect silencieux du virus. Depuis, les nombreuses études qui ont été réalisées sur le sujet ne permettant toujours pas de conclure de manière claire sur les interactions entre le HBV et l'immunité cellulaire. En effet, alors qu'une étude montre que l'ARNpg est un substrat de RIG-I et induit l'expression d'IFN de type III (Sato *et al.*, 2015), d'autres études affirment que le HBV n'induit pas du tout de réponse interféron au cours de l'infection (Mutz *et al.*, 2018 ; Suslov *et al.*, 2018).

Il a également été suggéré que des protéines virales interfèrent avec les voies de l'immunité innée cellulaire. Pour exemple, il a été montré que AgHBe inhiberait les voies de signalisation induites par les TLR, que AgHBc inhibe l'expression de certains ISG ou encore que Pol interfère avec la voie de signalisation RIG-I/MAVS (Visvanathan *et al.*, 2007 ; Wang et Ryu, 2010 ; Yu *et al.*, 2010b). Ces données suggèrent que le HBV induit effectivement une réponse antivirale et que le HBV a développé des mécanismes d'échappement à l'immunité cellulaire.

Un autre mécanisme d'échappement proposé est que le HBV ne soit pas reconnu par les PRR (Suslov *et al.*, 2018) et en particulier par le senseur cGAS. Néanmoins, les interactions entre la voie de signalisation cGAS/STING et le HBV restent peu décrites et même controversées. Une hypothèse est que le génome viral serait protégé au sein de la capside lors de son transport dans le cytoplasme,

évitant ainsi l'activation de la cascade de signalisation des senseurs cytosoliques (Wieland *et al.*, 2004). D'autre part, il a été décrit que la protéine virale Pol inhibe l'activation de TBK1, effecteur de la voie cGAS/STING (Yu *et al.*, 2010b). Enfin, il faut noter la faible expression de l'effecteur secondaire STING dans les hépatocytes, ce qui pourrait expliquer la faible réponse IFN après infection (Thomsen *et al.*, 2016). Pourtant, une récente étude a démontré que le génome viral du HBV pouvait activer la voie de signalisation cGAS *in vitro* et ainsi inhiber la réplication et l'assemblage du virus (Dansako *et al.*, 2016).

L'immunité innée est le premier rempart de défense de l'organisme face à un pathogène. L'activité antivirale de l'hôte est effective grâce à une succession d'interférence entre le virus et les effecteurs de l'immunité innée. Dans le cas du HBV, nous avons donc décrit que ces interactions sont encore floues et même très controversées. Pourtant, une meilleure connaissance de ces interactions pourrait permettre de découvrir de nouvelles cibles thérapeutiques prometteuses, afin de tendre vers une guérison totale des patients infectées par le HBV.

## INTERACTIONS VIRUS-HOTE ET NOUVELLES CIBLES THERAPEUTIQUES

Une connaissance fine des étapes du cycle viral ainsi que des interactions virus-hôte est indispensable dans le cadre de la découverte de nouvelles thérapies innovantes. En effet, en parallèle des traitements conventionnels actuellement approuvés, de nouvelles stratégies antivirales ciblant le virus, les DAA (pour *direct acting antiviral*) ou l'hôte, les HTA (pour *host-targeting agent)* sont actuellement activement explorées **Figure 14.**

Ces nouvelles stratégies ont pour objectif d'inhiber complètement la réplication virale, de stimuler les réponses immunitaires innées et adaptatives ou de ne cibler que les cellules infectées et en particulier directement l'ADNccc pour ainsi tendre vers une guérison totale de l'infection par le HBV.

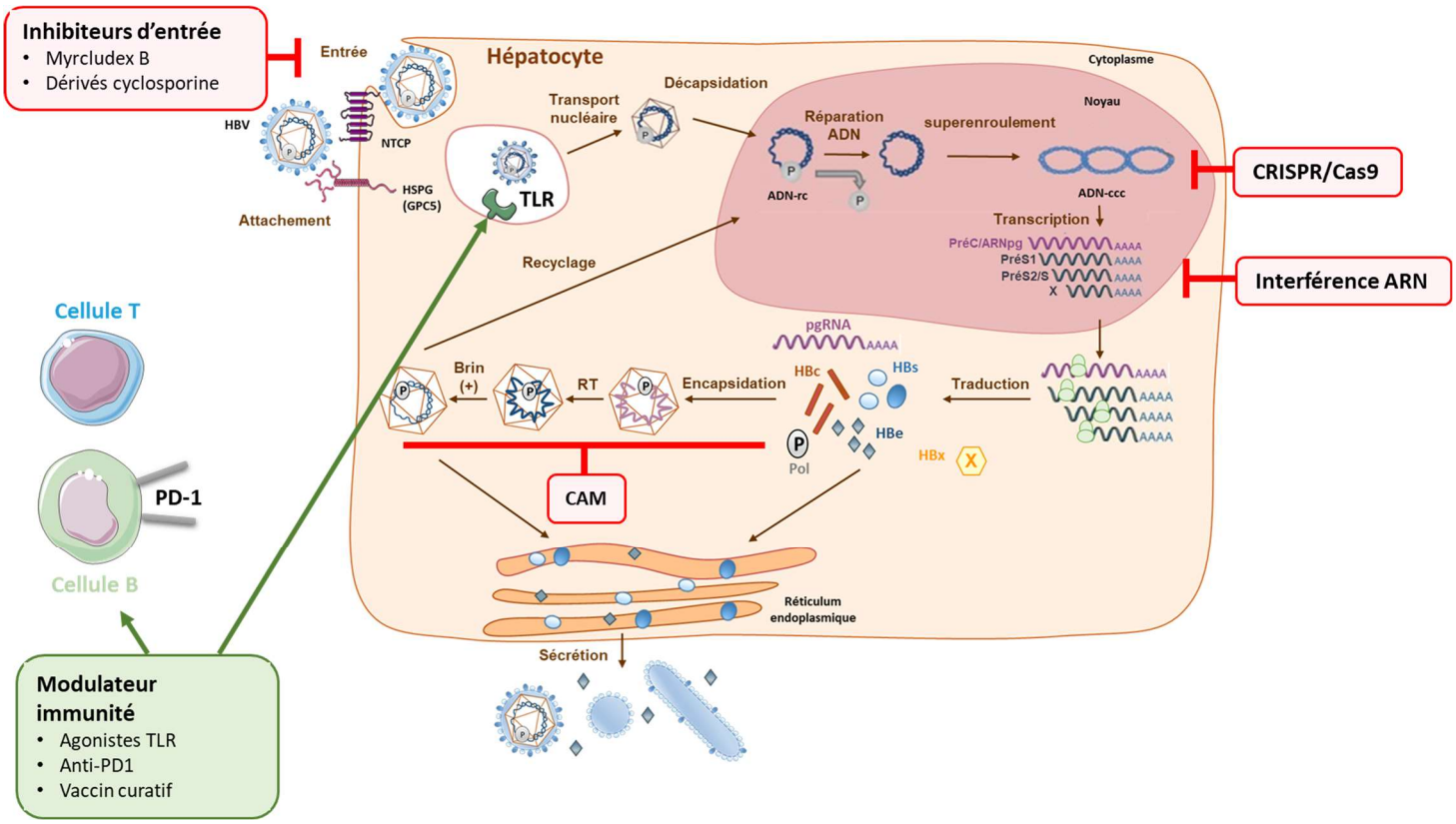


**Figure 14 : Schéma des nouvelles stratégies thérapeutiques contre le HBV**, d'après (Zoulim *et al.*, 2016)**.** De nouvelles molécules antivirales sont en cours de développement. Certaines ciblent le virus avec des outils moléculaires de modulation de l'expression des gènes viraux (interférence ARN, CRISPR/Cas9) ou des modulateurs de l'assemblage de la capside (CAM). D'autres ciblent l'hôte avec des inhibiteurs d'entrée (Myrcludex B/Bulevirtide, dérivés de cyclosporine) ou modulent la réponse immunitaire anti-HBV innée ou adaptative avec les agonistes de TLR, les anticorps anti-PD1 ou le vaccin curatif.

### 1/ Les antiviraux à action directe : DAA

L'avantage des agents antiviraux ciblant le virus est basé sur la faible réaction croisée avec les facteurs cellulaires, permettant ainsi de réduire la toxicité. Ainsi, les traitements actuels de l'infection chronique par le HBV sont basés sur l'administration de DAA, les NUC.

D'autres DAA sont en cours de développement comme les modulateurs d'assemblage de la capside ou CAM (pour *capsid assembly modulator*). En effet, la formation de la nucléocapside et l'assemblage sont des étapes importantes du cycle viral du HBV. Deux classes de CAM sont actuellement en développement. Les CAMs de type I interfèrent avec l'encapsidation de l'ARNpg et accélèrent la formation de capsides immatures vides (Campagna *et al.*, 2013). En parallèle, les CAMs de type II induisent la formation de structures capsidiques aberrantes, voire de leur destruction (Schlicksup *et al.*, 2018). De nombreux CAM sont actuellement en cours d'étude par des compagnies pharmaceutiques et présentent des activités antivirales prometteuses (Nijampatnam et Liotta, 2019).

Une autre stratégie thérapeutique en cours de développement est basée sur l'interférence ciblant le virus *via* des siARN (pour *small interference* ARN). Différents siARN ciblant le HBV sont en

cours de développement ou en cours d'essai clinique. Des études de pharmacocinétique et de pharmaco-toxicité sont encore nécessaires pour s'assurer de l'efficacité et de la sécurité d'un tel traitement (Gish *et al.*, 2015).

La mise en évidence de l'outil d'édition du génome CRISPR/Cas9 (pour *clustered regularly interspaced short palindromic repeats associated nuclease 9*) a ouvert la possibilité de nombreuses applications dans le domaine de la thérapie génique. Différentes études sont en cours de développement pour l'utilisation de guide d'ARN (sgARN pour *single guide* ARN) ciblant l'ADNccc ou les séquences d'ADN viral intégrées dans le génome cellulaire (Seeger et Sohn, 2014). Néanmoins, des effets non spécifiques ou des dommages au niveau de séquences oncogènes de l'ADN cellulaire ont été observés avec ces molécules (Anderson *et al.*, 2018 ; Haapaniemi *et al.*, 2018).

### 2/ Les antiviraux ciblant l'hôte : HTA

Les molécules ciblant l'hôte sont déjà utilisées pour le traitement d'infections virales telles que le maraviroc qui inhibe l'entrée du VIH dans la cellule hôte.

La caractérisation récente des mécanismes moléculaires impliqués dans le cycle viral ont permis le développement de nouvelles molécules ciblant l'hôte (Baumert *et al.*, 2015). La récente découverte du récepteur du HBV/HDV a permis l'émergence de plusieurs HTA ciblant NTCP (Verrier *et al.*, 2016c). Le peptide myristoylé Bulevirtide (anciennement Myrcludex B), dérivé du domaine PréS1 de l'AgHBs L, est actuellement en phase clinique 3 pour le traitement du HDV avec une autorisation particulière d'utilisation en France, en Allemagne et en Autriche dans le cas de co-infection HBV/HDV (Kang et Syed, 2020). Cet inhibiteur compétitif du HBV se lie au récepteur NTCP et inhibe ainsi d'entrée du HBV et du HDV dans les cellules hôtes (Ni *et al.*, 2014).

Un traitement alternatif serait de cibler le système immunitaire et ainsi de restaurer l'immunité antivirale innée et adaptative contre le HBV. Ainsi, différentes stratégies sont en cours de développement. D'une part, des d'agonistes des TLR (pour *toll-like receptor*) – récepteurs impliqués dans la reconnaissance des bactéries et des virus dont le HBV – sont en cours de développement et permettent de moduler l'immunité innée anti-HBV. D'autre part, des anticorps anti-PD1 (pour anti-*programmed cell death protein 1*) sont actuellement en phase clinique pour le traitement de patients chroniquement infectés par le HBV avec un CHC (Pu *et al.*, 2020). En effet, chez les patients chroniquement infectés, les cellules T CD8+ surexpriment des inhibiteurs de l'immunostimulation tels que PD1, ce qui expliquerait leur inefficacité à contrôler l'infection virale. Enfin, une stratégie de vaccin curatif est également en cours de développement basée sur la stimulation des cellules T spécifiques du HBV chez les patients infectés par le HBV (Lok *et al.*, 2016).

Finalement et malgré le fait que le HBV ait été découvert il y a plus de 50 ans, les connaissances sur les facteurs impliqués dans le cycle viral restent limitées. Ce manque d'information à propos des

interactions virus-hôte peut être expliqué par l'absence de modèle pertinent pour l'étude physiologique de l'infection par le HBV.

## ETUDE GENOMIQUE FONCTIONNELLE POUR L'IDENTIFICATION DES INTERACTIONS VIRUS-HOTE

Au cours de l'infection virale, le virus et l'hôte interagissent grâce à des mécanismes directs ou indirects. Pour l'hôte, ces interactions ont pour but de contrôler l'infection et pour le virus de favoriser la réplication virale et d'échapper au système immunitaire. Les nouvelles connaissances de ces interactions permettent de développer des nouveaux outils d'étude de l'infection virale et de développer des traitements curatifs ciblant l'hôte. En effet, de nouvelles stratégies thérapeutiques visent à cibler les différentes étapes du cycle viral du HBV telles que l'entrée, la formation et l'activité de l'ADNccc, la réplication ou l'expression des protéines virales (Levrero *et al.*, 2016). Cette stratégie a l'avantage de limiter les résistances au traitement et potentiellement de cibler un plus large spectre de virus.

La génomique fonctionnelle est une discipline visant à étudier l'expression des gènes et leur fonction en analysant le transcriptome et/ou le protéome de la cellule. Le terme de génomique est directement lié à l'utilisation de méthodes à haut débit, telles quel le séquençage des génomes ou des transcriptomes complets par NGS (pour *next generation sequencing*). Au sein de cette discipline, la génétique inverse est une approche particulière étudiant l'impact de la modification de l'expression d'un gène sur un phénotype donné. Dans l'étude des interaction hôte-virus, l'utilisation de la génétique inverse à haut débit à l'aide de banques de petits ARN interférents (siRNA) a permis notamment l'identification de nombreux facteurs cellulaires impliqués dans l'infection par le VIH (Brass *et al.*, 2008 ; Zhou *et al.*, 2008). Depuis, de nombreux criblages ont permis l'identification de facteurs d'hôte impliqués dans le cycle viral de différents virus tels que le virus de la Dengue, de Chikungunya, de la grippe, de la fièvre jaune ou Zika (Le Sommer *et al.*, 2012 ; Marceau *et al.*, 2016 ; Hafirassou *et al.*, 2017 ; Li *et al.*, 2019b ; Dirmeier *et al.*, 2020 ; Labeau *et al.*, 2020 ; Li *et al.*, 2020), ainsi que les virus hépatiques (Lupberger *et al.*, 2011 ; Marceau *et al.*, 2016 ; Verrier *et al.*, 2016a).

L'étude du cycle viral du HBV par génomique fonctionnelle a pendant longtemps été limitée par le manque de modèle *in vitro* permettant des criblages à haut débit. La découverte du récepteur NTCP et la mise au point de modèles cellulaires permettant un cycle viral complet du HBV a ouvert de nombreuses possibilités d'étude (Yan *et al.*, 2012 ; Ni *et al.*, 2014). Depuis, des criblages utilisant la technologie de CRISPR/Cas9 ont mis en évidence de nouveaux facteurs d'hôte interagissant avec le HBV lors de la réplication virale (Hyrina *et al.*, 2019 ; Mueller *et al.*, 2019).

La plupart de ces études sont basées sur des méthodes de génomique fonctionnelle en perte de fonction. En effet, l'utilisation de banques de lentivirus ou de puces de siARN, shARN ou sgARN se sont montrées efficaces pour découvrir des facteurs d'hôte. Néanmoins, les études fonctionnelles utilisant des siARN et shARN sont limitées par leur efficacité à diminuer l'expression des gènes. De

plus et de manière générale, la méthode de criblage par perte de fonction présente l'inconvénient d'identifier des faux positifs dus aux effets non spécifiques, et à la nécessité d'un modèle d'infection robuste. Une étude sur le VIH a également démontré un faible taux de reproductibilité entre les criblages utilisant les stratégies de pertes de fonction (Bushman *et al.*, 2009). En parallèle et afin de compléter les stratégies de perte de fonction, des méthodes de gain de fonction ont été développées. Ainsi, la stratégie de criblage en gain de fonction a récemment été utilisée dans l'étude des interactions entre les facteurs de l'hôte et des virus de la famille des *flaviridae* (Petrova *et al.*, 2019). En 2011, une banque de lentivirus d'expression de 16 000 ORFs de l'Homme a été élaborée et validé dans le domaine de la cancérologie (Yang *et al.*, 2011 ; Duffy *et al.*, 2016). Cette banque d'ORF permet de réaliser des études de surexpression de gènes humains à haut débit.

Les nouvelles stratégies d'étude du HBV, impliquant des études de génomique fonctionnelle, couplées aux modèles d'études actuellement disponibles, permettent de mettre en évidence de nouvelles interactions entre le HBV et l'hôte, pour *in fine* développer des traitements antiviraux permettant la guérison totale des patients infectés. En parallèle, les nouvelles connaissances permettent également de développer des modèles *in vivo* et *in vitro* se rapprochant le plus de l'état physiologique de l'infection virale.

## MODELE D'ETUDE DU HBV

Depuis la découverte du HBV en 1965, de nombreuses connaissances ont été acquises grâce à des études réalisées sur des prélèvements de patients infectés. Néanmoins, l'étude des interactions entre le virus et l'hôte, ainsi que le développement de nouvelles stratégies thérapeutiques nécessitent des modèles expérimentaux *in vivo* et *in vitro*.

### 1/ Modèles d'étude *in vivo*

Le HBV possède un tropisme d'espèce très restreint. Comme précédemment décrit, seuls les humains, certains primates non-humains, ainsi que le Toupaye de Belanger sont sensibles et permissifs au HBV. Ces modèles *in* vivo permettent de se rapprocher au mieux des conditions physiologiques et de mieux comprendre la biologie de l'infection virale et l'évolution de la maladie (Ortega-Prieto *et al.*, 2018).

#### a) Le chimpanzé et autres singes

En 1972, une étude a démontré qu'il était possible d'induire une infection chronique par le HBV chez le chimpanzé – qui partage près de 98% d'homologie génétique avec l'homme – à partir de

sérum de patients infectés (Maynard *et al.*, 1972). Par la suite, ce modèle de primate non-humain a été crucial dans les avancées scientifiques portant sur le HBV telles que l'étude de l'immunité antivirale, le développement du vaccin et les mécanismes de chronicité (Ortega-Prieto *et al.*, 2018). Au contraire, l'infection chronique du chimpanzé n'induit pas de maladie hépatique et l'étude des maladies progressives du foie est donc impossible sur ce modèle.

La règlementation sur l'éthique de l'expérimentation animale, ainsi que les contraintes de gestion de tels modèles ont rendu délicate puis interdite l'utilisation des primates non-humains pour l'étude du HBV. En effet, une directive européenne de 2010, amendée en 2019, interdit l'utilisation du chimpanzé pour les études biomédicales (Directive 2010/63/EU – Régulation 2019/1010).

L'infection par le HBV a également été observée chez d'autres primates tels que le gibbon, le singe laineux ou encore le babouin (Bancroft *et al.*, 1977 ; Kedda *et al.*, 2000 ; Lanford *et al.*, 2000).

b) Le Toupaye de Belanger

C'est en 1996 que la première infection *in vivo* et *in vitro* du Toupaye de Belanger par le HBV a été décrite (Walter *et al.*, 1996). En effet, les hépatocytes de ce petit mammifère présentent une permissivité à ce virus dans des conditions de compétence immunologique. Néanmoins, les principales limitations à l'utilisation de cette musaraigne arboricole dans les études biomédicales sont les difficultés de reproduction en captivité, le risque de co-infection dans le cas de spécimen provenant de leur habitat naturel et le manque d'outils de biologie moléculaire.

c) Modèles animaux d'hepadnavirus autres que HBV

Des modèles viraux parallèles au HBV tels que le HBV de la marmotte, WHBV (pour *Woodchuck hepatits B virus*) ou du canard, DHBV (pour *Duck hepatitis B virus*) ont largement permis de mieux comprendre le cycle viral des hepadnavirus et du HBV (Summers *et al.*, 1978 ; Mason *et al.*, 1980).

Le WHBV, découvert en 1970, possède une forte homologie génétique, morphologique et biologique avec le HBV (Galibert *et al.*, 1982 ; Menne et Cote, 2007). Comme le HBV, le WHBV est capable d'induire une infection chronique et le développement de maladies progressives et de cancer du foie. Ce modèle a précédemment permis de comprendre le cycle viral et l'émergence de résistances associées au traitement par un analogue de nucléos(t)ide (Summers et Mason, 2004). Néanmoins, la difficulté d'obtention de ces animaux ainsi que leur besoin d'hibernation rendent ce modèle difficile à utiliser dans la recherche.

Le modèle viral du DHBV a été découvert dans les années 1980 et a largement contribué aux connaissances du HBV (Omata *et al.*, 1983 ; Cova et Zoulim, 2004). En effet, ce modèle *in vivo* a permis de mettre en évidence la réplication du HBV par une transcriptase inverse ou encore la structure moléculaire de l'ADNccc et son rôle de matrice (Funk *et al.*, 2007). Contrairement au WHBV, le virus du canard n'induit pas les mêmes degrés de pathologie liée à l'immunité que le HBV. Malgré tout, ce modèle *in vivo* possède de réels atouts comme sa facilité d'utilisation et d'obtention mais également son faible coût d'élevage.

De même que le Toupaye de Belanger, une des difficultés majeures dans l'utilisation de ces modèles dans la recherche biomédicale est le manque d'outils de biologie moléculaire.

d) Les modèles murins

Les souris ne sont pas sensibles à l'infection par le HBV, y compris les modèles transgéniques exprimant la version humaine du récepteur NTCP (Li *et al.*, 2014). Cependant, différents modèles de souris transgéniques exprimant les protéines du HBV ont été développés (Chisari *et al.*, 1985 ; Farza *et al.*, 1988 ; Milich *et al.*, 1990 ; Guidotti *et al.*, 1994). De plus, d'autres modèles d'étude de la réplication du HBV sans la tolérance immunitaire consiste en la transfection de souris immunocompétentes avec de l'ADN viral *via* des vecteurs adénoviraux (Ortega-Prieto *et al.*, 2018). Ces modèles de souris ont permis l'étude de nombreux composés antiviraux et dans un modèle de souris immunocompétent, l'immunité innée face au HBV (Dandri *et al.*, 2012 ; Dion *et al.*, 2013).

Enfin, le modèle des souris humanisées avec des PHH (pour *primary human hepatocytes*) permet l'étude du cycle complet du HBV (Bissig *et al.*, 2010). Il est basé sur la transplantation intra-splénique de PHH dans des souris chimères exprimant l'uPa (pour *urokinase plasminogen activator*), ce qui induit la dégénérescence des hépatocytes murins (Brown *et al.*, 2000). Ce modèle a déjà largement été utilisé pour l'étude des interactions HBV/HDV et en particulier pour la démonstration de l'efficacité de Myrcludex B/Bulevirtide *in vivo* (Lutgehetmann *et al.*, 2012 ; Volz *et al.*, 2013). Malheureusement, ce modèle implique une forte immunodéficience pour la prise de la xénogreffe. Afin de pallier cette limite immunologique, des souris chimériques alliant les hépatocytes et les cellules du système immunitaire du même donneur ont été développées (Strick-Marchand *et al.*, 2015).

Ainsi, de nombreux modèles *in vivo* ont été développés afin de permettre l'étude de tous les aspects du cycle viral du HBV **(Tableau 2).**

| |  Human |  Chimpanzé |  Tupaye de Belanger |  Marmotte |  Canard |  Souris transgéniqu e VHB |  Souris transfectées |  Souris chimérique foie humain |  Souris chimérique foie-SI humain |
|---|---|---|---|---|---|---|---|---|---|
| **Entrée virale** | **VHB** | **VHB** | **VHB** | **WHBV** | **DHBV** | **Non** | **Non** | **VHB** | **VHB** |
| **Formation de l'ADNccc** | **Oui** | **Oui** | **Oui** | **Oui** | **Oui** | **Non** | **Oui/Non** | **Oui** | **Oui** |
| **Sécrétion particules infectieuses** | **Oui** | **Oui** | **Oui** | **Oui** | **Oui** | **Oui** | **Oui** | **Oui** | **Oui** |
| **Suceptibilité isolats patients** | **Oui** | **Oui** | **Oui** | **Non** | **Non** | **Non** | **Non** | **Oui** | **Oui** |
| **Type d'infection** | **Aigüe/ Chronique** | **Chronique** | **Chronique** | **Chronique** | **Chonique** | **Chronique** | **Aigüe/ Chronique** | **Chronique** | **Chronique** |
| **Réponse immunitaire** | **Oui** | **Oui** | **Oui** | **Oui** | **Oui** | **Non** | **Oui** | **Non** | **Oui** |
| **Outils bio-moléculaires** | **++** | **+** | **-** | **-** | **-** | **++** | **++** | **++** | **++** |

**Tableau 2 : Tableau comparatif des modèles *in vivo* d'étude du HBV adapté de (Ortega-Prieto *et al.*, 2019)**

### 2/ Modèles d'étude *in vitro*

Les modèles *in vitro* d'étude du HBV sont très limités du fait de la spécificité d'hôte du virus et de son important tropisme hépatocytaire. Pendant de nombreuses années, aucune lignée cellulaire ne permettait l'étude du cycle viral complet du HBV.

a) Les hépatocytes primaires

Les hépatocytes primaires sont les cellules hôtes naturelles du HBV et constituent le modèle cellulaire le plus physiologique actuellement disponible. En effet, les hépatocytes primaires humains (PHH pour *primary human hepatocytes)* sont sensibles et permissifs au HBV (Galle *et al.*, 1994). Pendant longtemps, les PHH étaient le seul modèle disponible pour l'étude du cycle viral complet du HBV *in vitro.* Néanmoins, leur utilisation en routine reste limitée. En effet, les PHH ne présentent pas ou peu de croissance cellulaire après leur mise en culture, leur durée de vie reste limitée et leur culture est réalisée dans des conditions particulières nécessitant un milieu complexe (Zeisel *et al.*, 2015). De plus, le taux d'infection des cultures primaires est variable selon le donneur et nécessite une grande quantité de particules virales et la présence de dimétylsulfoxyde (DMSO) (Gripon *et al.*, 1988). Cette caractéristique est également due au fait qu'une fois en culture, les PHH perdent leur polarisation avec en particulier comme conséquence la perte de l'expression de NTCP et donc la diminution de la sensibilité des PHH au HBV. Enfin, les PHH sont isolés à partir de résections chirurgicales de patient ayant une pathologie du foie et présentant donc une variabilité génétique pouvant impacter le cycle viral (Allweiss et Dandri, 2016). Dernièrement, la mise au point d'une nouvelle forme de culture en 3D, combinant les PHH avec les cellules non-parenchymateuses, permet de se rapprocher un peu plus des conditions physiologiques pour l'étude des interactions entre le virus et les cellules du foie (Ortega-Prieto *et al.*, 2018).

Les hépatocytes de Toupaye de Belanger (PTH pour *primary tupaia hepatocytes)* sont également un modèle *in vitro* de cellules primaires supportant l'infection par le HBV. Ce modèle a d'ailleurs permis de découvrir l'unique récepteur connu du HBV en 2012 ou encore d'étudier le mécanisme d'action des antiviraux ou les phénotypes des variants viraux isolés de patients (Kock *et al.*, 2003 ; Baumert *et al.*, 2005 ; Yan *et al.*, 2012).

Les cellules primaires possèdent notamment des voies de l'immunité innée fonctionnelles permettant l'étude de la réponse antivirale face à l'infection par le HBV. Néanmoins, ce modèle *in vitro* reste difficile à obtenir et nécessite des collaborations avec des centres de recherche spécialisés ou des structures vétérinaires.

b) Lignées cellulaires hépatocytaires

Les cellules HepG2 - dérivées d'un hépatoblastome d'un patient masculin caucasien de 15 ans - et les Huh7 - dérivées d'un hépatocarcinome d'un patient masculin japonais de 57 ans - ont pendant de nombreuses années été utilisées comme modèle d'étude principal de la réplication du HBV *in vitro* (Nakabayashi *et al.*, 1982 ; Lopez-Terrada *et al.*, 2009). Néanmoins, ces lignées hépatocytaires

n'expriment pas le récepteur NTCP et ne sont donc pas sensibles à l'infection par le HBV (Yan *et al.*, 2012). Ainsi, seule la transfection de l'ADN viral dans ces lignées permettait l'étude des étapes de la transcription à la sécrétion des particules virales (Seeger *et al.*, 2000 ; Pollicino *et al.*, 2006).

Afin de produire de façon constitutive des virions complets du HBV, les lignées inductibles HepAD38 et HepG2.2.15, dérivées des HepG2, ont été produites par intégration du génome du HBV (Sells *et al.*, 1988 ; Ladner *et al.*, 1997). Ces lignées sont couramment utilisées pour la production de particules infectieuses *in vitro* utilisées lors de l'infection de cellules sensibles. Elles sont également intéressantes pour l'étude des interactions virus-hôte et le criblage de molécules ciblant la réplication du HBV (Koniger *et al.*, 2014 ; Verrier *et al.*, 2016a).

La découverte du récepteur du HBV, NTCP et sa surexpression dans la lignée cellulaire HepG2, ont permis d'obtenir un nouveau modèle d'étude du cycle complet du HBV, de l'entrée à la sécrétion (Yan *et al.*, 2012 ; Ni *et al.*, 2014). Comme les PHH, ce modèle d'infection nécessite des conditions de culture et d'infection particulières avec l'utilisation de DMSO, de PEG 8000 et d'une grande quantité de particules virales (Ni *et al.*, 2014 ; Verrier *et al.*, 2016a ; Michailidis *et al.*, 2017). Ce modèle *in vitro* a déjà permis de caractériser les interactions moléculaires virus-hôte dont celles décrites dans ce manuscrit (Verrier *et al.*, 2016a). Néanmoins, ce modèle ne permet pas d'obtenir une propagation efficace de l'infection. Dans ce but, une équipe a développé un clone de HepG2-NTCP capable de propager l'infection *in vitro* (Konig *et al.*, 2019). Enfin, ce modèle cellulaire hépatocytaire est limité par les modifications génétiques induites par la cancérisation et qui peuvent modifier les interactions entre le virus et la cellule.

c) Les HepaRG

Avant la découverte de NTCP, la seule lignée cellulaire disponible pour l'étude complète du cycle viral du HBV était les cellules immortalisées non cancéreuses HepaRG. Il s'agit d'une lignée de cellules progénitrices du foie dérivées d'un carcinome hépatocellulaire induit par le HCV (Gripon *et al.*, 2002). Ces cellules ont la capacité de se différencier en deux types phénotypiques de cellules hépatiques via l'ajout de DMSO : des cellules de type hépatocyte ou des cellules de type biliaire (Marion *et al.*, 2010). Une fois différenciées, les cellules de type hépatocyte présentent des caractéristiques similaires aux hépatocytes humains, incluant une expression constitutive du récepteur NTCP et une réponse immunitaire innée fonctionnelle (Maire *et al.*, 2008 ; Ni *et al.*, 2014). Ce modèle est une bonne alternative aux PHH mais nécessite des conditions de culture contraignantes et une longue différenciation. De plus, le taux d'infection est faible, et peu d'amplification du nombre de copies d'ADNccc via la voie de recyclage est observée (Hantz *et al.*, 2009). Ce modèle cellulaire a permis la découverte de nombreux facteurs d'hôte tels que les HSPG, le récepteur NTCP ou la protéine PLK1 impliquée dans la réplication du HBV (Schulze *et al.*, 2007 ; Ni *et al.*, 2014 ; Diab *et al.*, 2017).

d) Hépatocytes dérivés de progéniteurs

La mise en évidence de la différenciation des cellules souches pluripotentes *iPS* (pour *induced pluripotent stem*) en une multitude de types cellulaires, dont les cellules hépatocytaires, est un autre modèle pour l'étude du HBV *in vitro* (Takahashi et Yamanaka, 2006 ; Kaneko *et al.*, 2016). En effet, une fois différenciées, ces cellules présentent une morphologie et un phénotype hépatique avec par exemple l'expression de facteurs spécifiques tels que NTCP ou HNF4α (Sullivan *et al.*, 2010 ; Shlomai *et al.*, 2014). Ce modèle cellulaire se rapproche du modèle des cellules primaires avec l'avantage de conserver sa permissivité pendant plusieurs semaines et permet la propagation de l'infection d'une cellule à l'autre (Xia *et al.*, 2017). Néanmoins, il nécessite du matériel biologique spécifique pour l'isolement des iPS et une longue différenciation pour obtenir des cellules hépatocytaires.

# OBJECTIF DE LA THESE

L'infection chronique par le HBV est donc un problème majeur de santé publique et reste la première cause du développement du cancer du foie (Lozano *et al.*, 2012). Malgré l'objectif de l'OMS d'obtenir une éradication des hépatites virales dans le monde d'ici 2030, aucun traitement actuel ne permet efficacement la guérison totale des personnes chroniquement infectées par le HBV. Alors que les stratégies thérapeutiques basées sur l'utilisation de HTA pour traiter les infections virales sont prometteuses, elles reposent néanmoins sur une connaissance fine des interactions moléculaires entre le virus et son hôte.

Jusqu'à récemment, la caractérisation des facteurs cellulaires impliquées dans le cycle viral complet était limitée par l'absence de modèle robuste d'étude de l'infection *in vitro* et *in vivo*. La découverte du transporteur NTCP comme récepteur d'entrée du HBV et du HDV a permis le développement de modèles cellulaires sensibles et permissifs à l'infection virale (Yan *et al.* 2012 ; Ni *el al.,* 2014).

C'est dans ce contexte que s'est inscrite cette thèse, qui a eu pour objectif de tirer parti des nouveaux modèles cellulaires permettant l'étude du HBV afin de caractériser de nouvelles cibles antivirales.

Ainsi, les objectifs de ma thèse ont été :

(1) La mise en place des modèles cellulaires d'infection par le HBV, non disponibles à cette époque dans la communauté scientifique et basés sur la surexpression de NTCP dans des lignées hépatocytaires, ainsi que la mise au point de toutes les techniques d'étude et de détection de l'infection virale.
(2) L'application de ces modèles dans des études pilotes pour valider leur intérêt dans la compréhension des différentes étapes de l'infection virale.
(3) L'utilisation de ces modèles d'étude du HBV dans un criblage à haut débit pour l'identification de nouveaux facteurs cellulaires impliqués dans la réplication du virus.

# RESULTATS

# PARTIE I

## Mise au point et validation des modèles cellulaires pour l'étude du virus de l'hépatite B (HBV)

## Un criblage fonctionnel d'interférence ARN identifie le glypican 5 comme facteur d'entrée des virus de l'hépatite B et D

## *A targeted functional RNA interference screen uncovers glypican 5 as an entry factor for hepatitis B and D viruses*

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## Mise en évidence du récepteur d'entrée du HBV NTCP comme régulateur de la réponse immunitaire ciblant le HCV

## *Solute carrier NTCP regulates innate antiviral immune responses targeting hepatitis C virus infection of hepatocytes*

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# 1. Résultats

La découverte du seul récepteur connu pour l'entrée du HBV et du HDV, le transporteur des acides biliaires NTCP, a permis le développement de nouveaux modèles *in vitro* pour l'études de ces virus hépatiques (Yan *et al.*, 2012 ; Ni *et al.*, 2014). En effet, la surexpression de ce transporteur dans des lignées hépatocytaires qui ne l'expriment pas naturellement pas (Huh7, HepG2), leur confère une sensibilité au HBV et au HDV.

La première partie de mes travaux de thèse a consisté en la mise au point des modèles cellulaires pour l'étude du HBV. En effet, les modèles robustes d'infection pour l'étude du cycle viral du HBV, ainsi que les méthodes de production de particules virales recombinantes infectieuses n'étaient pas disponibles au début de ma thèse. Le développement de ces modèles a donc été basé sur la surexpression du gène *SLC10A1* - codant pour le récepteur NTCP - à la surface de cellules de la lignée hépatocytaire HepG2 **(Figure 1.1. A-B)**. En parallèle, j'ai développé au laboratoire la production de HBV recombinant à partir des cellules HepAD38, lignée inductible provenant d'un hépatoblastome humain et ayant intégré le génome du HBV (Ladner *et al.*, 1997) **(Figure 1.1 C)**. Afin d'analyser et de quantifier l'infection virale par le HBV dans notre modèle cellulaire et de permettre des études à haut débit, j'ai mis au point les techniques de détection de l'infection par le HBV dans la lignée hépatocytaire HepG2 surexprimant NTCP et dans les hépatocytes primaires humains. Ainsi, l'infection par le HBV peut être observée par immunofluorescence (IF) avec détection intracellulaire de AgHBs **(Figure 1.1. D)** et par quantification des marqueurs de réplication AgHBe dans le surnageant par chemiluminescence (CLIA) et ARNpg par qRT-PCR **(Figure 1.1. E)**.

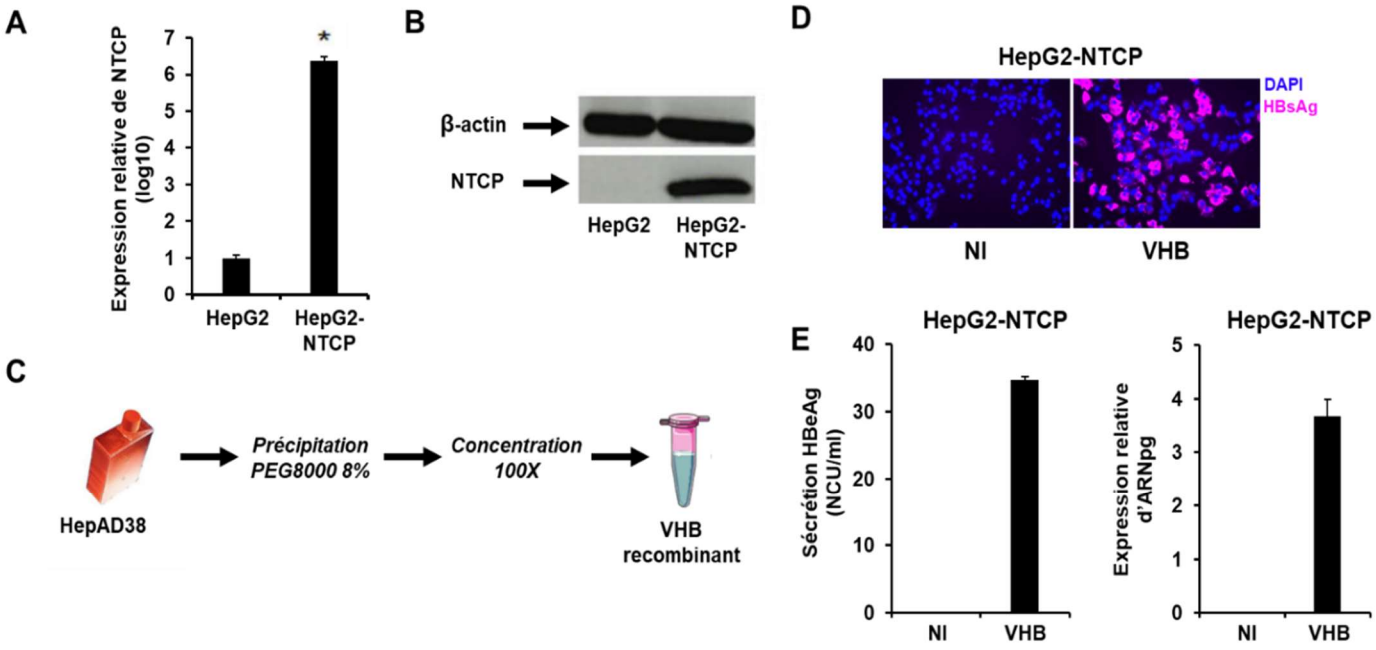


**Figure 1.1 : Production des modèles cellulaires d'étude et de production du HBV.** (A) Expression relative de NTCP dans la lignée HepG2-NTCP comparée à la lignée parentale HepG2 par qRT-PCR. (B) Détection de la protéine NTCP par western blot. (C) Schéma de production de particules virales recombinantes du HBV. Le surnageant de cellules HepAD38 cultivées en *hyperflask* est précipité sur la nuit avec du PEG8000 à 8% avant d'être concentré par centrifugation. (D-E) Infection des HepG2-NTCP par le HBV recombinant. L'infection est détectée 10 jours post-infection.

(D) Les cellules positives au HBV sont visualisées par IF en rose après immunomarquage des protéines AgHBs avec un anticorps monoclonal de souris anti-AgHBs. Le noyau des cellules est détecté en bleu par du 4',6-diamidino-2-phénylindole (DAPI). (E) La réplication du HBV déterminée par quantification du AgHBe dans le surnageant par CLIA et de l'ARNpg par qRT-PCR.

Ce modèle cellulaire d'étude du HBV, ainsi que la méthode de production de virus recombinant infectieux, ont été utilisés et validés dans une première étude menée au laboratoire en 2015. Ces travaux ont permis l'identification d'un nouveau facteur d'entrée impliqué dans le cycle viral du HBV, le GPC5. En effet, l'entrée virale du HBV a été démontré comme débutant par une étape d'attachement aux HSPG à la surface des hépatocytes (Sureau *et al.*, 2013). Néanmoins, le ou les membres des HSPG impliqués dans cette étape étaient méconnus. Un criblage siRNA ciblé sur les protéines de la famille des HSPG et un modèle d'infection du HDV – utilisé comme pseudoparticules du HBV car enveloppé de ses glycoprotéines de surface - a mis en évidence GPC5 comme un facteur de l'hôte impliqué dans l'attachement du HDV et du HBV à la surface des hépatocytes (Verrier *et al.*, 2016a).

Dans cette étude, j'ai utilisé la technique de diminution de l'expression de gènes en utilisant un siRNA ciblant GPC5 lors de l'infection des HepG2-NTCP par le HBV **(Figure 1.2. A-C)**. Ces expériences ont permis de confirmer qu'en plus du HDV, GPC5 avait également un rôle dans le cycle viral du HBV. En utilisant la lignée productrice de HBV, les HepAD38, j'ai confirmé le rôle précoce de GPC5 dans l'infection par le HBV **(Figure 1.2. D-E)**. Ainsi, mes travaux lors de cette étude ont permis d'étendre le rôle de GPC5 dans l'attachement du HBV lors de l'entrée virale. La publication associée à cette étude est présentée en **annexe 2** du manuscrit (Verrier *et al.*, 2016a).

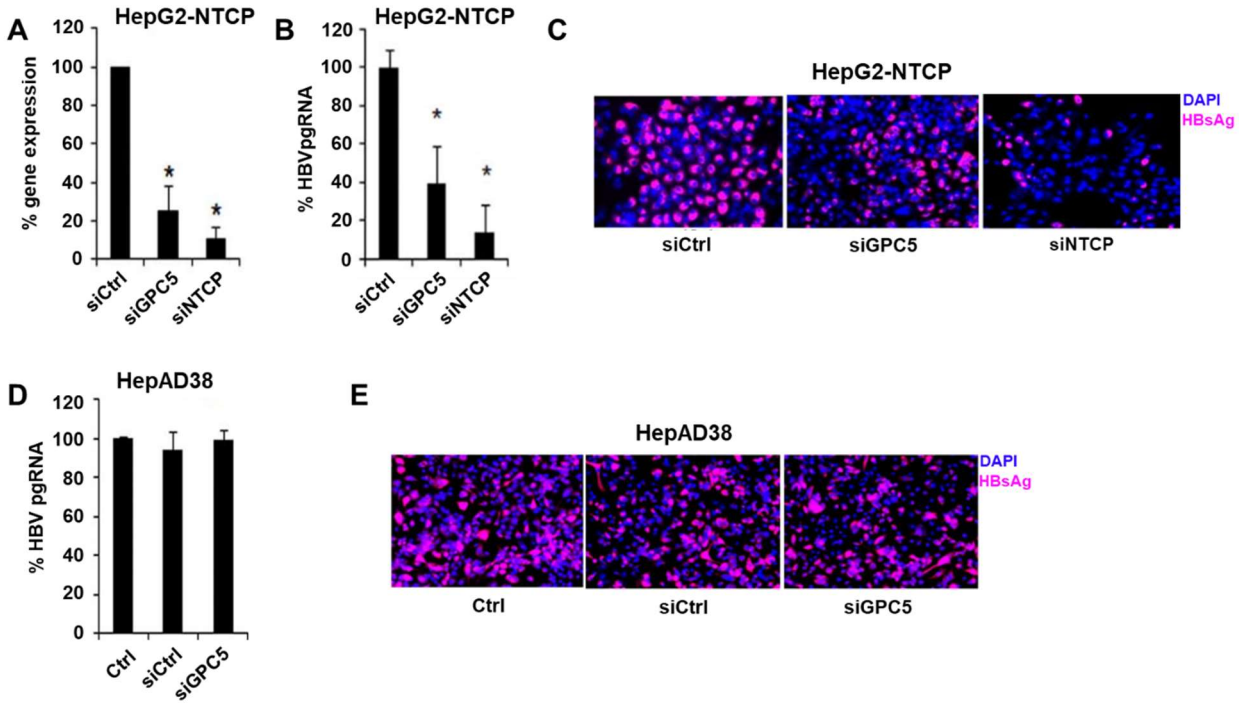


**Figure 1.2 : GPC5 est un facteur d'entrée du HBV.** (A-C) La diminution de l'expression de GPC5 par siARN inhibe l'entrée du HBV dans les HepG2-NTCP. L'efficacité de la diminution de l'expression de GPC5 a été quantifiée par qRT-PCR (A). L'infection des HepG2-NTCP après transfection des siARN a été quantifiée par détection de l'ARNpg par qRT-PCR et de l'AgHBs intracellulaire par IF. (D-E) Absence d'effet de la diminution de l'expression de GPC5 sur la production de HBV dans les HepAD38. La réplication du HBV dans les HepAD38 a été quantifié par détection de l'ARNpg par qRT-PCR et de l'AgHBs intracellulaire par IF.

Dans l'étude décrivant NTCP comme étant un récepteur d'entrée du HBV et du HDV, les auteurs ont également montré que lors de l'infection virale, les acides biliaires - ligands naturels du transporteur – entraient en compétition avec les virus (Yan *et al.*, 2012). Au contraire, les acides biliaires ont été identifiés comme favorisant la réplication du HCV par un mécanisme encore non déterminé (Chang et George, 2007 ; Chhatwal *et al.*, 2012). Néanmoins, le rôle des transporteurs des acides biliaires, tels que NTCP, dans l'infection par d'autres virus hépatotropes n'était alors pas encore clair.

Dans l'étude présentée en **annexe 3** de ce manuscrit, nous avons identifié le rôle de NTCP dans la régulation de la réponse immunitaire innée ciblant le HCV (Verrier *et al.*, 2016b).

Mes premiers travaux de thèse ont conduit à la mise au point d'un modèle de lignée cellulaire hépatocytaire surexprimant NTCP (HepG2-NTCP) susceptible à l'infection par le HBV. Cette lignée a été utilisée dans deux autres études réalisées au laboratoire et auxquelles j'ai participé. J'ai en particulier montré que le transport des acides biliaires via le transporteur NTCP induisait une modulation de l'expression des ISG tel que IFITM3 (pour *interferon-induced transmembrane protein 3*), qui affecte alors l'infection des hépatocytes par le HCV. Le transporteur NTCP a donc un rôle dans le cycle viral de trois virus hépatotropes : HBV, HDV et HCV (Verrier *et al.*, 2016b).

Cette première partie de mes travaux, à l'origine de la première étude sur le HBV, issue de notre laboratoire, a permis de valider nos modèles cellulaires d'infection et la pertinence de leur utilisation pour la découverte de nouveaux facteurs cellulaires impliqués dans les interactions virus-hôte.

# PARTIE II

## Echappement du virus de l'hépatite B à la détection par le senseur de guanosine monophosphate-adénosine monophosphate cyclique synthase dans les hépatocytes humains

## *Hepatitis B virus evasion from cyclic guanosine monophosphate-adenosine monophosphate synthase sensing in human hepatocytes*

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* ont contribué à part égale à ce travail comme premiers auteurs

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# 1. Objectifs

Le rôle de l'immunité innée au cours de l'infection par HBV reste mal connu. L'hypothèse dominante, soutenue par de nombreuses données *in vivo* et *in vitro,* suggère que le HBV est un virus « silencieux » induisant peu ou pas de réponse immunitaire innée (Rehermann et Nascimbeni, 2005). Cependant, certains travaux soutiennent que les ARN du HBV peuvent être détectés par RIG-I (Sato *et al.*, 2015) ou MDA5 (Lu et Liao, 2013) ou au contraire, que la réponse immunitaire innée serait inhibée par les protéines virales du HBV (Bertoletti *et al.*, 2012). En 2013, le senseur cellulaire cGAS a été décrit comme pouvant détecter l'ADN des rétrovirus tel que le VIH (Gao *et al.*, 2013). En 2014, Schoggins *et al.* ont notamment décrit cGAS comme un acteur majeur de la réponse antivirale innée, possédant une activité antivirale contre un large spectre de virus à ADN et ARN (Schoggins *et al.*, 2014). Néanmoins, aucune donnée ne décrivait les interactions entre cGAS et le HBV. Ainsi, à l'aide de nos modèles d'infection *in cellulo,* nous avons étudié le rôle potentiel de cGAS dans la détection par les mécanismes de l'immunité innée du HBV lors de l'infection.

Après avoir démontré que notre modèle cellulaire HepG2-NTCP permettaient l'étude de l'immunité innée avec activation des voies de signalisation liées à la détection de l'ADN, nous avons confirmé que le HBV n'induisait pas l'expression des IFN de type I et III ou de tout autre ISG. En collaboration avec le Dr Seung-Ae Yim, étudiante en thèse travaillant également sur ce sujet, j'ai réalisé des études fonctionnelles utilisant des stratégies de perte et de gain de fonction combinées avec un profilage de l'expression de plus de 36 gènes effecteurs de la voie de signalisation de cGAS.

Mes résultats expérimentaux ont montré que contrairement à la forme encapsidée de l'ADN viral du HBV, l'ADN-rc nu est reconnu par cGAS et induit l'expression d'ISG. Nous avons également démontré que cette activation de l'immunité innée par l'ADN-rc nu n'était plus détectée après extinction par KO (pour *knock-out*) du gène *MD21D1,* codant pour la protéine cGAS. Ces résultats sont en faveur d'un échappement du HBV à la détection de cGAS probablement due à la protection du génome au sein de la nucléocapside lors de son transport vers le noyau. J'ai par la suite confirmé ces résultats en utilisant des PHH.

De manière intéressante, mes expériences de perte de fonction des effecteurs de la voie cGAS-STING ont mis en évidence une activité antivirale fonctionnelle dépendant de cette voie contre l'infection par le HBV. Ces données ont été confirmées par gain de fonction par le Dr Yim et les résultats ont montré une diminution de la quantité d'ADNccc dans les cellules infectées. Nos résultats suggèrent donc que la voie de signalisation cGAS/STING serait active contre le HBV sans détection de l'ADN viral, mais via l'activation de la voie cGAS/STING non viro-induite par un ligand inconnu. Dans cette étude, nous avons également mis en évidence dans les HepG2-NTCP et dans les souris chimériques infectées par le HBV que la voie de signalisation cGAS était elle-même inhibée par l'infection virale.

Toutes ces données ont permis de suggérer un échappement du HBV à l'immunité innée par (1) l'impossibilité de la détection de l'ADN viral par les senseurs et (2) par un rétrocontrôle négatif de l'expression de cGAS par le HBV lui-même.

La compréhension des interactions entre les acteurs de l'immunité innée et le HBV permettent de mieux comprendre les interactions virus-hôte et d'imaginer de nouvelles stratégies de thérapie basées sur la stimulation de l'immunité cellulaire et donc une meilleure prise en charge de cette maladie chronique virale.

Mes travaux expérimentaux ont permis de mettre en évidence de nouvelles interactions entre le HBV et la cellule hôte et ont contribué à la publication d'un article dans Hepatology en 2018 (Verrier *et al.*, 2018).

## 2. Publication

### Hepatitis B virus evasion from cyclic guanosine monophosphate-adenosine monophosphate synthase sensing in human hepatocytes

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Key words antiviral, capsid, innate immunity, liver, recognition.

**ABSTRACT**

Chronic hepatitis B virus (HBV) infection is a major cause of chronic liver disease and cancer worldwide. The mechanisms of viral genome sensing and the evasion of innate immune responses by HBV infection are still poorly understood. Recently, the cyclic GMP-AMP synthase (cGAS) was identified as a DNA sensor. In this study, we aimed to investigate the functional role of cGAS in sensing of HBV infection and elucidate the mechanisms of viral evasion. We performed functional studies including loss- and gain-of-function experiments combined with cGAS effector gene expression profiling in an infectious cell culture model, primary human hepatocytes and HBV-infected human liver chimeric mice. Here we show that cGAS is expressed in the human liver, primary human hepatocytes and human liver chimeric mice. While naked relaxed-circular HBV DNA is sensed in a cGAS-dependent manner in hepatoma cell lines and primary human hepatocytes, host cell recognition of viral nucleic acids is abolished during HBV infection, suggesting escape from sensing, likely during packaging of the genome into the viral capsid. While the hepatocyte cGAS pathway is functionally active, as shown by reduction of viral cccDNA levels in gain-of-function studies, HBV infection suppressed cGAS expression and function in cell culture models and humanized mice. ***Conclusion:*** HBV exploits multiple strategies to evade sensing and antiviral activity of cGAS and its effector pathways.

**INTRODUCTION**

With more than 250 million chronically infected patients, hepatitis B virus (HBV) infection is a leading cause of liver disease and hepatocellular carcinoma [1, 2]. Current antiviral therapies effectively control viral load, but largely fail to cure [3]. HBV is a partially double stranded DNA (dsDNA) virus infecting human hepatocytes after initial attachment to heparan sulfate proteoglycans (HSPG) and its receptor Na+/taurocholate cotransporting polypeptide (NTCP; reviewed in [4]). Following uncoating, the viral nucleocapsid is released into the cytoplasm. The viral genome is imported into the nucleus through mechanisms which are still poorly understood. The viral genome it is converted in the nucleus into a covalently closed circular DNA (cccDNA) [5]. This minichromosome serves as a template for both pregenomic RNA (pgRNA) and viral mRNA transcription. While recent studies suggested sensing of the pgRNA or other HBV RNAs by either MDA5 [6] or RIG-I [7], the recognition of the viral nucleic acids by the regular pattern recognition receptors (PRR) still remains elusive. In general, HBV does not or only marginally activate innate immune responses in cell culture models and in vivo [8-14], leading to the concept that HBV behaves like a “stealth” virus avoiding viral DNA and RNA sensing [15]. Other studies have suggested an active inhibition of the innate immune responses by HBV proteins [16]. Consequently, the interaction of HBV and the innate immune system of hepatocytes, and in particular the sensing of HBV DNA, is only poorly understood.

Foreign DNA recognition by cytosolic DNA sensors triggers an early antiviral innate immune response, including type I and type III IFN production [17]. Recently, the cyclic GMP-AMP (cGAMP) synthase

(cGAS) was identified as a DNA sensor exhibiting an antiviral activity against a broad range of DNA and RNA viruses [18-20]. cGAS is encoded by MB21D1 gene and directly binds to dsDNAs inducing the production of cGAMP which is recognized by the stimulator of IFN genes (STING, encoded by TMEM173) triggering the expression of IFN-stimulated genes (ISG) through TBK1 activation [21-23]. While two studies have investigated cGAS-HBV interactions in viral replication and assembly [24, 25], the functional role of cGAS in sensing of the viral genome during natural infection of human hepatocytes remains unknown.

The understanding of HBV-host interactions, including innate immune response after infection, has been hampered for long time by the absence of robust cell culture model system for the study of viral infection [26]. The development of HBV-susceptible NTCP-overexpressing hepatoma cells, such as HepG2-NTCP cells, allows the study of the full life cycle in a robust and easy-to-use cell culture model [26]. HepG2 cells are capable of mounting an efficient innate immune response after infection by hepatitis C virus [27]. Moreover, another study took advantage of HBV-infected HepG2-NTCP for studying the interaction between RIG-I and HBV RNA [7], suggesting that this cell line is suitable for the study of innate immune response after HBV infection. Here, we aimed to understand the functional role of cGAS for the HBV life cycle in human hepatocytes and unravel the mechanisms of viral evasion using loss- and gain-of-function experiments combined with cGAS effector gene expression profiling in human liver chimeric mice.

## EXPERIMENTAL PROCEDURES

**Human subjects.** Human material including liver tissue from patients undergoing surgical resection or HBV-positive serum was obtained with informed consent from all patients. Protocols were approved by the Ethics Committee of the University of Strasbourg Hospitals, France (CPP 10-17 and DC-2016-2616).

**Animal Experimentation.** All mice were kept in a specific pathogen-free animal housing facility at Inserm U1110. The respective protocols were approved by the Ethics Committee of the University of Strasbourg Hospitals and authorized by the French Ministry of Research (number 02014120416254981AL/02/19/08/12, AL/01/18/08/1202014120416254981, 0201412051105 4408). Mice were kept in individual ventilated cages, with bedding composed of irradiated sawdust and chips from spruce and pine and enriched with cotton cocoon and aspen bricks. The animal diet consists of 25kGy irradiated RM3(E) (SDS) and mice were not fasted. Primary human hepatocytes (PHH) were transplanted into 3 week-old uPA/SCID-bg mice (male and female) by intrasplenic injection as described [28]. Engraftment and viability of PHH were assessed by quantification of human serum albumin by ELISA (E80-129, Bethyl Laboratories; [28]). uPA/SCID-bg mice were then infected with serum-derived HBV and sacrificed 16 weeks after virus inoculation. Serum HBV load was determined by qPCR (Realtime HBV viral load kit, Abbott) before sacrifice. Interventions were all performed during light cycle.

**Cell lines and human hepatocytes.** HEK 293T [29] and HepG2-NTCP [30] cells and isolation of PHH have been described [29].

**Reagents and plasmids.** DMSO, PEG 8000 (polyethylene glycol), Poly (I:C) and calf thymus DNA (control dsDNA) were obtained from Sigma-Aldrich, pReceiver-Lv151 plasmid from GeneCopoeia™ and lentiCas9-Blast and lentiGuide-Puro plasmids were gifts from Feng Zhang (Addgene #52962 and #52963).

**Small interfering RNAs for functional studies.** Pools of ON-TARGET plus (Dharmacon) small interfering RNA (siRNA) targeting MB21D1 (cGAS), TMEM173 (STING), TBK1, and IFI16 expression were reverse-transfected into HepG2-NTCP using Lipofectamine RNAi-MAX (Invitrogen) as described [29]. RNA was purified from cells harvested two days after transfection and gene expression was analyzed using qRT-PCR.

**HepG2-NTCP-cGAS overexpressing and MB21D knock-out cells.** Lentivirus particles were generated in HEK 293T cells by cotransfection of plasmids expressing the human immunodeficiency virus (HIV) gap-pol, the vesicular stomatitis virus glycoprotein (VSV-G) and either the human MB21D1 full open reading frame (ORF) encoding plasmid, or the MB21D1-targeting single-guide RNA (sgRNA) encoding plasmids, or the Cas9 expressing plasmid in the ratio of 10:3:10. HepG2-NTCP cells were then plated and transduced with lentivirus encoding either the human MB21D1 ORF or the eGFP ORF in pReceiver-Lv151 vector (GeneCopoeia™). After 3 days, transduced cells were selected with 200 $\mu$ g/ml of neomycin (G418). The cGAS-over-expressing and control HepG2-NTCP cells were then further cultured in presence of G418 at 200 $\mu$ g/ml. For the generation of MB21D1 knock-out cell lines, one MB21D1-targeting sgRNA was designed using CRISPR Design Tool (Broad Institute: http://www.genome-engineering.org/crispr/?page_id=41). The sgRNA sequence targeting the exon 1 of MB21D1 (sgcGAS 5'-CACCGCGGCCCCCATTCTCGTACGG-3') was inserted into lentiGuide-Puro plasmid [31]. We first generated Cas9 expressing HepG2-NTCP cells after transduction of cells with the lentiCas9-Blast plasmid [31]. Cells were then selected with 6 µg/ml blasticidin for 10 days. HepG2-NTCP-Cas9 cells were seeded in six-well plates at 50% confluency 24 h prior to transduction with the sgcGAS-encoding plasmid. Subpopulations of cells were selected from the whole population and cultured independently. cGAS expression was controlled by Western blot. Finally, two cGAS-deficient cell lines (cGAS_KO#1 and cGAS_KO#2) were selected.

**Analysis of gene expression using qRT-PCR.** Total RNA was extracted using ReliaPrep™ RNA Miniprep Systems (Promega) and reverse transcribed into cDNA using Maxima First Strand cDNA Synthesis Kit (Thermo Scientific) according to the manufacturer's instructions. Gene expression was then quantified by qPCR using a CFX96 thermocycler (Bio-Rad). Primers and TaqMan® probes for MB21D1 (cGAS), TMEM173 (STING), TBK1, IFI16, IFNB1, IFNL1, and GAPDH mRNA detection were obtained from ThermoFisher (TaqMan® Gene expression Assay, Applied Biosystems). All values were normalized to GAPDH expression.

**Protein expression.** The expression of cGAS, STING, and $\beta$-actin proteins was assessed by Western blot as described [30] using two polyclonal rabbit anti-cGAS antibodies (HPA031700, Sigma

& NBP1-86761, Novus Biologicals, see Supporting Information), a polyclonal rabbit anti-STING antibody (19851-1-AP, Proteintech), and monoclonal anti-β-actin antibody (mAbcam8226, Abcam). Protein expression was quantified using ImageJ software.

**Infection of HepG2-NTCP cells and PHH.** The purification of infectious recombinant HBV particles from HepAD38 cells and infection of HepG2-NTCP cells has been described [30]. Briefly, HepG2-NTCP and derived cells were plated one day prior to incubation with HBV in presence of 4% PEG at multiplicity of infection (MOI) ~ 500 genome equivalent/cell (GEq/cell) except otherwise stated. Sixteen hours after HBV inoculation, cells were washed with PBS and then cultured in 3.5% DMSO primary hepatocyte maintenance medium (PMM) for ten days. HBV infection was assessed by quantification of HBV pgRNA using qRT-PCR or HBV total DNA using qPCR as described [30], or by immunofluorescence (IF) using anti-HBsAg antibody (1044/329, Bio-Techne) and AF647-labelled goat antibody targeting mouse IgGs (115-605-003, Jackson Research) as described [30]. Southern blot detection of HBV cccDNA was performed using DIG-labelled (Roche) specific probes as described [32]. Total DNA from HBV-infected cells was extracted using the previously described HIRT method [33]. Specific DIG-labelled probes for the detection of HBV and mitochondrial DNAs were synthetized using the PCR DIG Probe Synthesis Kit (Roche) and the primers indicated in Table S1. PHH were plated one day prior to incubation with a HBV preS1- or a control peptide for one hour at 37° C as described [30]. PHH were then infected with recombinant HBV particles for ten days. HBV infection was assessed by quantification of HBV pgRNA using qRT-PCR and immunofluorescence as described above.

**Sendai virus (SeV) infection.** HepG2-NTCP cells and PHH were infected with SeV DI-H4 at an MOI of 10 as described [13].

**Extraction of HBV rcDNA from HBV infectious particles.** HBV rcDNA was extracted from HBV preparations using QiaAMP DNA MiniKit protocol (Qiagen). PEG-precipitated cell supernatants from naive HepG2-NTCP cells were used as non-virion controls. The presence of HBV DNA was confirmed by PCR and quantified by qPCR as described [30] (see Supporting Information). One μg of rcDNA or dsDNA (calf thymus DNA) was transfected in cells using Lipofectamine 2000 (Invitrogen) and CalPhos Mammalian Transfection Kit (Clonetech) according to manufacturer's instructions. Cells transfected with HepG2-NTCP control supernatants were used as a control. Three days after transfection, total RNA was extracted and purified as described above.

Transcriptomic analysis by digital multiplexed gene profiling using nCounter NanoString. Transcriptomic analyses using nCounter NanoString were performed according to manufacturer's instructions. Specific probes for a set of 36 innate antiviral response-related (IAR) genes (according to [20] and additional genes listed in Table S2) were obtained from the manufacturer. HepG2-NTCP cells, HepG2-NTCP derived cell lines and PHH were either infected with HBV or SeV, or were transfected with Poly (I:C) (100ng) for two days. Alternatively, HepG2-NTCP-Cas9 and HepG2-NTCP-KO_cGAS#2 cells were transfected with rcDNA (1μg) or dsDNA (calf thymus DNA, 1μg) for three

days. Total RNA was then extracted and subjected to nCounter Digital Analyzer system (NanoString). Alternatively, total liver RNA was extracted from HBV-infected mice and gene expression was assessed by either qRT-PCR (MB21D1 expression) or nCounter Digital Analyzer system. The 36 genes were considered as an artificial gene set termed innate antiviral response (IAR) gene set. Its perturbation by infection, transfection or gain-/loss-of -function studies was assessed through Gene Set Enrichment Analysis (GSEA [34]). GSEA determines whether an a priori defined set of genes shows statistically significant differences between two biological states. False discovery rate (FDR) < 0.05 was considered statistically significant. Heatmaps illustrating the induction (red) or repression (blue) of the genes of the IAR compared to control were illustrated using Morpheus software (Broad Institute of MIT and Harvard, Cambridge, MA, USA). The heatmap illustrating the induction (red) or repression (blue) of individual genes in chimeric mouse livers were designed using GenePattern (Broad Institute of MIT and Harvard, Cambridge, MA, USA).

**FISH analyses.** Fluorescence in situ hybridization (FISH) analyses were performed as described [28, 35]. Briefly, liver samples were collected from mice and then immediately embedded into optimal cutting temperature compound (OCT). OCT-embedded liver sections were cryosectioned (10 $\mu$m) using a cryostat (Leica). Upon fixation with 4% formaldehyde at 4° C, washing, and dehydration in ethanol, tissue sections were boiled at 90-95° C for 1 min in a pretreatment solution (Affymetrix-Panomics), followed by a 10 min digestion in protease QF (Affymetrix-Panomics) at 40° C. Sections were then hybridized using specific probe sets targeting HBV (target region nucleotides 483-1473 of HBV [Genotype D, GenBank V01460]) and human MD21D1 (VA1-3013492-VC, Affymetrix-Panomics). Pre-amplification, amplification and detection of bound probes were performed according to the manufacturer's instructions. Finally, pictures were acquired by LSCM (LSM710, Carl Zeiss Microscopy) and Zen2 software.

**Statistical Analysis.** Except otherwise stated, cell culture experiments were performed at least three times in an independent manner. Statistical comparisons of the samples were performed using a two-tailed Mann-Whitney U test. For in vivo experiments, a two-tailed unpaired Student's t-test was performed for comparing gene expression from non-infected and HBV-infected mice. $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***) were considered significant. Significant p values are indicated by asterisks in the figures. Each digital multiplexed gene profiling experiment was performed using three biological replicates per condition and the induction or repression of the gene set was analyzed using GSEA. FDR < 0.05 was considered statistically significant.

## RESULTS

**Expression of cGAS in primary human hepatocytes and an infectious HBV cell culture model.** Prior to its functional characterization, we studied cGAS/MB21D1 expression in primary hepatocytes and HBV permissive cell lines. As shown in **Figure 2.1. A**, cGAS protein expression was easily detectable in PHH from three independent donors. Since HBV infection of primary cells is highly variable and does not allow robust perturbation studies, we used an HBV infectious cell culture model

based on differentiated hepatocyte-derived HepG2 cells overexpressing NTCP [30] – a key HBV entry factor. As shown in **Figure 2.1. A**, cGAS protein is expressed in HepG2-NTCP cells. We validated the specificity of cGAS detection using a siRNA specifically targeting the MD21B1 expression (sicGAS) and Western blots applying two antibodies (**Figure 2.1. A, Figure S2.1**). Moreover, we generated CRISPR-mediated MB21D1 knock-out (KO) cells using a specific sgRNA **(Figure 2.1. B)**. Two cell lines, KO_cGAS#1 and KO_cGAS#2 were selected for further studies **(Figure 2.1. B)**. Interestingly, the adaptor STING was also detected in HepG2-NTCP cells, suggesting a fully functional cGAS-STING pathway **(Figure 2.1. C)**. To test the suitability of these cells as a model to analyze cGAS-mediated innate immune response after virus infection, we stimulated cells with different analogs of viral nucleic acids. Stimulation by Poly (I:C) or dsDNA transfection elicited a dose-dependent IFNB1 expression in HepG2-NTCP cells **(Figure 2.1. D)**. These results suggest that the cGAS sensing machinery is present, functional, even if it is less efficient that the RNA sensing complex. Moreover, cGAS protein expression was induced by both Poly (I:C) and dsDNA stimulation confirming an efficient IFN response through the upregulation of ISG such as MB21D1 [36] after RNA or DNA stimulation **(Figure 2.1. E)**. Collectively, these data show that the HepG2-NTCP model is suitable to study innate immune responses.

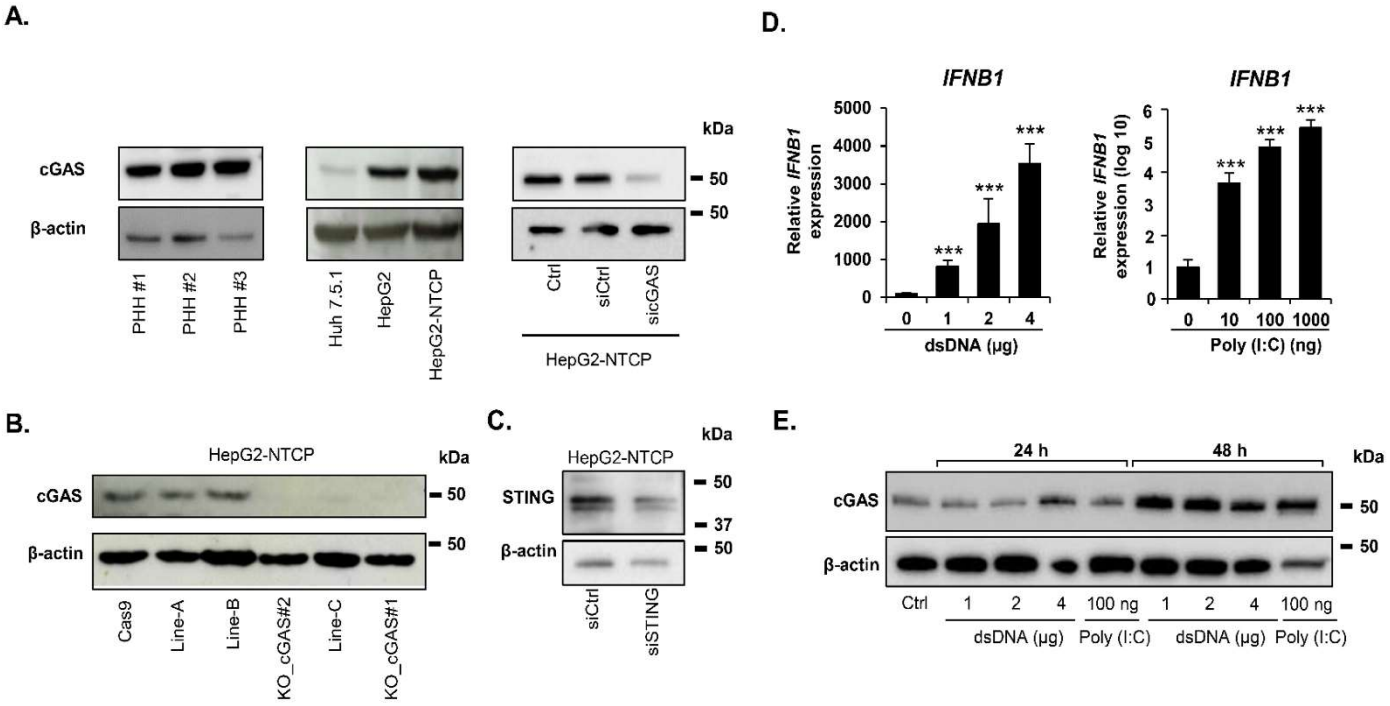


**Figure 2.1. cGAS expression and function in human hepatocytes and in a cell culture model for HBV infection.** (A) Detection of endogenous cGAS protein expression in different cellular models by Western blot. Cell lysates from Huh7.5.1, HepG2, HepG2-NTCP cells, and PHH from three independent donors were used. HepG2-NTCP cells were reverse transfected with a siRNA targeting *MB21D1* (sicGAS) or a non-targeting siRNA control (siCtrl) two days before cGAS detection. β-actin was used as a Western blot control. Individual representative experiments are shown. (B) Generation of *MB21D1* knock out (KO) cells. *MB21D1* KO HepG2-NTCP cell lines were generated via CRISPR/Cas9 technology. The absence or presence of cGAS protein was controlled by Western blot using the HPA031700 anti-cGAS antibody in Cas9-expressing HepG2-NTCP cells (Cas9) and in different cell lines after transduction with the sgRNA targeting *MB21D1* (line-A, line-B, line-C, cGAS_KO#1 and cGAS_KO#2). One experiment is

(C) Detection of endogenous STING protein in HepG2-NTCP cells. siRNA targeting *TMEM173* (siSTING) or a non-targeting siRNA (siCtrl) were reverse-transfected into HepG2-NTCP cells. Silencing efficacy was assessed by Western blot. One experiment is shown. (D-E) Poly (I:C) and dsDNA transfection induce *IFNB1* and *MB21D1* expression in HepG2-NTCP cells. HepG2-NTCP cells were transfected with increasing doses of Poly (I:C) or calf thymus DNA at the indicated concentrations. *IFNB1* mRNA expression was quantified by qRT-PCR 24 h after transfection and cGAS protein expression was assessed by Western blot 24 h and 48 h after transfection. qRT-PCR data (D) are expressed as means ± SD relative *IFNB1* expression (log10) compared to non-transfected control (0, set at 100) from four independent experiments performed in triplicate (dsDNA) or from three independent experiments performed in triplicate (Poly I:C). One representative Western blot experiment is shown (E).

**HBV evades cGAS sensing.** Next, we investigated whether HBV was sensed in HBV permissive cells. To address this question, we infected HepG2-NTCP cells with recombinant HBV (MOI: 500 GEq/cell) and studied the expression of IFNB1 at early time points after HBV infection. As it has been described that HBV infection may induce the expression of type III IFN [7], IFNL1 expression was also quantified. As shown in **Figure 2.2. A-B**, the lack of increase in IFNB1 and IFNL1 expression in spite of efficient infection **(Figure S2)** indicates poor or absent detection of HBV by cellular sensors. In contrast, SeV, known to induce a strong IFN response in hepatocytes [13], strongly induced IFNB1 and IFNL1 expression **(Figure 2.2. A-B)**. Since cGAS has been shown to induce the expression of a large set of innate effector genes (such as OAS2 or IFI44, see [20]), the analysis of expression of a single effector gene such IFNB1 may not be sufficient to evaluate cGAS sensing. Therefore, we designed a 36 innate antiviral response gene set (named IAR), comprising 29 ISG whose expression is modulated by cGAS activity described by Schoggins and Rice in [20] as well as 7 established innate immune response effector genes (Table S2). We then infected HepG2-NTCP cells with HBV or SeV, and measured the innate antiviral immune response at day 2 post infection by analysis of IAR gene expression using digital multiplexed gene profiling (nCounter NanoString) and GSEA-based analysis. Whereas Poly (I:C) transfection and SeV infection induced a marked modulation of cGAS effector/IAR gene expression (FDR = 0.004 and < 0.001, respectively), no significant modulation of IAR gene expression was observed in HBV-infected cells **(Figure 2.2. C)**, as further illustrated by the expression of IFNB1 and IFI44 **(Figure 2.2. E)**. To measure the impact of cGAS expression on cellular response to HBV infection, we then infected cGAS-depleted (KO_cGAS#2) and -overexpressing (cGAS_OE) HepG2-NTCP cells with HBV and analyzed the expression of the cGAS-related genes after infection. As shown in **Figure 2.2. D**, no significant modulation of the IAR signature was observed in HBV-infected samples, as further illustrated by absent modulation of IFNB1 and IFI44 expression **(Figure 2.2. F)**.

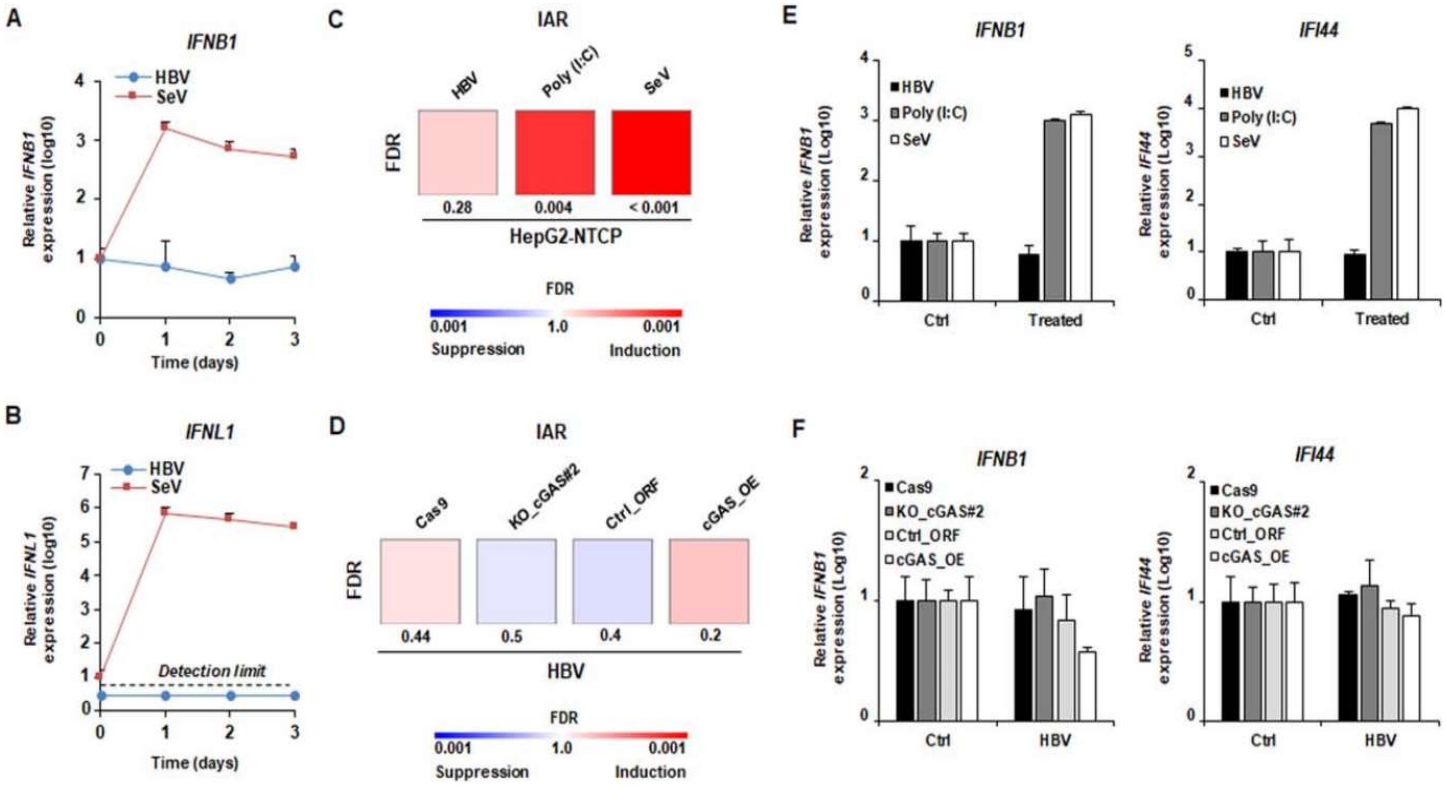


**Figure 2.2. Impaired cGAS-mediated sensing of HBV infection in HepG2-NTCP cells.** (A-B) HBV infection does not induce *IFNB1* or *IFNL1* expression. HepG2-NTCP cells were infected with HBV (MOI: 500) or SeV (MOI: 10) and total RNA was extracted every day for 3 days. RNA extracted from naive cells before infection was used as a control (D0). *IFNB1* (A) and *IFNL1* (B) expression was then assessed by qRT-PCR. Results are expressed as means ± SD *IFNB1/IFNL1* relative expression (log10) compared to controls (D0, all set at 1) from three independent experiments performed at least in duplicate (SeV) or four independent experiments performed in duplicate (HBV). No robust *IFNL1* expression was detected in HBV-infected samples (representative dots are presented under the "detection limit" dotted line). (C, E) cGAS-related ISG are not affected by HBV infection. HepG2-NTCP cells were infected with HBV or SeV. Alternatively, HepG2-NTCP cells were transfected with Poly (I:C) (100ng). Two days after infection or transfection, total RNA was extracted. Gene expression of IAR signature was then analyzed using multiplexed gene profiling. Results were analyzed by GSEA enrichment compared to non-transfected or non-infected controls (C) or by *IFNB1* and *IFI44* gene expression (log10) compared to non-transfected or non-infected controls (set at 1) (E). One experiment performed in triplicate is shown. (D, F) cGAS expression level does not affect the cellular response to HBV infection. HepG2-NTCP-Cas9 (Cas9), HepG2-NTCP-KO_cGAS#2 (Ko_cGAS#2), HepG2-NTCP-Ctrl_ORF (Ctrl_ORF), and HepG2-NTCP-cGAS_OE (cGAS_OE) were infected with HBV. Two days after infection, total RNA was extracted. Gene expression of IAR signature gene set was then analyzed using multiplexed gene profiling. Results were analyzed by GSEA enrichment compared to non-transfected or non-infected controls (D) or by *IFNB1* and *IFI44* gene expression (log10) compared to non-transfected or non-infected controls (set at 1) (F). One experiment performed in triplicate is shown. IAR: innate antiviral response gene set.

To exclude the possibility that low HBV infection could be the reason for the absence of IFN induction, we performed additional experiments using increasing MOIs. As shown in **Figure 2.3. A-C**, no induction of IFNB1 **(Figure 2.3. A)** was observed even at a MOI 10000, despite very high infection efficiency as shown by quantitation of pre-genomic RNA and immunofluorescence of HBsAg **(Figure**

**2.3. B-C)**. To investigate whether IFN induction occurs potentially at a very early step of viral infection and was missed in the experimental design shown above, we performed a time course studying IFN response to HBV within the first 24 hours post infection. As shown in Figure 2.3. D, HBV infection did not induce a measurable IFN response during early steps of HBV infection. In contrast, SeV, an established inducer of IFN showed robust induction of IFN responses in HepG2-NTCP cells **(Figure 2.3. D)**. Taken together, these data suggest an absence of sensing of HBV infection by the cGAS-STING pathway in HepG2-NTCP cells.

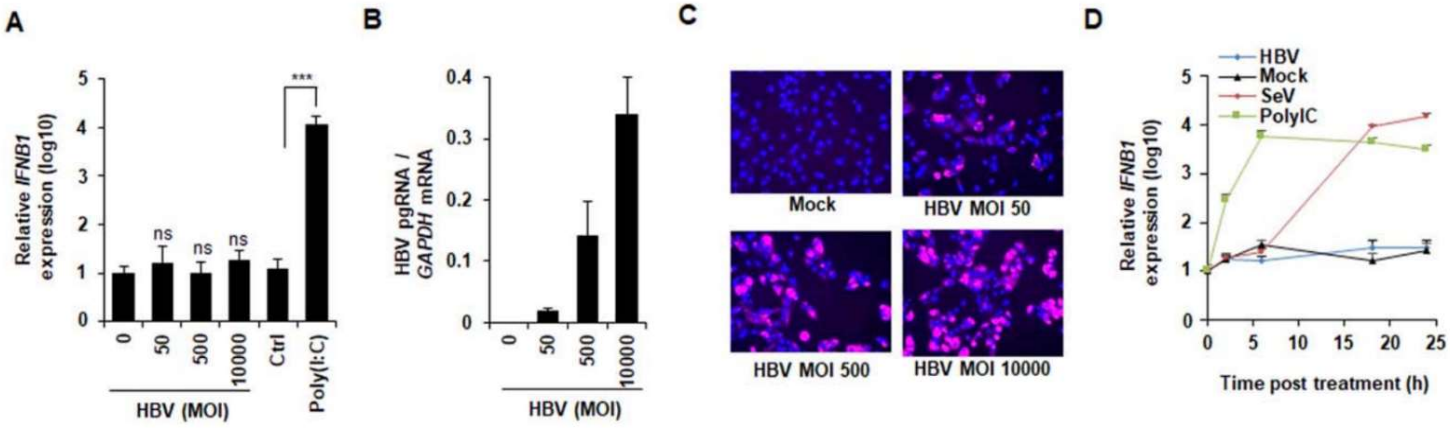


**Figure 2.3. Impaired sensing of HBV infection at high MOIs or early time points of infection.** (A-C) HepG2-NTCP cells were infected with HBV at increasing MOIs (0, 50, 500, and 10000 GEq/cell) or transfected with Poly (I:C) (100 ng). Two days after infection or transfection, cells were lysed, total RNA was extracted, and *IFNB1* expression (A) as well as HBV pgRNA levels (B) were quantified by qRT-PCR. (A) Results are expressed as means ± SD relative *IFNB1* expression (log10) compared to mock infected cells (MOI 0, set at 1) from three independent experiments performed in triplicate. (B) Results are expressed means ± SD relative HBV pgRNA levels compared to mock infected cells (MOI 0, set at 100%) from three independent experiments performed in triplicate. Alternatively, HBV infection was assessed at 10 days post infection by IF of HBsAg (C). One representative experiment is shown. (D) HBV infection does not induce *IFNB1* expression at early time points. HepG2-NTCP cells were either infected by HBV or SeV, or transfected with Poly (I:C). Total RNA was extracted at 2 h, 6 h, 18 h, and 24 h post infection/transfection and *IFNB1* expression was assessed by qPCR. Results are expressed as means ± SD relative *IFNB1* expression (log10) compared to naive cells (0, set at 1) from three independent experiments performed in triplicate.

As the HBV genome is packaged into the nucleocapsid [37], we investigated whether packaging shields virion DNA from cGAS recognition. We purified HBV genomic rcDNA from HBV infectious particles **(Figure S2.3)** and transfected the naked viral genome into HepG2-NTCP cells (1 µg, corresponding to approximately 106-107 HBV DNA copies/µL). As shown **in Figure 2.4. A**, a significant (FDR = 0.02) induction of the IAR signature (illustrated by IFNB1 and IFI44 expression**, Figure 2.4. B**) was observed after both rcDNA and dsDNA transfection, suggesting sensing of the naked HBV genome. Interestingly, the amount of cellular HBV DNA copies was higher in HBV-infected cells compared to rcDNA transfected cells **(Figure 2.4. C)**, confirming that the levels of HBV DNA in HBV infected cells are sufficient to trigger IFN signaling and the absence of HBV sensing in infected

cells was not due to low MOIs. Moreover, the induction of the IAR gene expression was absent in HepG2-NTCP-KO_cGAS#2 cells, suggesting a cGAS-specific activation of innate immunity by both dsDNA and rcDNA transfection in our model.

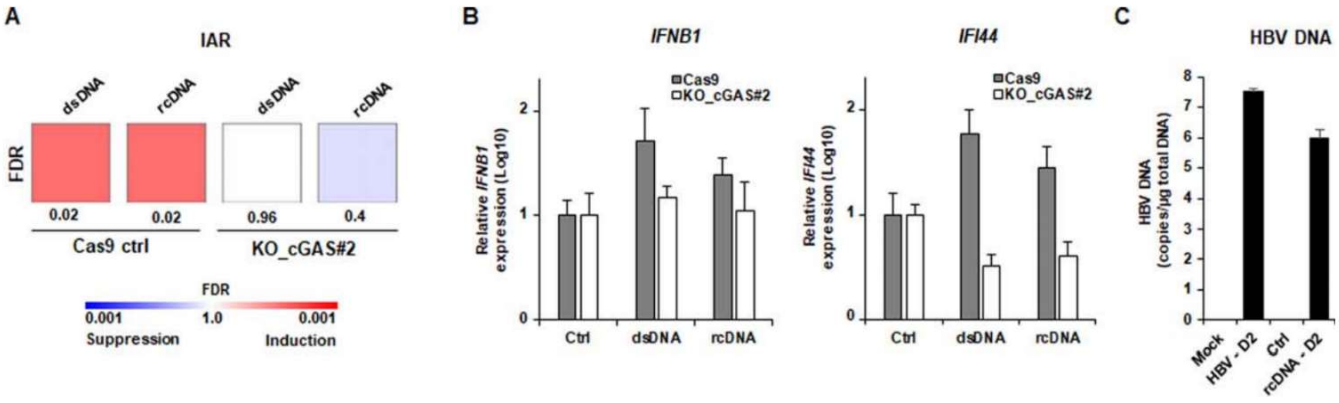


**Figure 2.4. Sensing of HBV rcDNA in HepG2-NTCP cells**. (A-B) Transfection of purified HBV rcDNA genome induces a cGAS-mediated innate immune response. HBV rcDNA was extracted from recombinant HBV virions as described in Experimental Procedures and quantified by qPCR (Figure S2.3). HBV rcDNA (1 µg) and positive control dsDNA (1 µg) were transfected into HepG2-NTCP-Cas9 and HepG2-NTCP-KO_cGAS#2 cells. Three days after transfection, total RNA was extracted. Gene expression of IAR set was then analyzed using multiplexed gene profiling. The transcripts were analyzed by GSEA enrichment compared to non-transfected control (A) or by *IFNB1* and *IFI44* gene expression (log10) compared to non-transfected control (set at 1) (B). One experiment in triplicate is shown. (C) HBV DNA in HepG2-NTCP cells after rcDNA transfection and HBV infection are similar. HepG2-NTCP were infected with HBV or transfected with HBV rcDNA (1µg). At day 2 after transfection/infection, DNA was extracted and total HBV DNA was quantified by qPCR. Results are expressed as means ± SD total DNA copies per µg of total DNA (log10) from three independent experiments performed in triplicate (HBV infection) and two independent experiments performed in triplicate (HBV rcDNA transfection). IAR: innate antiviral response gene set.

To validate these observations in a more physiological model, we infected PHH with HBV and control cGAS gene expression after two days of infection. Interestingly, while SeV strongly induced IAR gene expression **(Figure 2.5 B)**, a highly efficient HBV infection **(Figure 2.5. A)** did not induce the induction of the expression of innate antiviral response genes **(Figure 2.5. B)**. In contrast, the transfection of rcDNA and dsRNA into PHH from four different donors robustly triggered the expression of IFNB1 and IFNL1 **(Figure 2.5. C)**, suggesting a robust sensing of viral DNA in human hepatocytes. Collectively, these data suggest that non-encapsidated HBV DNA is sensed by cGAS, but this sensing is impaired during HBV infection.

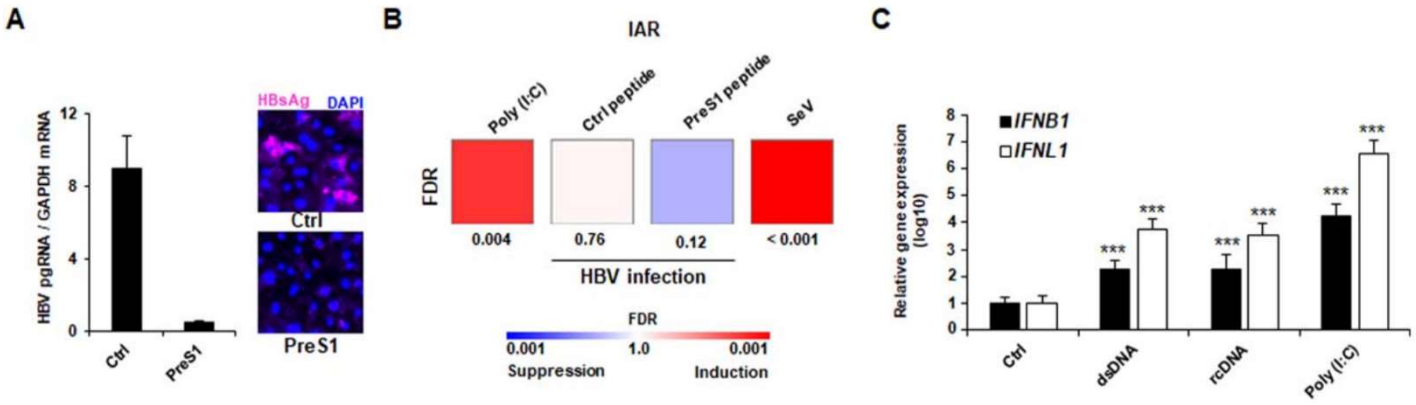


**Figure 2.5. Sensing of naked HBV rcDNA but not infectious HBV virions in primary human hepatocytes.** (A-B) HBV infection does not induce ISG expression in PHH. PHH were treated with a preS1 peptide (PreS1) or a scrambled peptide (Ctrl) for one hour before infection with HBV. As positive controls, PHH were transfected with Poly (I:C) (100ng) or infected with SeV (MOI ~ 10). HBV infection of PHH was assessed 10 days post infection by qRT-PCR (A, left panel) or immunofluorescence (A, right panel). qRT-PCR results are expressed as means ± SD ratio HBV pgRNA RNA / GAPDH mRNA from one experiment corresponding to the gene profiling experiment and performed in duplicate. Two days after infection, total RNA was extracted and gene expression of IAR was then analyzed using multiplexed gene profiling. Results were analyzed by GSEA enrichment compared to non-transfected control (B). One experiment in triplicate is shown. (C) Induction of *IFBN1* and *IFNL1* expression in PHH. PHH were transfected with dsDNA (4 µg) or HBV rcDNA (4 µg). *IFNB1*- and *IFNL1* expression was assessed 24 h after transfection by qRT-PCR. Results are expressed as means ± SD log10 *IFNB1* or *IFNL1* expression compared to non-transfected control cells (Ctrl, set at 1) from five independent experiments performed at least in duplicate. IAR: innate antiviral response gene set.

**The cGAS-STING pathway exhibits robust antiviral activity against HBV infection with reduction of cccDNA levels.** As cGAS exhibits an antiviral activity against a broad range of DNA and RNA viruses (meaning even in absence of direct viral sensing) [20], we then investigated the antiviral effect of the cGAS-STING signaling pathway in HBV infection. We silenced the expression MB21D1, TMEM173 (encoding the STING protein), TBK1 and IFI16 (encoding the gamma-interferon-inducible protein 16, another cytoplasmic DNA sensor able to directly activate STING [17]) in HepG2-NTCP cells prior to infection with HBV. As shown in **Figure 2.6. A-B**, silencing of MB21D1, TMEM173 and TBK1 expression induced a marked increase in HBV infection. In contrast, the silencing of IFI16 had no effect on HBV infection. CRISPR/Cas9-mediated KO or overexpression of cGAS protein resulted in a marked increase or decrease in HBV infection and HBV cccDNA levels – the key viral nucleic acid responsible for viral persistence **(Figure 2.6. C-E)**. Notably, the overexpression of cGAS did not affect NTCP expression at the cell surface, suggesting that the susceptibilities of the different cell lines to HBV infection are equivalent **(Figure S2.4)**. Taken together, our results suggest that cGAS is functional and exerts antiviral activity in HBV permissive cells.

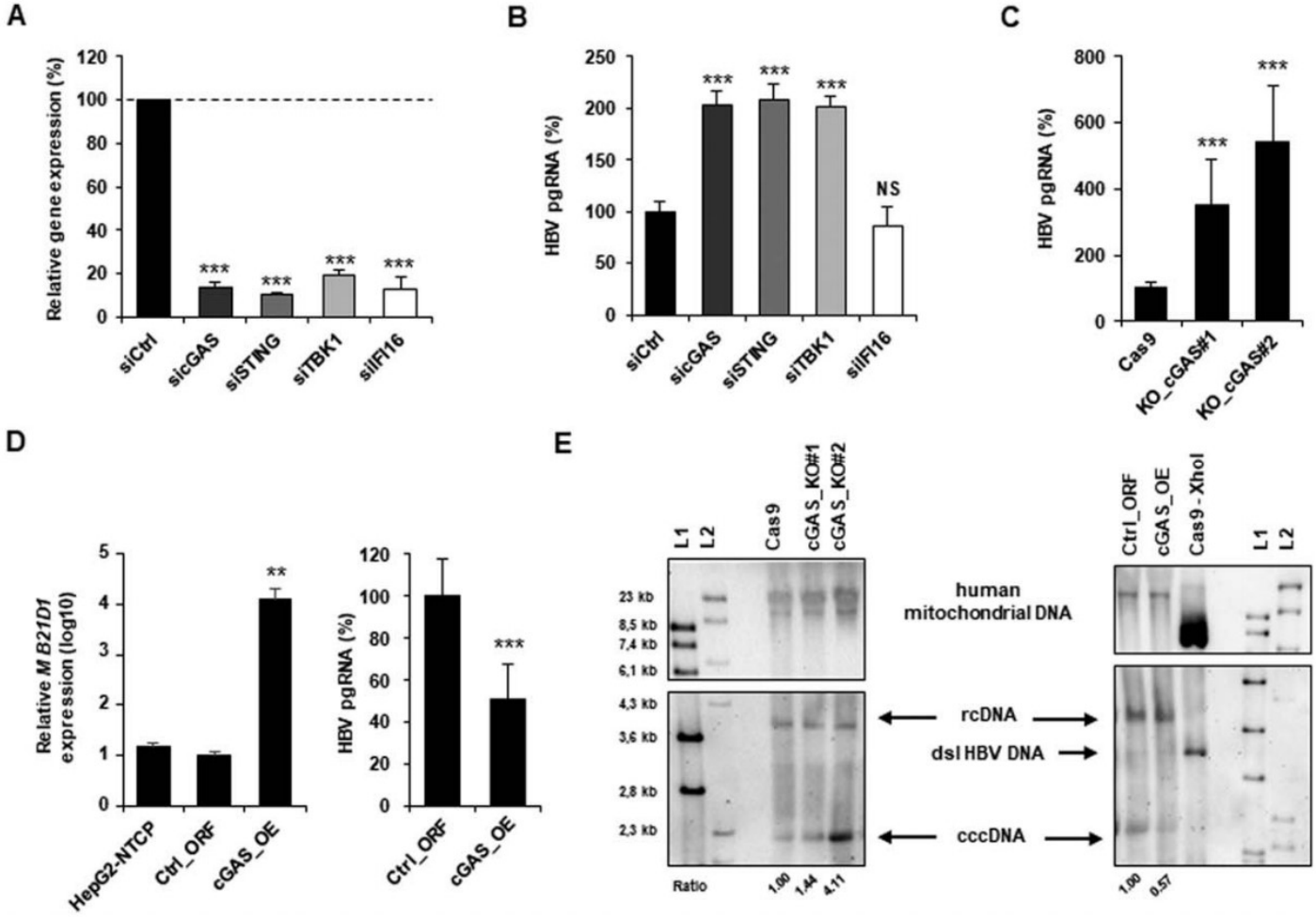


**Figure 2.6. Antiviral activity of cGAS results in reduction of HBV cccDNA.** (A-B) Silencing of cGAS-related gene expression increases HBV infection. siRNA targeting *MB21D1* (sicGAS), *TMEM173* (siSTING), *TBK1* (siTBK1), *IFI16* (siIFI16) or a non-targeting siRNA (siCtrl) were reverse-transfected into HepG2-NTCP cells 2 days prior to HBV infection. Silencing efficacy was assessed by qRT-PCR 2 days after transfection (A). Results are expressed as means ± SD % gene expression relative to siCtrl (set at 100%) from four independent experiments performed in technical duplicate. HBV infection was assessed by quantification of HBV pgRNA by qRT-PCR 10 days after infection (B). Results are expressed as means ± SD % HBV pgRNA expression relative to siCtrl (set at 100%) from four independent experiments performed in technical duplicate. (C) KO of *MB21D1* gene increases HBV infection. cGAS_KO#1, cGAS_KO#2, and the control Cas9 cells were then infected with HBV and viral infection was assessed 10 days after infection as described above. Results are expressed as means ± SD % HBV pgRNA expression relative to control cell line (Cas9, set at 100%) from three independent experiments performed in triplicate. (D) cGAS overexpression reduces HBV infection. HepG2-NTCP cells were transduced with lentivirus encoding either a control plasmid (Ctrl_ORF) or a plasmid encoding the full length *MB21D1* ORF (cGAS_OE). *MB21D1* expression was assessed by qRT-PCR (left panel). Results are expressed as means ± SD % relative *MB21D1* expression (log10) relative to control cell line (Ctrl_ORF, set at 1) from three independent experiments performed in duplicate. HepG2-NTCP, Ctrl_ORF, and cGAS_OE cells were then infected with HBV for ten days and HBV infection was assessed as described above. Results are expressed as means ± SD % HBV pgRNA expression relative to control cell line (Ctrl_ORF, set at 100%) from three independent experiments performed in triplicate. (E) Detection of HBV cccDNA by Southern blot. HepG2-NTCP-derived cGAS_KO- or cGAS_overexpressing cell lines were infected for 10 days with HBV. Total DNA from indicated HBV infected cells was extracted and HBV DNA were detected by Southern blot. Two different DNA ladders (L1 & L2) were used. *XhoI* digestion of DNA extracted from HBV-infected HepG2-NTCP-Cas9 cells was used as a control and resulted in a single 3.2 kb band (dsl HBV DNA). Mitochondrial DNA (mt DNA) was detected as a loading control. One experiment is shown.

**HBV infection induces repression of cGAS and its effector gene expression in cell culture and in liver chimeric mice.** As several reports have suggested that HBV proteins can inhibit IFN-signaling pathways [16], we next investigated whether HBV infection interferes with the expression of cGAS-related gene by quantifying MB21D1/cGAS mRNA and protein expression **(Figure 2.7. A-B)**. Interestingly, cGAS protein expression **(Figure 2.7. B)** as well as the expression of MB21D1, TMEM173, and TBK1 mRNA **(Figure 2.7. C)** were significantly inhibited in HBV-infected cells. To confirm this observation in vivo, we then investigated the expression of human MB21D1 expression in HBV-infected human liver chimeric mice. MB21D1 was expressed at low but detectable levels **(Figure 2.7. D)**. As shown in **Figure 2.7. E**, MB21D1 expression was significantly ($p = 0.013$) downregulated in HBV-infected mice compared to non-infected control mice, confirming our results in the cell culture model. Importantly, MB21D1 expression levels did not correlate with HBV genotype **(Table 2.1)**. An absent correlation of human serum albumin with either MB21D1 expression **(Table 2.1, Figure 2.7. E)** or status of HBV infection (Table 1, t-test HBV versus Ctrl: $p = 0.26$) largely excludes that the observed differences in cGAS expression are due to different human hepatocyte repopulation levels or due to a decrease of human hepatocyte cell viability in individual animals. To investigate whether HBV modulates cGAS effector function, we analyzed virus-induced changes on cGAS effector gene expression using gene expression profiling in three control mice and the three HBV-infected mice exhibiting the lowest levels of MB21D1 expression (Table 1). As shown in **Figure 2.7. F**, HBV infection resulted in a significant (FDR = 0.047) down-regulation of the expression of cGAS effector genes in human hepatocytes in chimeric mice. The data showed that HBV represses expression of cGAS and its effector genes in vivo.

**Table 2.1. cGAS expression in HBV-infectedhuman liver chimeric mice**

| | Mouse | Albumin (μg/mL) | HBV (IU/mL) | *MB21D1* mRNA |
|---|---|---|---|---|
| Control | 6410 | 1,280 | — | 1.40E-03 |
| | **6472** | 2,720 | — | 1.40E-03 |
| | **6251** | 3,240 | — | 2.30E-03 |
| | **6254** | 7,870 | — | 1.90E-03 |
| HBV | | | | |
| Gt E | 4770 | 4,200 | 2.9E+07 | 1.30E-03 |
| | 4773 | 4,800 | 1.5E+08 | 1.70E-03 |
| | **4766** | 12,760 | 3.6E+06 | 3.20E-04 |
| | **4771** | 13,120 | 6.2E+05 | 3.70E-04 |
| Gt D | 4846 | 2,127 | 5.5E+05 | 3.80E-04 |
| | **4847** | 10,045 | 1.6E+08 | 1.70E-04 |
| | 4848 | 1,992 | 6.7E+06 | 7.50E-04 |

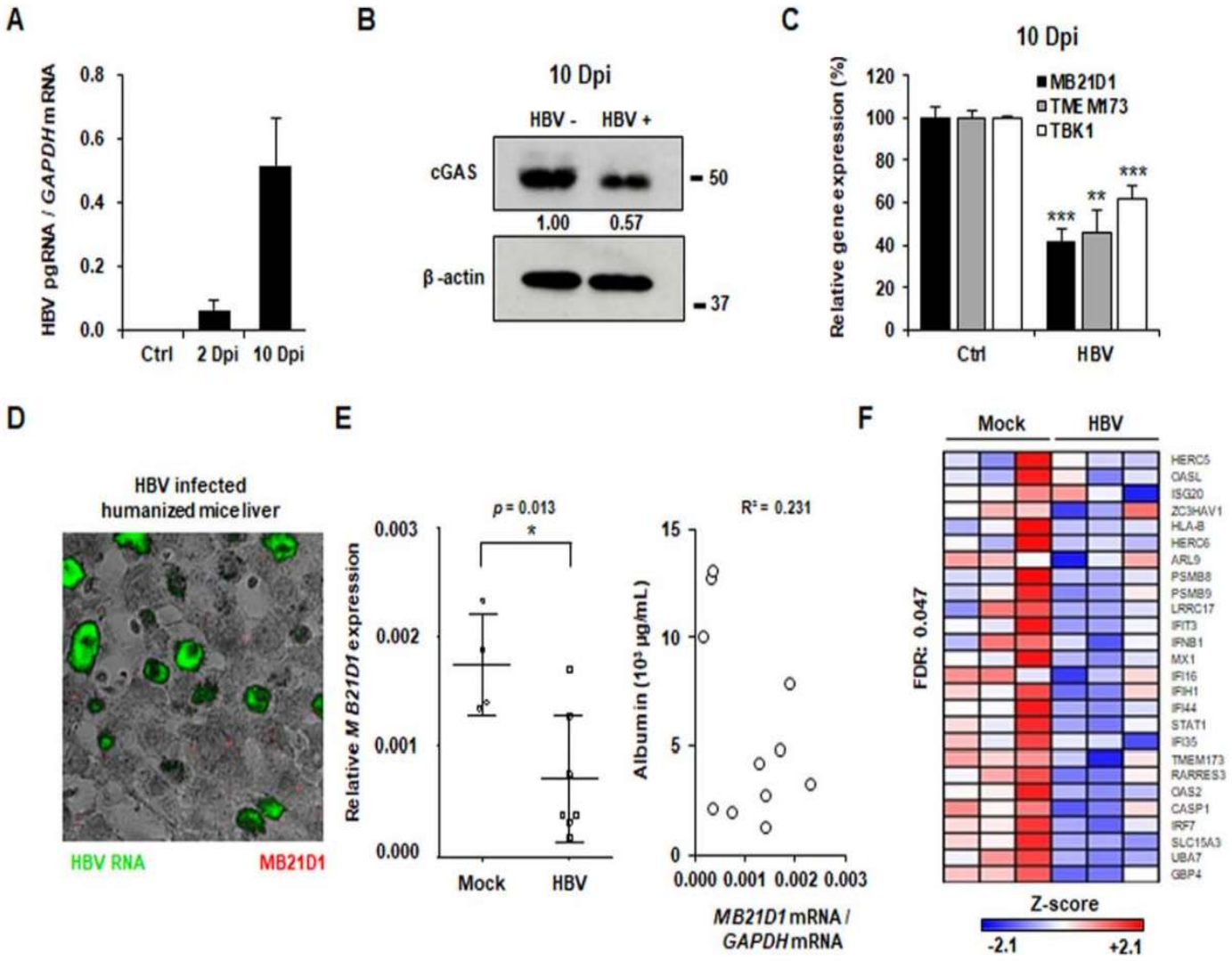


**Figure 2.7. HBV infection suppresses the expression of the cGAS-related genes cell culture and humanized liver chimeric mice *in vivo*.** (A-C) HepG2-NTCP cells were infected with HBV for 10 days. HBV infection was assessed by quantification of HBV pgRNA by qRT-PCR. Results are expressed as means ± SD from three experiments performed in triplicate. cGAS protein expression was assessed 10 days after infection (B, one experiment is shown). Gene expression relative to non-infected control cells of *MB21D1*, *TMEM173* and *TBK1* were assessed by qRT-PCR at day 10 after infection (C). Results are expressed as means ± SEM from three independent experiments performed in triplicate. (D-E) *MB21D1*- and IAR gene set expression is impaired in HBV-infected mice. uPA-SCID mice were infected with HBV for 16 weeks. Mice were then sacrificed and HBV infection was assessed by HBV RNA specific in situ hybridization (D) and quantification of HBV viral load in the serum (Table 1). Human *MB21D1* expression was detected in human hepatocytes by FISH from one HBV-infected mouse (D) and by qRT-PCR from 7 HBV-infected mice and 4 control mice (E, left panel). Results are expressed as the ratio *MB21D1* mRNA / *GAPDH* mRNA. All individual mice are presented as well as means ± SD for each group (Mock- and HBV-infected mice). The level of *MB21D1* expression was independent of the viability of engrafted human hepatocytes as indicated by an absent correlation between *MB21D1* expression and the human serum albumin expression in humanized mice ($R^2$ = 0.231, E, right panel). (F) The IAR gene set was analyzed using the nCounter NanoString in mice 6472, 6251, and 6254 (Mock-infected mice, Table 1) and 4766, 4771, and 4847 (HBV-infected mice, Table 1). A significant downregulation (FDR = 0.047) of the gene set was observed in HBV-infected mice compared to control mice. Individual Z-scores for the genes significantly modulated between the two groups according to GSEA analysis are presented. Negative Z-score (blue) and positive Z-score (red) correspond to repression and induction of the indicated genes, respectively. Dpi: days post infection.

The interaction between HBV and the innate immune system is a complex process still remaining elusive and controversial [15]. Collectively, our data demonstrate that in human hepatocytes (i) naked HBV genomic rcDNA is sensed in a cGAS-dependent manner whereas the packaged HBV genome appears not to be recognized during viral infection; (ii) cGAS-STING pathway exhibits antiviral activity against HBV infection including reduction of viral cccDNA levels; (iii) HBV infection suppresses both cGAS expression and function in cell culture and humanized liver chimeric mice.

The detection of HBV DNA by the cellular sensors within infected cells is still poorly understood and remains controversial. In vitro and in vivo data strongly suggest that HBV behaves like a stealth virus unable to trigger any innate immune response [8, 9, 11]. Other studies have suggested that HBV-derived dsDNA fragments [25] and viral nucleocapsid destabilization and disassembly [38, 39] could induce innate immune responses. Our results demonstrate, that in human hepatocytes - the natural target cell of HBV infection - the exposure of the naked HBV genome leads to the activation of innate antiviral immune responses. In contrast, sensing is largely absent during HBV infection, most likely due to packaging into the viral capsid. These results extend a previous observation in hepatoma cell lines transfected with replication-competent HBV DNA that the HBV genome itself can be recognized by the classical sensors [25].

Interestingly, the capsid of HIV-1 also prevents the sensing of HIV cDNA by cGAS following reverse-transcription up to integration, whereas HIV-2 capsid may unmask the cDNA leading to a stronger sensing by cGAS and a lower pathogenicity of the strain [40].

Another explanation of this absence of sensing would be the lack a functional STING protein in hepatocyte, as it has been recently reported [41]. In our study, rcDNA and dsDNA were sensed in a cGAS-dependent manner and were able to activate the cGAS-mediated antiviral response in HepG2-NTCP cells **(Figure 2.2)**. Moreover, we detected STING at the protein level in accordance with a recent study [42] and specific silencing of TMEM173 (STING) expression was associated with a significant increase in HBV infection **(Figure 2.6)**. Consequently, it is likely that STING is functionally active in HepG2 cells. The observed HBV DNA sensing in PHH **(Figure 2.5)** suggests that the foreign DNA detection pathways are active in PHH as well. This observed innate immune response in spite of a weak STING expression may suggest a STING-independent activity of cGAS as it has been recently reported [43], including in hepatocytes [44]. To understand the impact of the cGAS and STING expression on innate immune response to HBV infection, it would be of further interest to analyze the HBV-induced modulation of gene expression in Kupffer cells following phagocytosis, as they exhibit higher STING- and cGAS levels compared to hepatocytes [41, 45] and respond to HBV infection [11]. In the same vein, cGAMP has been shown to be packaged in viral particles [46]. It would be of interest to determine whether HBV particles can incorporate cGAMP during viral assembly and to test their ability to stimulate other cell types through this indirect pathway.

Moreover, our results show conclusive evidence that cGAS basal expression has antiviral activity against HBV infection including reduction of viral cccDNA. This finding extends a previous studies showing that cGAS exhibited an antiviral activity against a broad range of RNA and DNA viruses [20] and that the cGAS/STING pathway can impair HBV replication and assembly in transfection studies

[24, 25]. Schoggins and colleagues have proposed that the expression of cGAS may be responsible for the establishment of a basal antiviral level in the cells through its activation by an unknown ligand. cGAS-depleted cells may then be more susceptible to viral infections through the downregulation of the basal level of innate antiviral genes [20].

Given its antiviral function, cGAS is a target of choice for viruses in order to evade immune responses. It has been reported that the Kaposi's sarcoma-associated herpesvirus negatively regulated cGAS-dependent signaling pathway [47, 48]. In the same vein, HBV viral proteins have been shown to interfere with the JAK-STAT signaling pathway [16]. Our data suggest that HBV can repress the expression of the cGAS and its related genes, such as MB21D1, TMEM17 and TBK1. More interestingly, MB21D1 expression was downregulated in the liver of HBV-infected mice, validating the relevance of these findings in vivo. It still needs to be determined whether HBV can directly target cGAS and cGAS-related factors for an active inhibition of this signaling pathway. A recent study elegantly demonstrated an active inhibition of cGAS pathway by Dengue virus through NS2B protein [49]. On the other hand, MB21D1 (as a classical member of the ISG [20, 36]) downregulation may be the consequence of the global inhibition of the canonical IFN pathways by HBV as suggested by some investigators [16, 50], but not by others [11, 13, 14]. Given the antiviral activity of the cGAS-signaling pathway against HBV including reduction of HBV **cccDNA (Figure 2.5, [24, 25])** the virus-mediated restriction of MB21D1 expression may play an additional role in HBV immune evasion.

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Author names in bold designate shared co-first authorship.

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**Conflict of interest:** The authors declare no competing financial interests.

**Author contribution.** TFB initiated the study. TFB, ERV and CS designed and supervised research. ERV, SAY, LH, CB, VTL, SD, LM, JL, TC and DD performed the experiments. ERV, SAY, LH, CB, VTL, JL, DD, MBZ, NP, CS and TFB analyzed the data. PP provided liver resections for PHH isolation. NM, IH and NP made substantive intellectual contributions. ERV, SAY, CS and TFB wrote the paper.

**List of Abbreviations**

HBV: hepatitis B virus; cGAS: cyclic GMP-AMP synthase; HSPG: heparan sulfate proteoglycan; NTCP: Na+/taurocholate cotransporting polypeptide; rcDNA: relaxed circular DNA; cccDNA: covalently closed circular DNA; pgRNA: pregenomic RNA; PRR: pattern recognition receptors; cGAMP: cyclic GMP-AMP; IFN: interferon; STING: stimulator of IFN genes; ISG: IFN-stimulated gene; PHH: primary human hepatocyte; PEG: polyethylene glycol; siRNA: small interfering RNA; HIV: human immunodeficiency virus; VSV-G: vesicular stomatitis virus glycoprotein; sgRNA: single-guide RNA; PMM: primary hepatocyte maintenance medium; dslDNA: double stranded linear DNA; dsDNA: double stranded DNA; GSEA: gene set enrichment analysis; FDR: false discovery rate; FISH: fluorescence in situ hybridization; KO: knock-out. SeV: Sendai virus. IAR: innate antiviral response gene set. Dpi: days post infection.

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**Supplementary Experimental Procedures**

**Detection of cGAS protein expression using two independent antibodies**. HepG2-NTCP cells were transfected with a siRNA targeting MB21D1 expression (sicGAS) or with a non-targeting siRNA control (siCtrl). Three days after transfection, cells were lysed and cGAS protein expression was assessed as described in the Experimental Procedures section using two rabbit polyclonal anti-cGAS antibodies (HPA031700, Sigma & NBP1-86761, Novus Biologicals). Given the proximity of the bands, another western blot was run in parallel for the detection of $\beta$-actin as a control. Molecular weights were assessed using the Precision Plus Protein™ Standards molecular weight marker (Bio-Rad).

**Detection of HBV DNA by PCR and qPCR**. DNA was extracted using QiaAMP DNA MiniKit (Qiagen) following manufacturer's instructions. The presence of HBV DNA was confirmed by PCR using the following primers (expected band size: 148 bp) (1) : forward primer 5'-CACCTCGCCTAATCATC-3', reverse primer 5'-GGAAAGAAGTCAGAAGGCA-3'. For qPCR quantification of HBV DNA, The presence of HBV DNA was confirmed by PCR and quantified by qPCR using the following primers and probe (1) : forward primer 5'-CACCTCGCCTAATCATC-3', reverse primer 5'-GGAAAGAAGTCAGAAGGCA-3'; TaqMan probe 5'-[6FAM]-TGGAGGCTTCAACAGTAGGACATGAAC-[BHQ1]-3'. Copy number of HBV was determined using a standard curve.

**Detection of NTCP expression by flow cytometry**. HepG2 cells, HepG2-NTCP-Ctrl_ORF cells, and HepG2-NTCP-cGAS_OE cells were treated with the AF647-labelled pres1 peptide for one hour at 37° C as described (1). Cells were then fixed with 2% paraformaldehyde for 20 minutes at room temperature. NTCP expression was then quantified by flow cytometry using MacsQuant instrument (Miltenyi).

**Supplementary Figures**

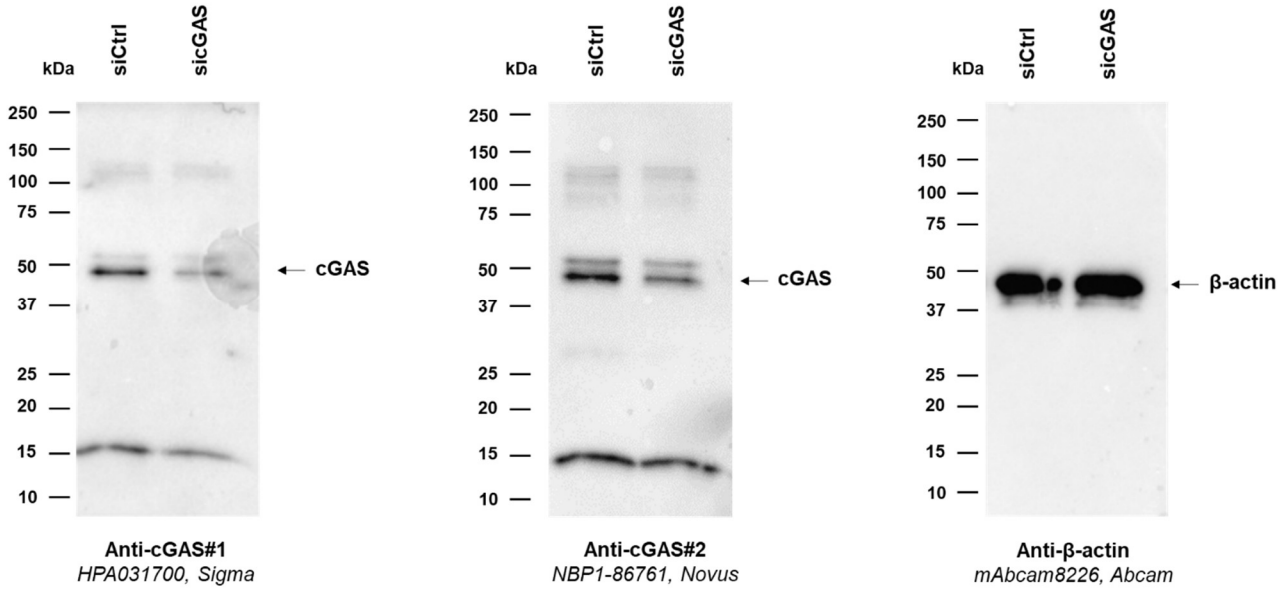


**Figure S2.1. Detection of cGAS protein in HepG2-NTCP cells (related to Figure. 1).** HepG2-NTCP cells were transfected for three days with a siRNA targeting *MB21D1* expression (sicGAS) or with a non-targeting siRNA control (siCtrl). Cells were then lysed and cGAS protein expression was assessed using two rabbit polyclonal anti-cGAS antibodies (HPA031700, Sigma, used in the main manuscript & NBP1-86761, Novus Biologicals). β-actin expression was assessed as a control. One representative experiment is shown.

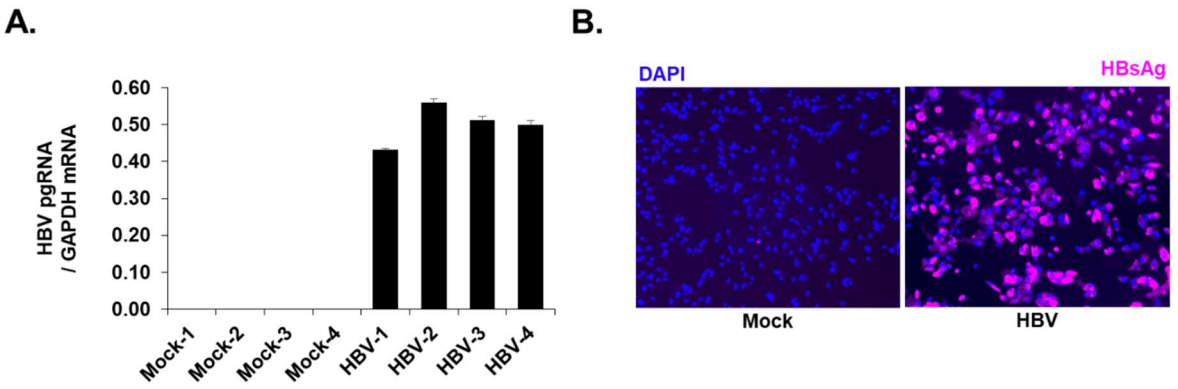


**Figure S2.2. Analysis of HBV infection in HBV time course samples with quantification of HBV pregenomic RNA and HBsAg expression (related to Figure 2).** HepG2-NTCP cells were infected with HBV as described in Experimental Procedures. 10 days after infection, total RNA was extracted and HBV infection was assessed by quantification of HBV pgRNA as described in Methods. Results are expressed as means ± SD HBV pgRNA / GAPDH mRNA from four independent experiments performed in duplicate (corresponding to the four experiments shown in

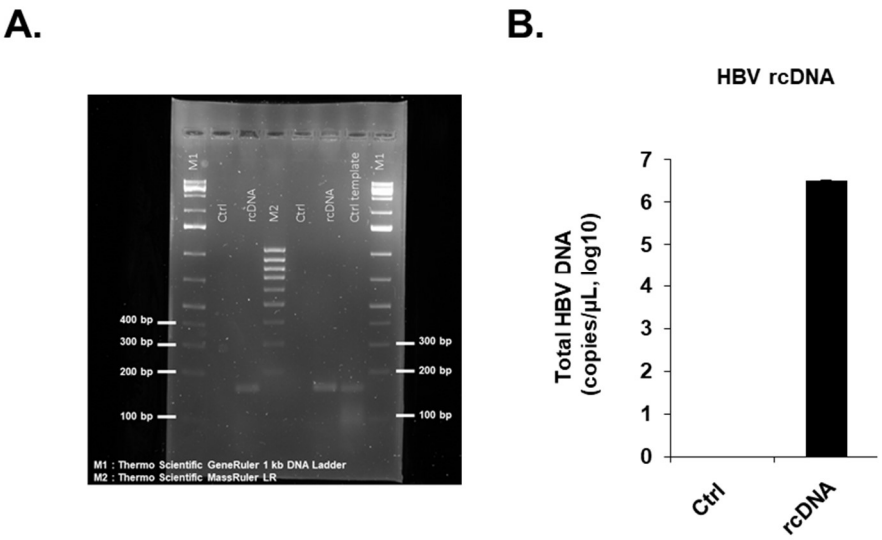


**Figure S2.3. Analysis and quantification of HBV DNA extracted from HBV infectious particles by PCR (related to Figure 4).** A-B. HBV genomic DNA (rcDNA) was extracted from cell culture-derived HBV virions. Extraction from naive HepG2-NTCP control supernatants without virus was used as a control (Ctrl). HBV DNA standard preparation used as a template for the calculation of HBV DNA concentration was used as a positive control (Ctrl template). The presence of HBV DNA was controlled by PCR (expected band size: 148 base pairs [bp]) (A) and quantified by qPCR (B). Two independent experiments (A) and one experiment performed in triplicate (B) are shown. C. Quantification of total HBV DNA in transfected or infected cells. HepG2-NTCP were infected with HBV or transfected with rcDNA. Three days after transfection/infection, DNA was extracted and total HBV DNA was quantified by qPCR.

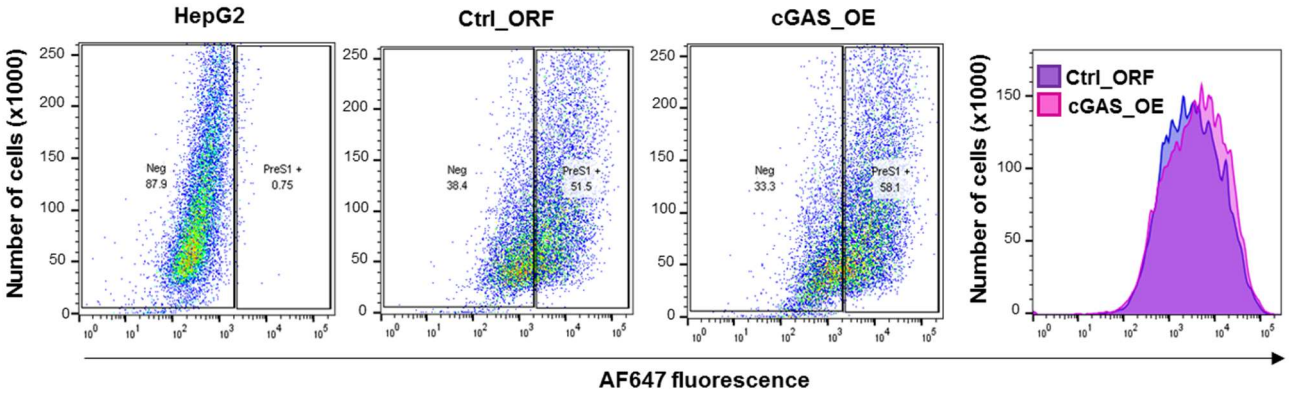


**Figure S2.4. PreS1-binding/NTCP cell surface expression is independent on cGAS expression.** HepG2 cells, HepG2-NTCP-Ctrl_ORF cells, and HepG2-NTCP-cGAS_OE cells were treated with the AF647-labelled preS1 peptide for one hour at 37°C. PreS1 binding corresponding to NTCP expression was quantified by flow cytometry.

### Supplementary Tables

**Tables S1:** Specific probes used for the detection of HBV and Mitochondrial DNA (Lucifora *et al.*, 2017).

| Target | Name | Sequence |
|---|---|---|
| HBV | HBV-F1 | TAGCGCCTCATTTTGTGGGT |
| | HBV-R1 | CTTCCTGTCTGGCGATTGGT |
| | HBV-F2 | TAGGACCCCTGCTCGTGTTA |
| | HBV-R2 | CCGTCCGAAGGTTTGGTACA |
| | HBV-F3 | ATGTGGTATTGGGGGCCAAG |
| | HBV-R3 | GGTTGCGTCAGCAAACACTT |
| | HBV-F4 | TGGAACCTTTTCGGCTCCTC |
| | HBV-R4 | GGGAGTCCGCGTAAAGAGAG |
| | HBV-F6 | TACTGCACTCAGGCAAGCAA |
| | HBV-R6 | TGCGAATCCACACTCCGAAA |
| | HBV-F8 | AGACGAAGGTCTCAATCGCC |
| | HBV-R8 | ACCCACAAAATGAGGCGCTA |
| Mitochondrial DNA | Fw_huND1 | CCCTACTTCTAACCTCCCTGTTCTTAT |
| | Rw_huND1 | CATAGGAGGTGTATGAGTTGGTCGTA |
| | Fw_huND5 | ATTTTATTTCTCCAACATACTCGGATT |
| | Rw_huND5 | GGGCAGGTTTTGGCTCGTA |
| | Fw_huATP6 | CATTTACACCAACCACCCAACTATC |
| | Rw-huATP6 | CGAAAGCCTATAATCACTGTGCC |

**Tables S2:** Specific probes of the IAR gene set for multiplexed gene profiling analysis.

| Gene | Accession Number | Target Sequence | Note |
|---|---|---|---|
| ATP5B | NM_001686.3 | GAAATTCTGGTGACTGGTATCAAGGTTGTCGATCTGCTA GCTCCCTATGCCAAGGGTGGCAAAATTGGGCTTTTTGGT GGTGCTGGAGTTGGCAAGACTG | HG |
| BAG6 | NM_001199698.1 | CATTGATCACGGGGCTAGAAGAGTATGTGCGGGAGAGTT TTTCCTTGGTGCAGGTTCAGCCAGGTGTGGACATCATCC GGACAAACCTGGAATTTCTCCA | HG |
| NDUFA2 | NM_001185012.1 | ATGGGCTAGGCTTTAGGGTCCGCGGTTGGTCAGACCGG AGCACTTGGCCTGAAGACCTGGAATTGGCGACTTCGATA TTAACAAGGATGGCGGCGGCCGC | HG |
| ARL9 | NM_206919.1 | CAGATATCCATGAAGCTTTGGCATTATCTGAAGTGGGAA ATGACAGGAAGATGTTCTTGTTTGGAACCTACCTGACTAA GAATGGCTCAGAGATACCCTC | Schoggins |
| CASP1 | NM_001223.3 | TGGAGACATCCCACAATGGGCTCTGTTTTTATTGGAAGA CTCATTGAACATATGCAAGAATATGCCTGTTCCTGTGATG TGGAGGAAATTTTCCGCAAGG | Schoggins |
| CXCL16 | NM_001100812.1 | CCATGGGTTCAGGAATTGATGAGCTGTCTTGATCTCAAA GAATGTGGACATGCTTACTCGGGGATTGTGGCCCACCAG AAGCATTTACTTCCTACCAGCC | Schoggins |
| CXCL8 | NM_000584.2 | ACAGCAGAGCACACAAGCTTCTAGGACAAGAGCCAGGA AGAAACCACCGGAAGGAACCATCTCACTGTGTGTAAACA TGACTTCCAAGCTGGCCGTGGCT | Schoggins |
| HERC5 | NM_016323.2 | TGGGCTGCTGTTTACTTTCGGTGCTGGAAAACATGGGCA ACTTGGTCATAATTCAACACAGAATGAGCTAAGACCCTGT TTGGTGGCTGAGCTTGTTGGG | Schoggins |
| HERC6 | NM_001165136.1 | TCCATCACCCAGATTTATACTTAGAGTCAGACGAAGTCG CCTGGTTAAAGATGCTCTGCGTCAATTAAGTCAAGCTGA AGCTACTGACTTCTGCAAAGTA | Schoggins |
| HLA-B | NM_005514.6 | CCCTGAGATGGGAGCCGTCTTCCCAGTCCACCGTCCCC ATCGTGGGCATTGTTGCTGGCCTGGCTGTCCTAGCAGTT GTGGTCATCGGAGCTGTGGTCGC | Schoggins |
| HLA-H | NR_001434.3 | GAGCGGGAGGGGCCGGAGTATTGGGACCGGAACACAC AGATCTGCAAGGCCCAAGCACGGACTGAACGAGAGAAC CTGCGGATCGCGCTCCGCTACTACA | Schoggins |
| IFI35 | NM_005533.3 | TGCCCTCTGCTTGCGGGCTCTGCTCTGATCACCTTTGAT GACCCCAAAGTGGCTGAGCAGGTGCTGCAACAAAAGGA GCACACGATCAACATGGAGGAGT | Schoggins |
| IFI44 | NM_006417.4 | GATGAAAGAAAGATAAAAGGGGTCATTGAGCTCAGGAAG AGCTTACTGTCTGCCTTGAGAACTTATGAACCATATGGAT CCCTGGTTCAACAAATACGAA | Schoggins |
| IFIH1 | NM_022168.2 | GCTTGGGAGAACCCTCTCCCTTCTCTGAGAAAGAAAGAT GTCGAATGGGTATTCCACAGACGAGAATTTCCGCTATCT CATCTCGTGCTTCAGGGCCAGG | Schoggins |
| IFIT3 | NM_001031683.2 | CGCCTGCTAAGGGATGCCCCTTCAGGCATAGGCAGTATT TTCCTGTCAGCATCTGAGCTTGAGGATGGTAGTGAGGAA ATGGGCCAGGGCGCAGTCAGCT | Schoggins |
| ISG20 | NM_002201.5 | AGCCCGCCGAGGGCTGCCCCGCCTGGCTGTGTCAGACT GAAGCCCCATCCAGCCCGTTCCGCAGGGACTAGAGGCT TTCGGCTTTTTGGGACAGCAACTA | Schoggins |
| LRRC17 | NM_001031692.1 | CAGCACAACCAGATCAAAGTCTTGACGGAGGAAGTGTTC ATTTACACACCTCTCTTGAGCTACCTGCGTCTTTATGACA ACCCCTGGCACTGTACTTGTG | Schoggins |
| MX1 | NM_002462.2 | GCCTTTAATCAGGACATCACTGCTCTCATGCAAGGAGAG GAAACTGTAGGGGAGGAAGACATTCGGCTGTTTACCAGA CTCCGACACGAGTTCCACAAAT | Schoggins |
| OAS2 | NM_016817.2 | TGAAAAACAATTTCGAGATCCAGAAGTCCCTTGATGGGTT CACCATCCAGGTGTTCACAAAAAATCAGAGAATCTCTTTC GAGGTGCTGGCCGCCTTCAA | Schoggins |
| OASL | NM_198213.1 | GGCGTTTCTGAGCTGTTTCCACAGCTTCCAGGAGGCAGC CAAGCATCACAAAGATGTTCTGAGGCTGATATGGAAAAC CATGTGGCAAAGCCAGGACCTG | Schoggins |
| PLCG2 | NM_002661.2 | GCTTGAAAATCTTACACCAGGAAGCGATGAATGCGTCCA CGCCCACCATTATCGAGAGTTGGCTGAGAAAGCAGATAT ATTCTGTGGATCAAACCAGAAG | Schoggins |

| | | | |
|---|---|---|---|
| PSMB8 | NM_004159.4 | ACTCACAGAGACAGCTATTCTGGAGGCGTTGTCAATATG TACCACATGAAGGAAGATGGTTGGGTGAAAGTAGAAAGT ACAGATGTCAGTGACCTGCTGC | Schoggins |
| PSMB9 | NM_002800.4 | TCAGGTATATGGAACCCTGGGAGGAATGCTGACTCGACA GCCTTTTGCCATTGGTGGCTCCGGCAGCACCTTTATCTA TGGTTATGTGGATGCAGCATAT | Schoggins |
| RARRES3 | NM_004585.3 | CTGACCCTCGTGCCCTGTCTCAGGCGTTCTCTAGATCCT TTCCTCTGTTTCCCTCTCTCGCTGGCAAAAGTATGATCTA ATTGAAACAAGACTGAAGGAT | Schoggins |
| SLC15A3 | NM_016582.1 | GCCGCTTCTTCAACTGGTTTTACTGGAGCATCAACCTGG GTGCTGTGCTGTCGCTGCTGGTGGTGGCGTTTATTCAGC AGAACATCAGCTTCCTGCTGGG | Schoggins |
| TNFRSF1B | NM_001066.2 | CCCAGCTGAAGGGAGCACTGGCGACTTCGCTCTTCCAG TTGGACTGATTGTGGGTGTGACAGCCTTGGGTCTACTAA TAATAGGAGTGGTGAACTGTGTC | Schoggins |
| UBA7 | NM_003335.2 | GCGGGAGGATGGGTCCCTGGAGATTGGAGACACAACAA CTTTCTCTCGGTACTTGCGTGGTGGGGCTATCACTGAAG TCAAGAGACCCAAGACTGTGAGA | Schoggins |
| UBE2L6 | NM_004223.3 | TGTTTCAAAACCACTTGCCATCCTGTTAGATTGCCAGTTC CTGGGACCAGGCCTCAGACTGTGAAGTATATATCCTCCA GCATTCAGTCCAGGGGGAGCC | Schoggins |
| ZC3HAV1 | NM_020119.3 | CTCCTTCTTCACATCGTAGAAACATGGCATATAGGGCTA GAAGCAAGAGTAGAGATCGGTTCTTTCAGGGCAGCCAAG AATTTCTTGCGTCTGCTTCAGC | Schoggins |
| ZMYND15 | NM_032265.1 | CCTCAGAGCGGCCGACAACTGCATGTCCTGGTACTGCAA TGCCTTCATCTTCCACCTGGTTTACAAGCCTGCTCAAGG GAGCGGGGCCCGCCCGGCGCCC | Schoggins |
| PMAIP1 | NM_021127.2 | CTAGTGTTTTTGCCGAAGATTACCGCTGGCCTACTGTGA AGGGAGATGACCTGTGATTAGACTGGGCGGCTGGGGAG AAACAGTTCAGTGCATTGTTGTT | Schoggins |
| GBP4 | NM_052941.4 | TTCTACAAGATATGCCATGGGCCTTTTCACAGGGGACAC AGGCTTCTTAAAACAACCCGGCTTCCTCACCCTATGTCCT TTATTTACAAAGCTGTGCTCC | Schoggins |
| TMEM173 | NM_198282.1 | CTGGCATGGTCATATTACATCGGATATCTGCGGCTGATC CTGCCAGAGCTCCAGGCCCGGATTCGAACTTACAATCAG CATTACAACAACCTGCTACGGG | STING |
| IFI16 | NM_005531.1 | ACGACTGAACACAATCAACTGTGAGGAAGGAGATAAACT GAAACTCACCAGCTTTGAATTGGCACCGAAAAGTGGGAA TACCGGGGAGTTGAGATCTGTA | |
| IFNB1 | NM_002176.2 | ACAGACTTACAGGTTACCTCCGAAACTGAAGATCTCCTA GCCTGTGCCTCTGGGACTGGACAATTGCTTCAAGCATTC TTCAACCAGCAGATGCTGTTTA | |
| IRF3 | NM_001571.5 | TCATGGCCCCAGGACCAGCCGTGGACCAAGAGGCTCGT GATGGTCAAGGTTGTGCCCACGTGCCTCAGGGCCTTGG TAGAAATGGCCCGGGTAGGGGGTG | |
| IRF7 | NM_001572.3 | CGCAGCGTGAGGGTGTGTCTTCCCTGGATAGCAGCAGC CTCAGCCTCTGCCTGTCCAGCGCCAACAGCCTCTATGAC GACATCGAGTGCTTCCTTATGGA | |
| STAT1 | NM_139266.1 | ACAGTGGTTAGAAAAGCAAGACTGGGAGCACGCTGCCA ATGATGTTTCATTTGCCACCATCCGTTTTCATGACCTCCT GTCACAGCTGGATGATCAATAT | |
| TBK1 | NM_013254.2 | ACCAGTCTTCAGGATATCGACAGCAGATTATCTCCAGGT GGATCACTGGCAGACGCATGGGCACATCAAGAAGGCAC TCATCCGAAAGACAGAAATGTAG | |

HG: Housekeeping genes
Schoggins: cGAS-related genes described by Shoggins *et al.*, (Schoggins *et al.*, 2014)

**Supplementary References**

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# PARTIE III

## Un criblage haut-débit en gain de fonction identifie CDKN2C comme nouveau facteur d'hôte du HBV

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

C. Eller*, **L. Heydmann*,** C.C. Colpitts, H. El Saghire, F. Piccioni, F. Jühling, K. Majzoub, C. Pons, C. Bach, J. Lucifora, J. Lupberger, M. Nassal, G.S. Cowley, N. Fujiwara, SY. Hsieh, Y. Hoshida, E. Felli, P. Pessaux, C. Sureau, S. Schuster, D.E. Root, E.R. Verrier**, T.F. Baumert**.

* ont contribué à part égale à ce travail comme premiers auteurs

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Travaux publiés dans

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# 1. Objectifs

Lors du développement des modèles d'études *in cellulo* du HBV et du HDV en surexprimant le transporteur NTCP à la surface de lignées hépatocytaires humaines, nous avons observé que les Huh7-NTCP (Huh-106) étaient très sensibles à l'infection par le HDV mais peu à l'infection par le HBV. Or, le HDV porte les antigènes de surface HBsAg du HBV. Tirant parti de cette observation, nous avons émis l'hypothèse que des facteurs cellulaires clés pour le cycle viral, absents dans la lignée Huh7, étaient responsables de cette différence. De plus, la caractérisation des différences transcriptomiques entre les deux lignées HepG2 et Huh7 permettrait l'identification de ces facteurs manquants et la caractérisation de nouvelles cibles thérapeutiques.

Dans ce contexte, j'ai réalisé un criblage à haut débit en gain de fonction à l'échelle du génome sur la lignée Huh7-106 infectée par le HBV, en utilisant une banque lentivirale permettant l'expression de plus de 16000 gènes. Ce criblage a permis d'identifier 47 facteurs favorisant l'infection virale. Parmi ces candidats, nous avons identifié des facteurs de transcription HNF4A ou HLF déjà décrits comme importants pour la réplication du HBV (Raney *et al.*, 1995 ; Ishida *et al.*, 2000). Ces candidats ont permis de confirmer la pertinence de notre approche pour l'identification de facteur de l'hôte importants pour l'infection virale.

Mes résultats de validation dans différents modèles cellulaires dont les PHH, combinés à la comparaison transcriptomique des deux lignées cellulaires, ont mis en évidence le facteur de l'hôte CDKN2C comme étant fortement surexprimé dans les HepG2 en comparaison aux cellules Huh7 et jouant un rôle clé dans la réplication virale de HBV. CDKN2C induit un arrêt du cycle cellulaire en phase G1 en inhibant les CDK4/6 (pour cyclin dependent kinase 4/6)(Guan *et al.*, 1994). Le Dr Carla Eller, étudiante en thèse et co-auteur de cette étude, a démontré que l'inhibition de CDK4/6 à l'aide de molécules thérapeutiques (dont le Palbociclib) induisait également la réplication virale. De plus, nous avons montré que CDKN2C stimule la transcription des ARN viraux à partir de l'ADNccc. Par ailleurs, j'ai montré que l'arrêt du cycle en phase G1 induite par la surexpression de CDKN2C est corrélé à l'induction de l'expression des facteurs favorisant la transcription du HBV, dont HNF4A et HLF.

Afin de déterminer l'importance clinique de CDKN2C, notre équipe a analysé l'expression de CDKN2C dans des hépatocytes primaires humains infectés, et en parallèle, à partir de bases de données, dans des tissus de foie de patients infectés par le HBV. De manière intéressante, l'expression de CDKN2C est induite par l'infection virale et il existe une corrélation entre la progression des maladies hépatiques et l'expression de la protéine chez les patients chroniquement infectés. Ces résultats suggèrent un rôle fonctionnel de CDKN2C dans le développement des maladies du foie induites par le HBV.

Ces données publiées dans le journal Nature Communications en 2020 et présentées ci-après démontrent la robustesse de nos modèles d'infection par le HBV et la pertinence de leur utilisation pour des stratégies de criblage à haut débit, dans le but d'identifier de nouveaux facteurs d'hôte du HBV et de découvrir de nouvelles cibles antivirales.

## 2. Publication

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

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

**ABSTRACT**

Chronic HBV infection is a major cause of liver disease and cancer worldwide. 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 to uncover host factors enhancing HBV infection. Validation studies in primary human hepatocytes identified CDKN2C as an important host factor for HBV replication. CDKN2C is overexpressed in highly permissive cells and HBV-infected patients. Mechanistic studies show a role for CDKN2C in inducing cell cycle G1 arrest through inhibition of CDK4/6 associated with the upregulation of HBV transcription enhancers. A correlation between CDKN2C expression and disease progression in HBV-infected patients suggests a role in HBV-induced liver disease. Taken together, we identify 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.

**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 (NUC) 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 levels are 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 NUC, are direct-acting antiviral (DAA) and allow the suppression of viral replication, but viral cure is rarely achieved. Innovative therapeutic strategies, such as host targeting agents (HTA), 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 perform 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 HBV host factors**. 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 **(Figure 3.1. a)**. However, in contrast to HepG2-NTCP cells, Huh-106 cells appear poorly permissive to HBV infection **(Figure 3.1. a)**, despite their ability to bind HBV particles **(figure 3.1. b)**. Furthermore, Huh-106 cells support the conversion of incoming HBV rcDNA to cccDNA, although to a much lesser extent than HepG2-NTCP cells **(Figure 3.1. c-d)**. Interestingly, the kinetics of cccDNA formation are similar in both cell lines **(Figure 3.1. e)**. 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 **(Figure 3.1. f-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.

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 **(Figure 3.2. a)**. 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, **Figure 3.2. a-b**) to the control population (HBV pre-sort, **Figure 3.2 a-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 **(Figure 3.2. c-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 **(Supplementary Table 3.1)**. 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

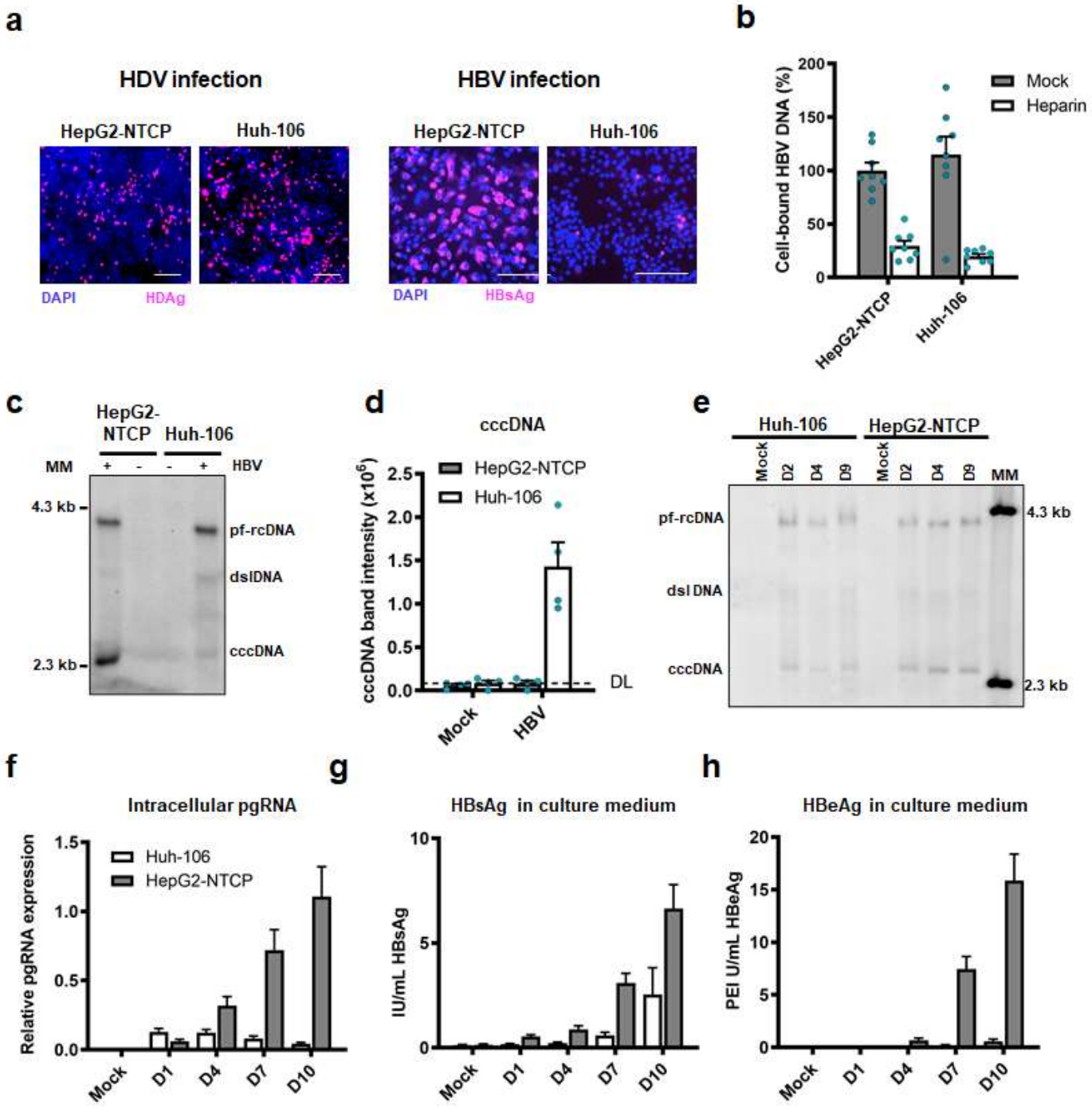

**Figure 3.1. Huh-106 are less permissive to HBV infection than HepG2-NTCP. a** HBV and HDV infection of HepG2-NTCP and Huh-106 cells and detection of HBsAg and HDAg by IF after 10 dpi. One representative experiment is shown. Scale bars: 100 µm. **b** Binding of HBV particles to HepG2-NTCP and Huh-106 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 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). MM: molecular marker. Source data are provided as a Source Data file.

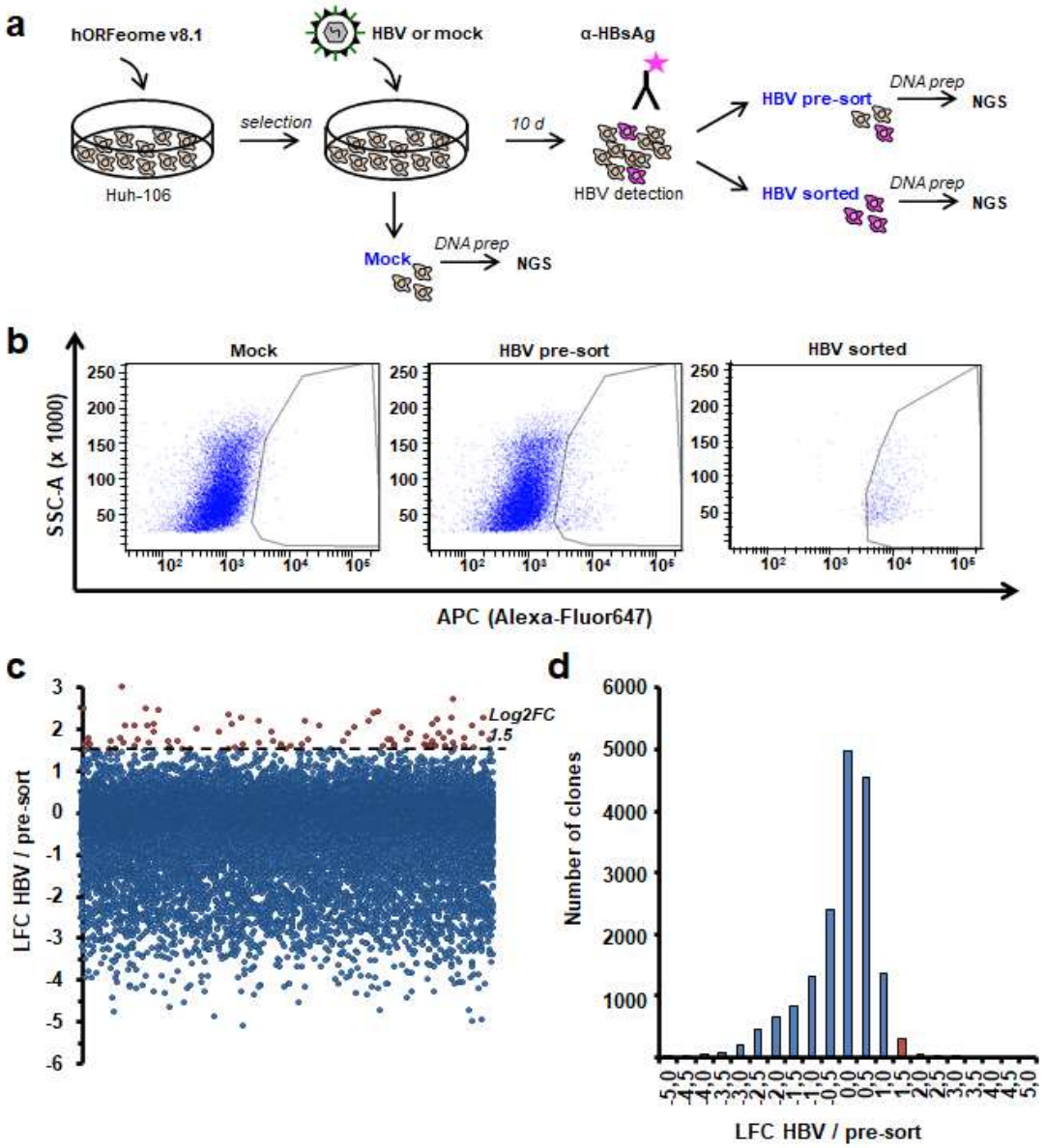

**Figure 3.2. Gain-of-function (GOF) screen in Huh-106 cells for the identification of HBV host factors. a** Schematic workflow of GOF-screen. **b** FACS for HBsAg-positive cells in Huh-106 transduced with an ORF-library (hORFeome v8.1) 10 days after HBV infection (HBV pre-sort). Flow-cytometric analysis 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. Source data are provided as a Source Data file.

**CDKN2C is a HBV host factor highly expressed in HepG2 cells.** 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 **(Supplementary Table 3.1)**. 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 **(Figure 3.3. a, Supplementary Figure 3.1)**. 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 **(Figure 3.3. b)**. Notably, IFN$\alpha$ 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).

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 **(Figure 3.3. c)**. 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 **(Figure 3.3. d, 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 **(Figure 3.4. a)**.

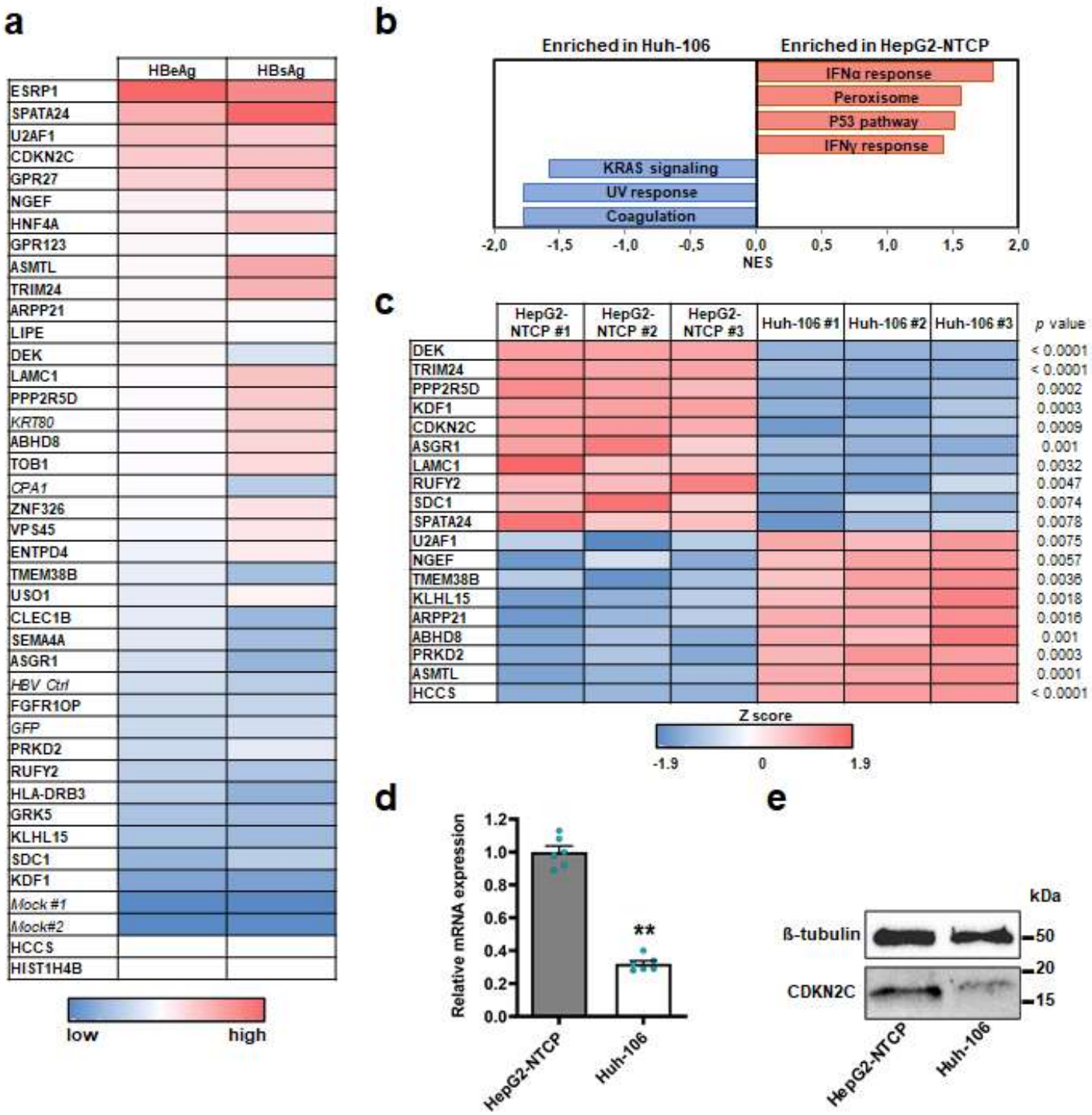


**Figure 3.3. CDKN2C is differentially expressed in HepG2-NTCP and Huh-106 cells. a** Heatmap of candidate validation. Huh-106 cells were transduced with the indicated ORF and infected with HBV. HBV infection was assessed at 10 dpi by CLIA quantification of secreted HBeAg and HBsAg. Results are expressed as means concentration of secreted HBeAg or HBsAg from 1 experiment (n=2). Genes in italic (*KRT80* and *CPA1*) correspond to negative controls which were not identified as candidates from the primary screen. Mock#1 and Mock#2: uninfected HepG2-NTCP cells. HBV ctrl: non-transduced HBV-infected HepG2-cells. GFP: GFP-transduced HBV-infected HepG2-NTCP cells. **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 is shown. *** $p < 0.01$ (two-tailed Mann-Whitney U test). Source data are provided as a Source Data file.

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 3.4 b-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 **(Figure 3.4. d)**. 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 **(Figure 3.4. e)**. 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 **(Figure 3.4. f)**. Consistent with our previous observations, the overexpression of HNF4A and CDKN2C in PHH resulted in a significant and marked increase in HBV infection **(Figure 3.4. g)**. Moreover, the silencing of CDKN2C expression using target-specific shRNA induced a significant and robust decrease in HBV infection **(Figure 3.4. h)**. 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 **(Supplementary Figure 3.2.)**. Detection of intracellular HBsAg by immunofluorescence (IF) **(Figure 3.5. a)** and its quantification by flow cytometric analysis **(Figure 3.5. b)** 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 **(Figure 3.5. b)**, suggesting that the two factors affect HBV infection through independent pathways. Interestingly, overexpression of both factors in Huh-106 cells markedly increased HBV infection but failed to reach levels observed in HepG2-NTCP **cells (Figure 3.5. b-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 3.5. d-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 **(Figure 3.5. f-g)**.

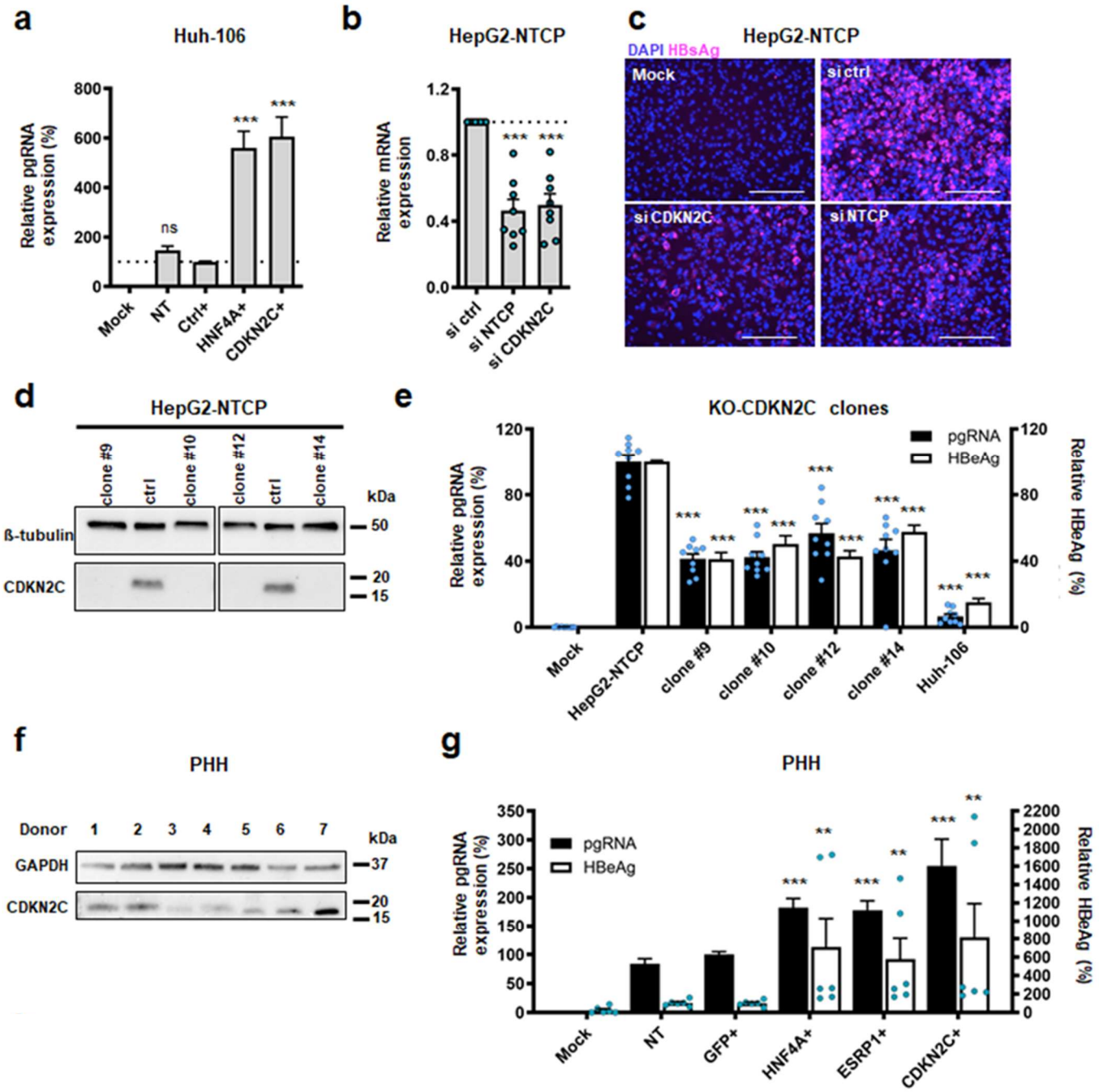


**Figure 3.4. CDKN2C is a HBV host factor. a** Individual ORF-overexpression in Huh-106 and HBV infection 3 days after transduction. Detection of HBV pgRNA by qRT-PCR 10 dpi. Results are expressed as means +/- SEM relative pgRNA expression (%) compared to ctrl (set as 100%) from 8 independent experiments (n=21). **b-c** siRNAs Transfection of HepG2-NTCP cells. **b** mRNA . Results are expressed as means +/- SEM relative expression compared to si ctrl (set to 1) from 4 independent experiments (n=8). **c** HBV infection after silencing was detected by IF 10 dpi. Scale bars: 100 µm. **d** Production of *CDKN2C*-knock-out 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 pgRNA qRT-PCR (black) and quantification of secreted HBeAg (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** Detection of endogenous CDKN2C expression in PHH from 7 donors. 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 (pgRNA, black; HBeAg, white) were detected 10 dpi. Results are expressed as means +/- SEM % HBV infection compared to ctrl (GFP) (set to 100%) from 3 independent experiments (n=12 for pgRNA; n=6 for HBeAg).

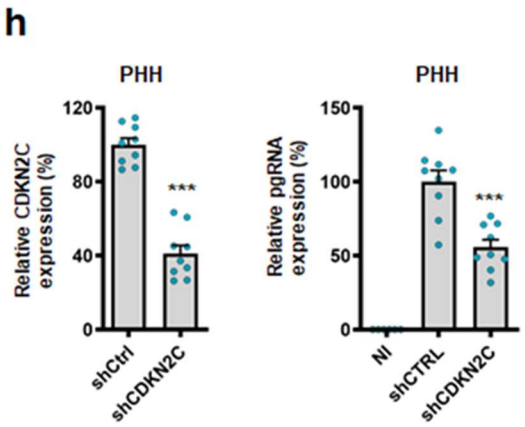


**Figure 3.4. (bis) h** PHH from 3 donors were transduced with lentiviruses containing CDKN2C-targeting shRNA or non-targeting shRNA control (sh ctrl). Silencing efficacy was assessed by qRT-PCR. Results are expressed as means +/- SEM % gene expression compared to sh ctrl (set to 100%) from 3 independent experiments (n=9). PHH were then infected with HBV and HBV infection was assessed by pgRNA qRT-PCR 8 dpi. Results are expressed as means +/- SEM relative pgRNA expression compared to sh ctrl (set to 100%) from 3 independent experiments (n=9). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ (two-tailed Mann-Whitney U test). Source data are provided as a Source Data file.

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 **(Figure 3.5. h)**. This suggests a role for CDKN2C in cccDNA-mediated transcription of HBV RNAs. To investigate whether the role of CDKN2C in transcription of HBV RNAs is linked to previously described HBV host factors, we quantified the expression of HNF4A, HLF and PPARA, known to enhance HBV transcription (19,20). Interestingly, CDKN2C overexpression in Huh-106 resulted in up-regulation of the expression of three HBV transcription factors **(Figure 3.5. i)**. Taken together, our results suggest that CDKN2C expression enhances transcription of HBV RNAs through the upregulation of HBV-related transcription factors.

**Enhanced supernatant infectivity of transduced HepAD38 cells**. Since a recent study suggested that HBV virion production was more efficient in quiescent cells (24), we then investigated whether modification of CDKN2C expression modulates the production of virus particles in HBV-expressing cells, and 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 **(Figure 3.6. a)**. While we observed a modest increase in the secretion of HBsAg and HBeAg is observed, CDKN2C overexpression in HepAD38 donor cells did not affect the levels of HBV DNA in the cell culture supernatant **(Figure 3.6. b-c)**. Interestingly, overexpression of CDKN2C in HepAD38 increased infection of HepG2-NTCP acceptor cells by about 3-fold suggesting that the supernatant of CDKN2C-transduced HepAD38 cells has a higher infectivity **(Figure 3.6. d-e)**.

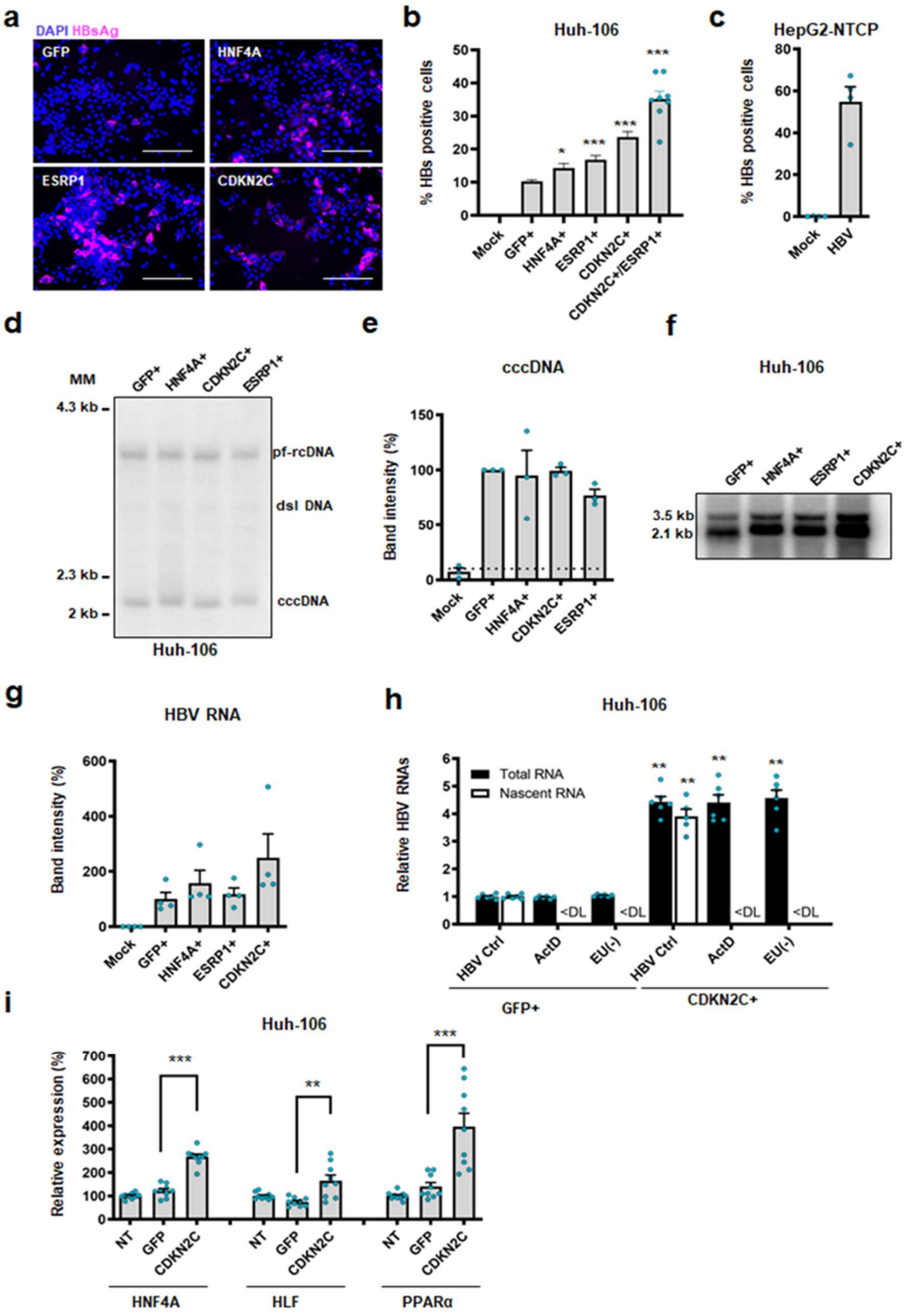


**Figure 3.5. CDKN2C stimulates HBV cccDNA-mediated transcription. a-b, d-g** Validation studies in Huh-106 overexpressing individual ORFs and infected with HBV for 10 days. **a** Detection of HBsAg by IF. Scale bars: 100 µm. **b** Flow-cytometric analysis for quantification of HBsAg-positive cells. 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 is shown.

**e** Quantification of cccDNA. 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 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). **i** *HNF4a, HLF and PPARα* mRNA expression in CDKN2C-overexpressing Huh-106 quantified by qRT-PCR. Results are expressed as means +/- SEM %

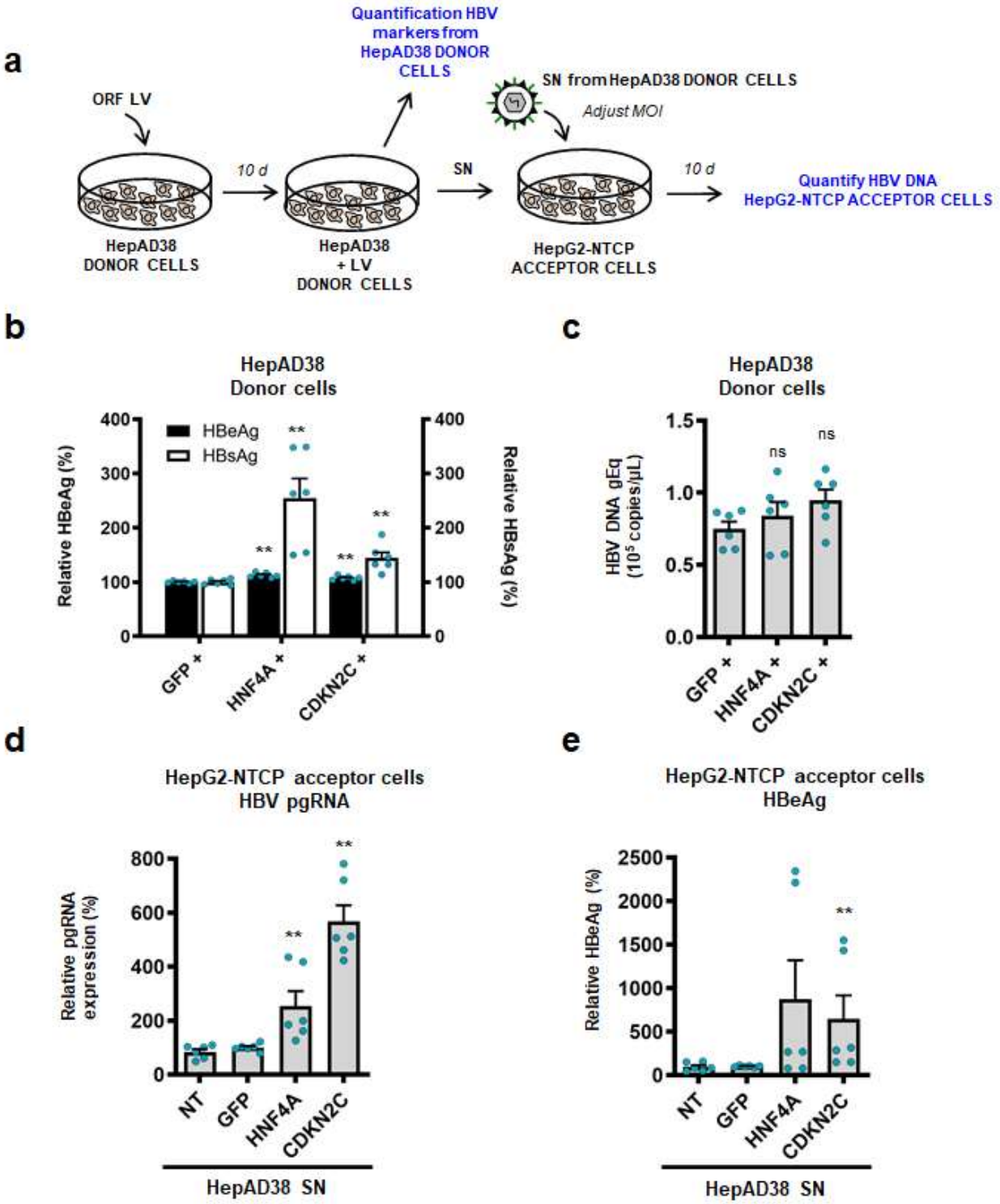

**Figure 3.6. *CDKN2C* overexpression results in enhanced infectivity of supernatants of transduced HepAD38 cells. a** Schematic workflow of experiments. HepAD38 cells in production medium (Donor cells) were non-transduced (NT) or transduced with ORF lentivirus for 10 days. **b-c** Supernatant (SN) from 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

**CDK4/6 inhibitors enhance HBV infection.** 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 **(Supplementary Figure S3.3.)**. To determine if this known function of CDKN2C is responsible for enhancing HBV infection, we performed functional studies using two clinically studied CDK4/6-specific small molecule inhibitors, Palbociclib (25) and LEE011 (26). Drug treatment of Huh-7 and Huh-106 cells induced a dose-dependent G1 cell cycle arrest associated with a decrease in cell proliferation **(Supplementary Figure S3.4. a-d)**, most likely due to drug-induced cytostatic effect associated with the accumulation of cells in G1 phase. At the reference concentration of 100 nM Palbociclib and LEE011 did not induce major cytotoxic effects as shown by the LDH-Glo cytotoxicity assay **(Supplementary Figure S3.4. b)**. We then determined HBV infection levels in Huh-106 cells treated with either of the inhibitors before and after HBV infection **(Figure 3.7 a, e)**. Visualization of intracellular HBsAg revealed a marked increase in HBV infection levels after treatment with Palbociclib or LEE011 **(Figure 3.7. b)**. Furthermore, quantification of HBV pgRNA and HBsAg-positive cells revealed a significant increase in HBV infection upon both Palbociclib and LEE011 treatment **(Figure 3.7. c)**. Similar results were obtained in PHH treated with CDK4/6 inhibitors at different concentrations (1, 10, 100 and 1,000 nM) confirming the proviral effect of Palbociclib and LEE011 **(Figure 3.7. d)**.
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 **(Figure 3.7. e)**. As shown in **Figure 3.7. f** and **Supplementary Figure S3.5**, 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 **(Figure 3.7. g)**.

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 **(Figure 3.8)**.

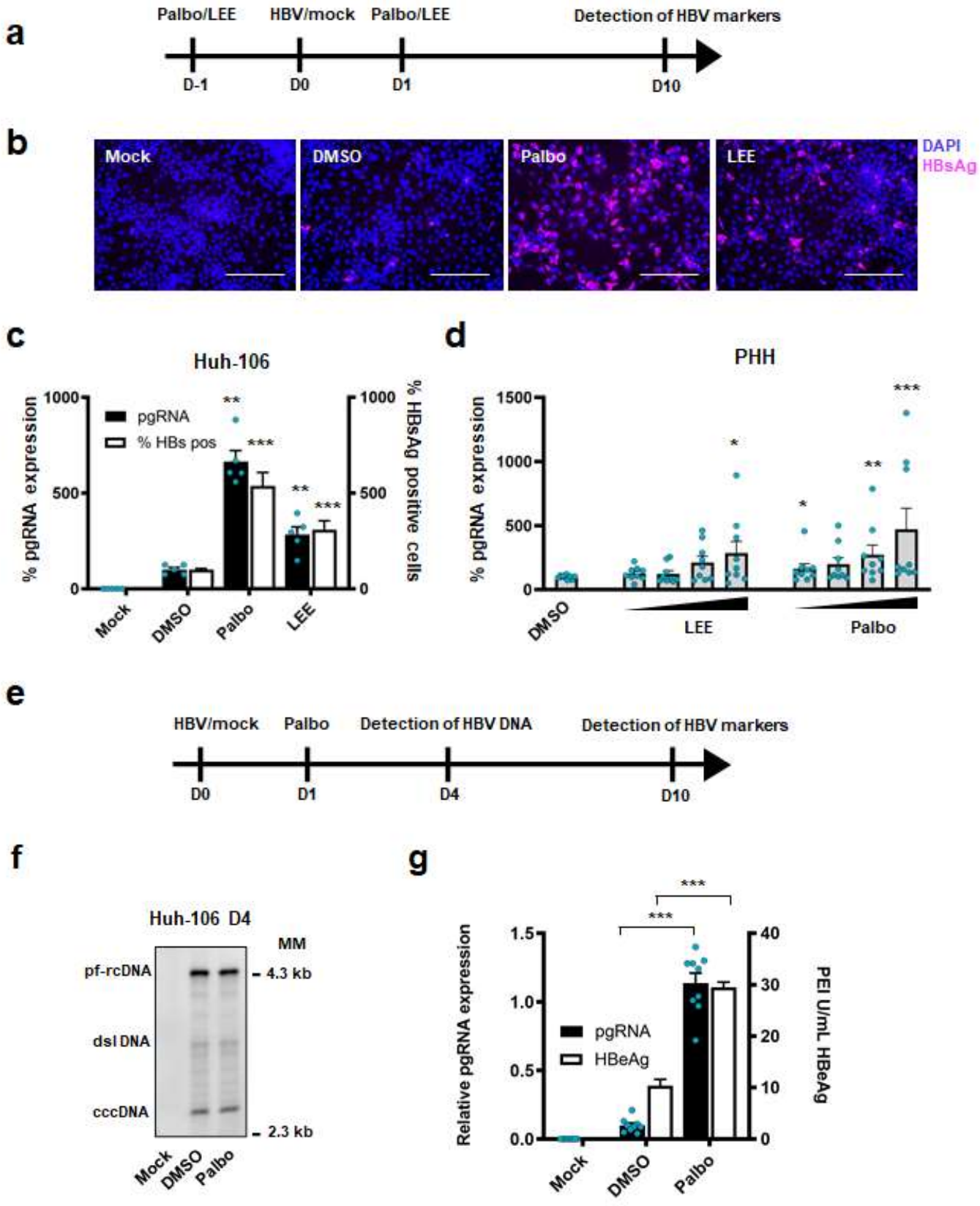


**Figure 3.7. CDKN2C-mediated stimulation of HBV transcription is correlated with a cell cycle arrest.** Effect of treatment with CDK4/6 inhibitors Palbociclib (Palbo) and LEE011 (LEE) on HBV infection. **a** Schematic workflow of experiments b-d. Detection of HBV markers in mock/HBV infected Huh-106 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 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. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ (two-tailed Mann-Whitney U test). MM: molecular marker. Source data are provided as a Source Data file.

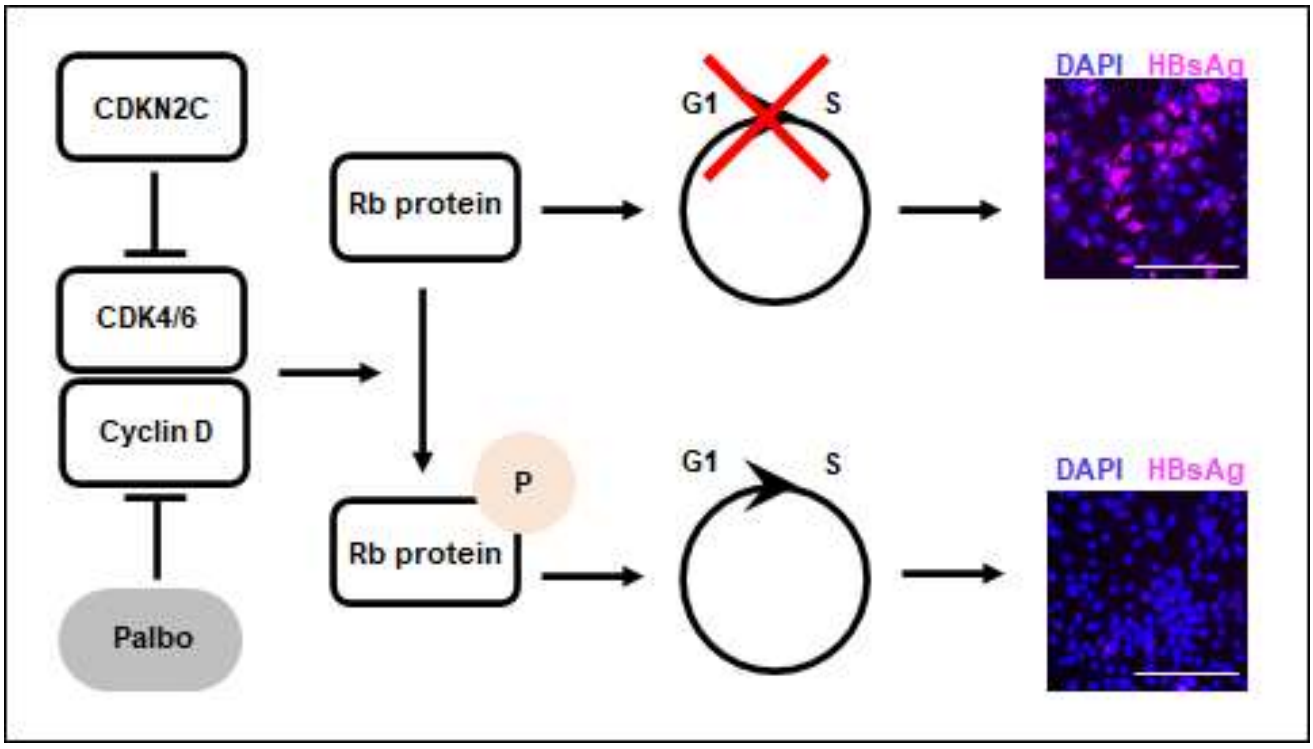


**Figure 3.8. Schematic model of the effect of CDKN2C expression and Palbociclib (Palbo) treatment on HBV infection.** CDKN2C and Palbociclib inhibit the CDK4/6 and Cyclin D mediated phosphorylation of Rb protein, leading to an accumulation of Rb protein in its unphosphorylated state. Unphosphorylated Rb protein induces a cell cycle G1 arrest resulting in increased HBV infection rates. Illustrative HBV infection pictures come from Fig. 6. Scale bars: 100 µm.

***CDKN2C* expression is associated with chronic liver disease.** 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 **(Figure 3.9. a)**. 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 **(Figure 3.9.**

**b)**. Moreover, a correlation was observed between HBV viral load and *CDKN2C* expression in liver tissues from 9 HBV-infected patients **(Supplementary Figure S3.6. a)**. Finally, *CDKN2C* expression appeared to be modulated in different stages of HBV infection **(Figure 3.9. c)**. 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 **(Supplementary Figure S3.6. b)**. Moreover, *CDKN2C* expression was significantly higher in tumor tissues from HBV-derived HCC compared to adjacent tissue **(Figure 3.9. d)**. 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 **(Figure 3.9. e)**, 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 **(Figure 3.9. f)**. 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. 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.

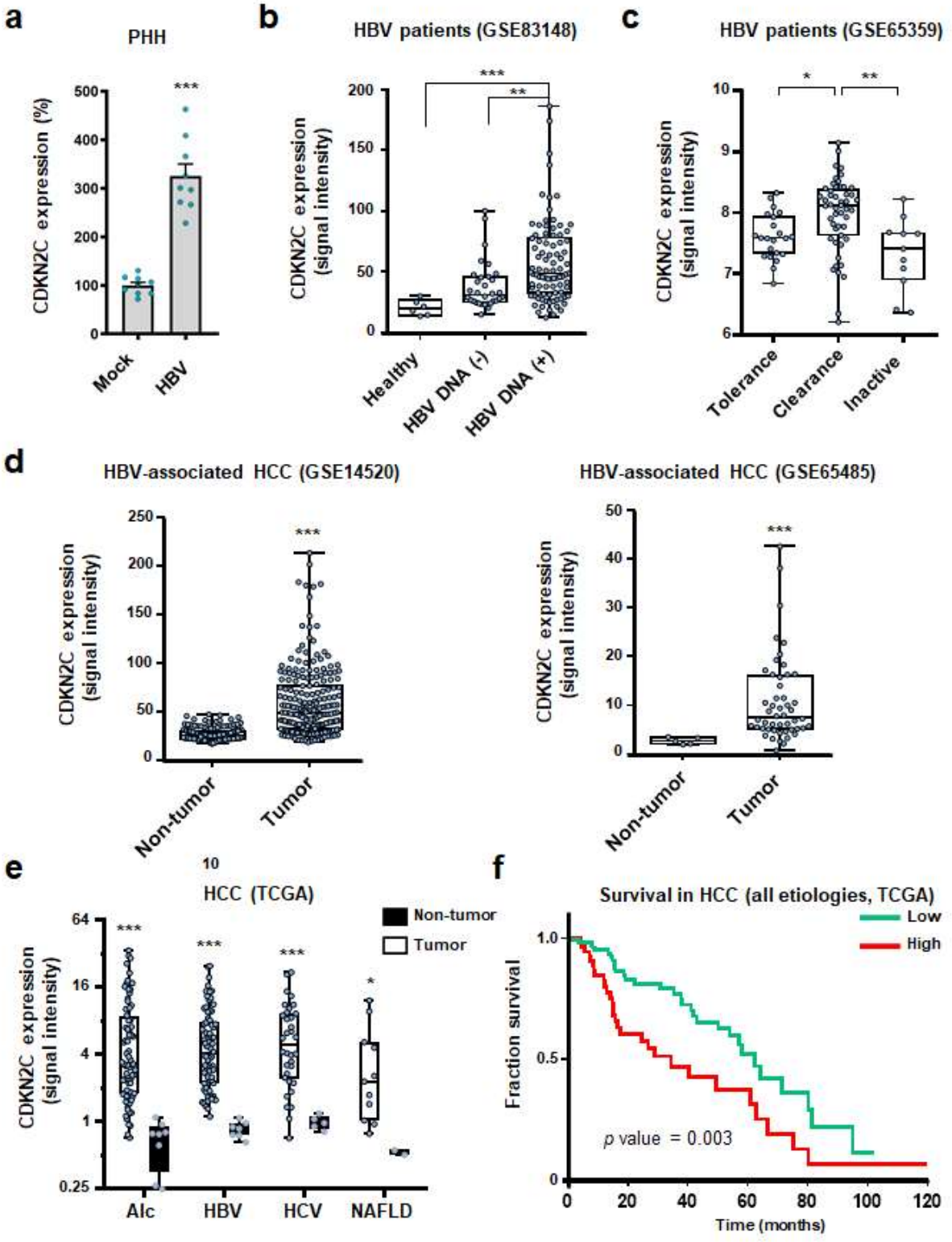

**Figure 3.9. CDKN2C expression is associated with HBV infection, liver disease and survival in patients. a** *CDKN2C* mRNA expression in HBV infected PHH from 3 different donors quantified by qRT-PCR. Results are expressed as means +/- SEM % relative CDKN2C expression compared to Mock (set to 100%) from 3 independent experiments (n=9). **b** *CDKN2C* expression in HBV-infected patients with undetectable (HBV DNA(-), n=32)or detectable (HBV DNA(+), n=90) HBV DNA compared to healthy patients (n=6)(cohorts described in Methods). **c** *CDKN2C* expression in HBV-infected patients depending on the stage of virus infection (cohorts described in Methods). Tolerance: n=22; Clearance: n=50; Inactive: n=11. **d** *CDKN2C* expression in tumor and adjacent tissues in HCC patients from two independent cohorts (see Methods) Non-tumor: n=198; Tumor: n=98 (left panel). Non-tumor: n=5; Tumor: n=50 (right panel)**. e** *CDKN2C* expression in tumor and non-tumor (normal) liver tissue from patients with alcoholic liver disease (Alc, Tumor: n=70; Non-tumor: n=8), HBV-infected patients (Tumor: n=76; Non-tumor: n=7), HCV-infected patients (Tumor: n=34; Non-tumor: n=5) and patients with non-alcoholic fatty liver disease (NAFLD, Tumor: n=11; Non-tumor: n=2) extracted from TCGA database as described in Methods. **f** Survival analysis for HCC patients with low or high CDKN2C expression (cohort see Methods). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ (**b-c**: Kruskal–Wallis H test adjusted for multiple comparisons; **d-e**: two-tailed Mann-Whitney U test). The details of the plots are presented in Supplementary Table 2 and Supplementary Table 3. Source data are provided as a Source Data file.

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. Indeed, CDKN2C overexpression is associated with an upregulation of transcription factors important for the HBV life cycle, such as HNF4A, HLF and PPARA **(Figure 3.5. i)**. Furthermore, we observed that overexpression of CDKN2C in HBV producer cells resulted in a supernatant containing HBV particles that appeared to be consistently more efficient in infecting naive recipient cells **(Figure 3.6)**. It is conceivable that CDKN2C overexpression and the subsequent modulation of expression of HBV host-dependency factors **(Figure 3.5. i)** results in differences in virion assembly which could explain this observation. Further experiments are needed to understand the functional role of CDKN2C for the formation of infectious particles. 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 **(Figure 3.1. a)**. 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 **(Figure 3.3. d, 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 **(Figure 3.5. d, 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 **(Figure 3.9)** 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 **(Figure 3.9)**. 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 (Figure 3.9) 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 **(Figure 3.9)**. 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). However, while HBV viral load and CDK2NC expression showed a positive correlation trend in a well-defined small cohort shown in **Supplementary Figure S3.6**, additional correlation analyses in other cohorts are needed to corroborate this finding. Collectively, it is likely that CDKNC2 expression is regulated by multiple and possibly different mechanisms in the different phases of HBV infection and disease and even more so in the context of HCCs.

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/proliferation was assessed using PrestoBlue Cell Viability Reagent (Invitrogen) according to the manufacturer's instructions. Cell toxicity was assessed using LDH-Glo Cytotoxicity Assay (Promega) in the supernatant 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 °C. 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, 1:100) and Alexa Fluor 647-labelled secondary antibody targeting mouse IgGs (Jackson Research, 1:200). 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 32P-labeled RNA probe specific for detection of HBV RNA of 1200 to 1944 bp of viral genome (3.5 kbp to 2.1 kbp). Quantification of HBV DNA- and RNA bands from blots was performed using Image Lab Version 5.2.1 (Bio-Rad). 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, 1:200) purified from serum of an HBV/HDV co-infected patient (64) and AF647-labeled secondary antibody targeting human IgGs (Jackson Research, 1:200) 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 $^{o}$C. 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, 1:1000) for 30 minutes at 4 $^{o}$C and then with an AF647-labelled secondary antibody targeting mouse IgGs (Jackson Research, 1:1000) for 30 minutes at 4 $^{o}$ C. For flow cytometry analysis of DNA content, cells were fixed in ice-cold 75% ethanol in water for 30 minutes at 4$^{o}$C. Cells were washed and resuspend and incubated in PBS 50 µg/mL propidium iodide (Invitrogen) and 50 µg/mL Ribonuclease A (Sigma-Aldrich, Merck) 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). The gating strategy is presented in **Supplemental Fig. 7**.

**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 (Supplementary Table 1, 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 **(Supplementary Table 1)**. 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

cotransfection 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; Fw: 5'-CACCGACACCGCCTGTGATTTGGCC-3', Re: 5'-AAACGGCCAAATCACAGGCGGTGTC-3'. guide 2; Fw: 5'-CACCGCACAGGCGGTGTCCCCCTTA-3', Re: 5'-AAACTAAGGGGGACACCGCCTGTGC-3'. 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 quantification of GAPDH, CDKN2C, SLC10A1 mRNA expression were obtained from ThermoFisher (TaqMan® Gene Expression Assays). Gene expression was quantified using iTaq Universal Probes Supermix (Bio-Rad). Primers for quantification of HNF4A (Fw: 5'-ACATTCGGCAAGAAGATT-3'; Re: ACTTGGCCCACTCAACGAG-3'), HLF (Fw: 5'CACCACGAAGACGATTTAG-3'; Re: 5'-CAAAAACTCCTCCAGGTCCA-3'), PPARA (Fw: 5'-GAGGGTCTCCACTGACGTG-3'; Re: 5'-ACACTGTGTATGGCTGAGAAG-3'), and GAPDH expression (Fw: 5'-GTCTCCTCTGACTTCAACAGCG-3'; Re: 5'-ACCACCCTGTTGCTGTAGCCAA-3') were obtained from Sigma-Aldrich (Merck). Gene expression was quantified using iTaq Universal SYBR Green Supermix (Bio-Rad).

**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, 1:1000), a rabbit polyclonal anti-β-tubulin antibody (GTX101279, Gentex, 1:3000) and a rabbit polyclonal anti-GAPDH (ab9485, Abcam, 1:2500), respectively. Peroxidase-AffiniPure Goat Anti-Rabbit IgG (H+L) (Jackson Research 111-035-144, 1:10000) 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).

**Statistics and Reproducibility**. Individual experiments were reproduced three times in an independent manner with similar results except otherwise stated. The precise number (n) of biologically independent samples used to derive statistics is indicated in the figure legends. 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 exact p-values are provided in the Source Data file. For $n < 10$, the corresponding data points are presented with the bar charts. 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 adjusted for multiple comparisons 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. Representative graphs and pictures presented in Fig. 1a, 1c, 1e, 4c, 5a, 5d, 5f, 7f are representative of three independent experiments with similar results. Representative graph presented in Fig. 3e is representative of two independent experiments with similar results. Graphs were designed using GraphPad PRISM 6 for Windows and Microsoft Excel for Microsoft Office 365 ProPlus (version 1911).

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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, CCC, and CB performed the validation experiments. CE, LH, CCC, KM, CB, 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.

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**Data availability**. The dataset generated in this study, including the results from the gain-of-function primary screen, are available within Supplementary information. Full immunoblots are provided in Supplementary Fig. 8. The microarray dataset is publicly available in the NCBI Gene Expression Omnibus database (accession number GSE132638 [https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE132638]). The source data underlying Figs 1, 2, 3, 4, 5, 6, 7, and 9 and Supplementary Figs 1, 4, 5 and 6 are provided as a Source Data file. The details of the box plots presented in Fig.9b-e and Supplementary Fig. 6b are presented in Supplementary Table S2 and Supplementary Table S3.

The rest of the data is available through the corresponding authors upon reasonable request. The following public databases were used in the study:

GSE83148 [https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE83148];
GSE65359 [https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE65359];
GSE84044 [https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE84044];
GSE65485 [https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE65485];
GSE14520 [https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE14520];
TCGA-LIHC [https://www.cancer.gov/about-nci/organization/ccg/research/structural-genomics/tcga].
The following public protocol was used: PoolQ [https://portals.broadinstitute.org/gpp/public/resources/protocols].

## SUPPLEMENTARY FIGURES

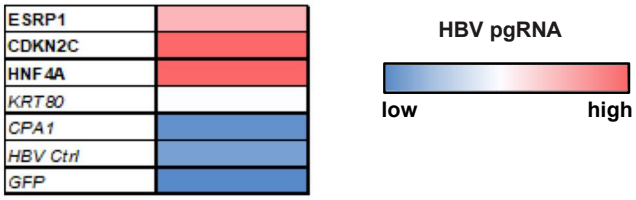



**Figure 3.S1. Heatmap of candidate validation by quantification of pgRNA** (related to **Figure 3a)**. Huh-106 cells were transduced with the indicated ORF and infected with HBV. HBV infection was assessed at 10 dpi by qRT-PCR quantification of pgRNA. Results are expressed as means relative pgRNA expression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

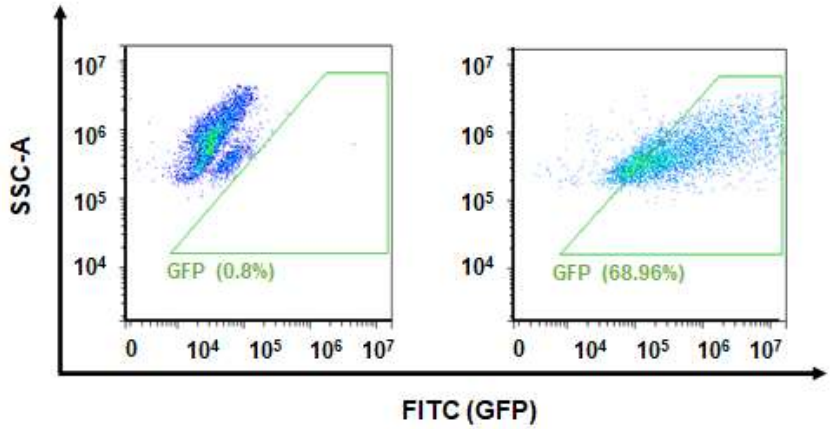



**Figure 3.S2. GFP control vector** (related to **Figure 5b**). Expression of GFP in Huh-106 transduced with lentivirus for GFP overexpression (GFP) or non-transduced (NT). Quantification of GFP-expressing cell population by flow cytometric analysis in HBV-infected cells 10 dpi.

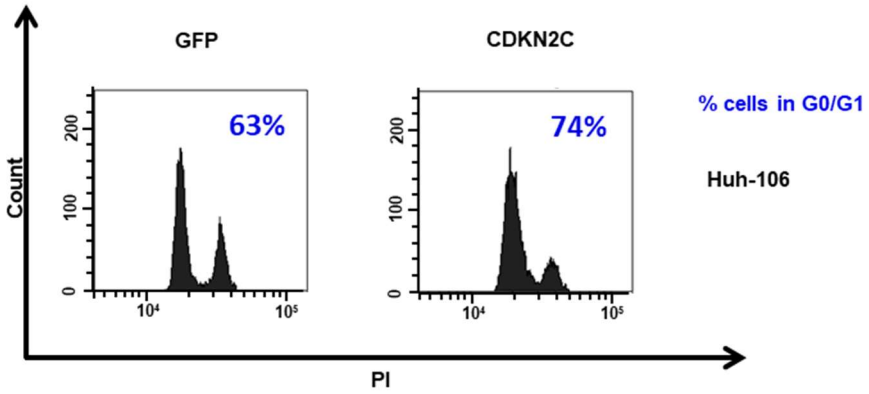



**Figure S3.3. Effect of *CDKN2C* overexpression on cell cycle** (related to **Figure 7**). Overexpression of GFP or CDKN2C in Huh-106 cells and cultivation in Williams Culture and 2% DMSO. Analysis of cell cycle using propidium iodide and flow cytometry after 3 days. One representative experiment out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

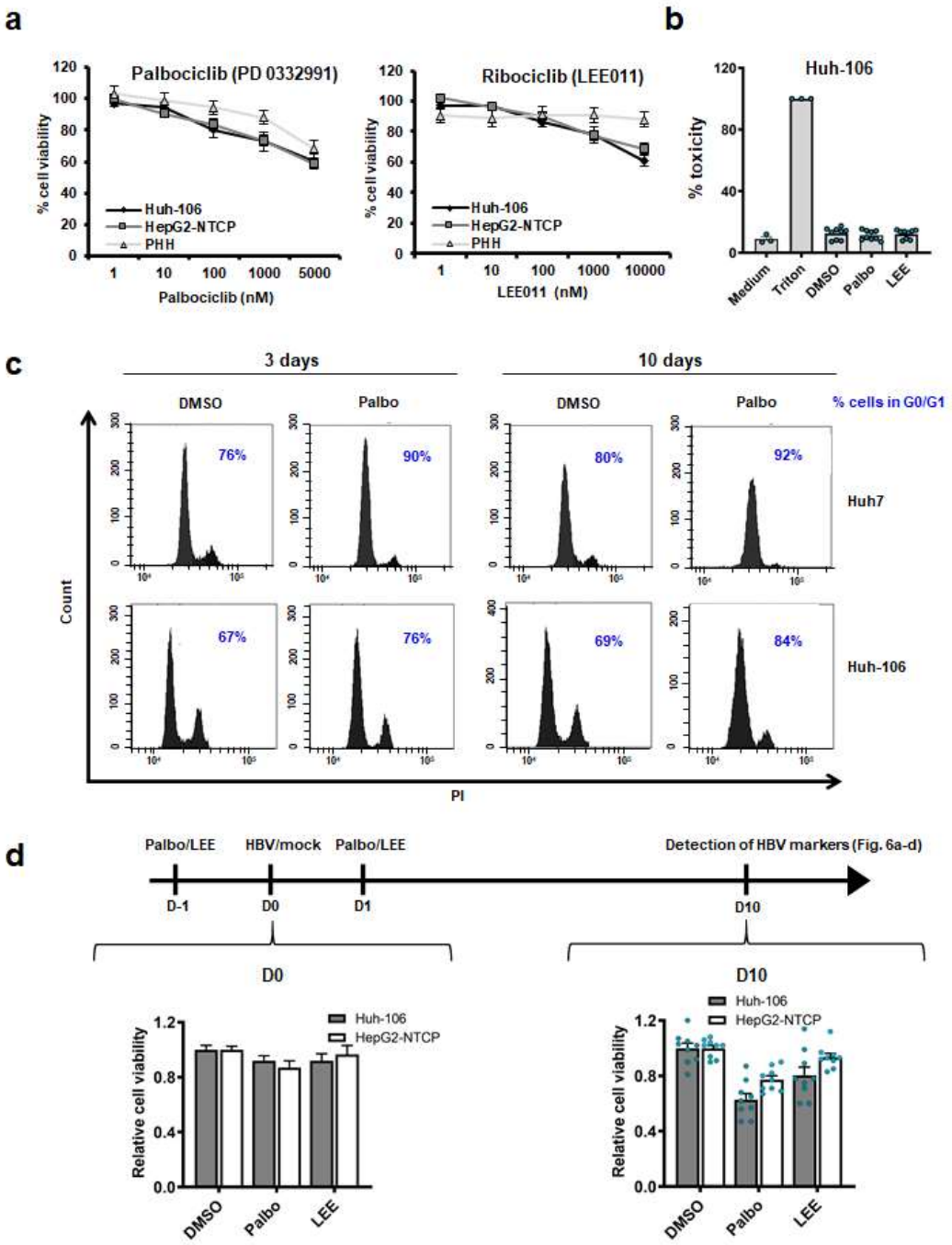



**Figure S3.4. Effect of treatment with CDK4/6 inhibitors Palbociclib (Palbo) and LEE011 on cell viability, cytotoxicity and cell cycle** (related to **Figure 7**). **a** Cell Viability. Cell viability was assessed by the PrestoBlue assay in Huh-106 cells, HepG2-NTCP cells and PHH from 3 different donors 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** Cytotoxicity of compounds in Huh-106 cells. Cytotoxicity was measured using the LDH-Glo cytotoxicity assay (Promega). The assay is based on the quantification of lactate dehydrogenase (LDH) released into the culture medium upon cell death. Cells were treated for three days with the compounds at a concentration of 100 nM or with 0.1% DMSO as a negative control. Results are expressed as means +/- SEM % cytotoxicity compared to 10% Triton (Triton) treated cells (set to 100%) from 3 independent experiments (n=9). Medium: cell culture medium control (basal detection). **c** Effect on cell cycle. Huh7 and Huh-106 cells were treated with DMSO as negative control or with 100 nM Palbociclib (Palbo) in Williams Culture and 2% DMSO. Analysis of cell cycle using propidium iodide and flow cytometry was performed after 3 days and 10 days. One representative experiment is shown (n=4). **d** Cell viability (PrestoBlue) in 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 relative cell viability compared to untreated HBV-infected cells (DMSO

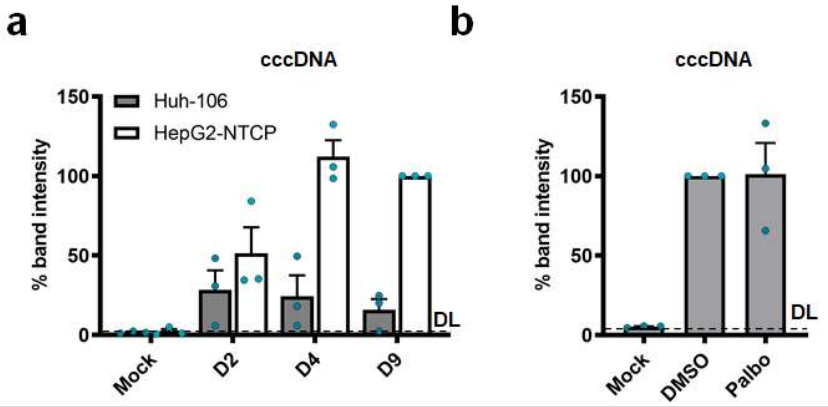



**Figure S3.5. Quantification of Southern Blot cccDNA band using Image Lab Version 5.2.1** (related to (a) **Figure 1e** and (b) **7f). a** Quantification of cccDNA bands in Mock or HBV infected Huh-106 and HepG2-NTCP cells 2 (D2), 4 (D4) or 9 (Mock, D9) days post HBV infection. Results are expressed 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**. Source data are provided as a Source Data file.

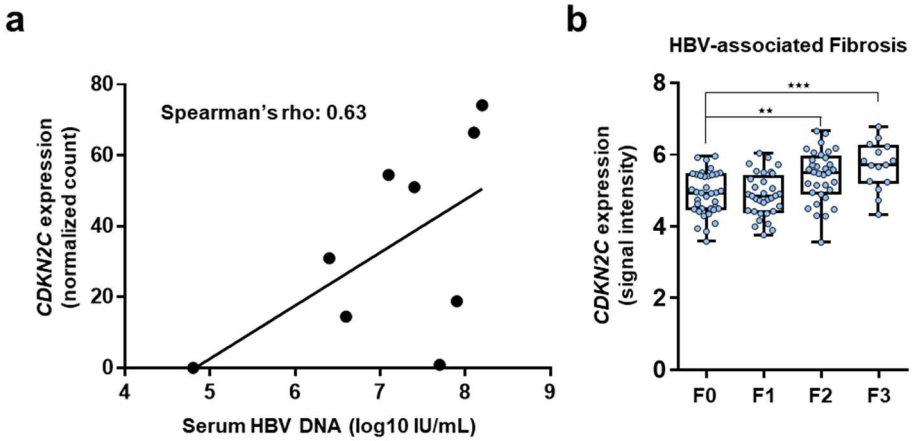



**Figure S3.6. CDKN2C expression is associated with HBV infection and survival in patients** (related to **Figure 9**). **a** Correlation between HBV DNA and *CDKN2C* expression in 9 HBV-infected patients. Serum HBV-DNA levels (log10 IU/mL) and liver tissue CDKN2C expressions showed a trend toward a positive correlation (Spearman's rho = 0.63, p = 0.076). **b** CDKN2C expression in patients with HBV-associated liver fibrosis at different stages of fibrosis (F0: n=37; F1: n=33; F2: n=34; F3: n=15). ★★ p < 0.01; ★★★ p < 0.001 (Kruskal–Wallis H test adjusted for multiple comparisons). Source data are provided as a Source Data file.

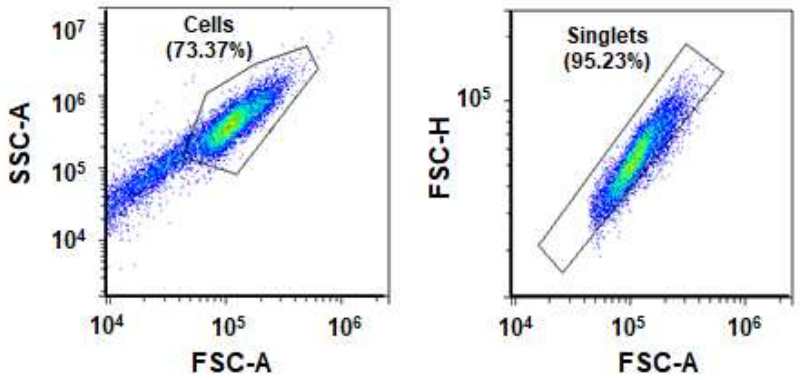
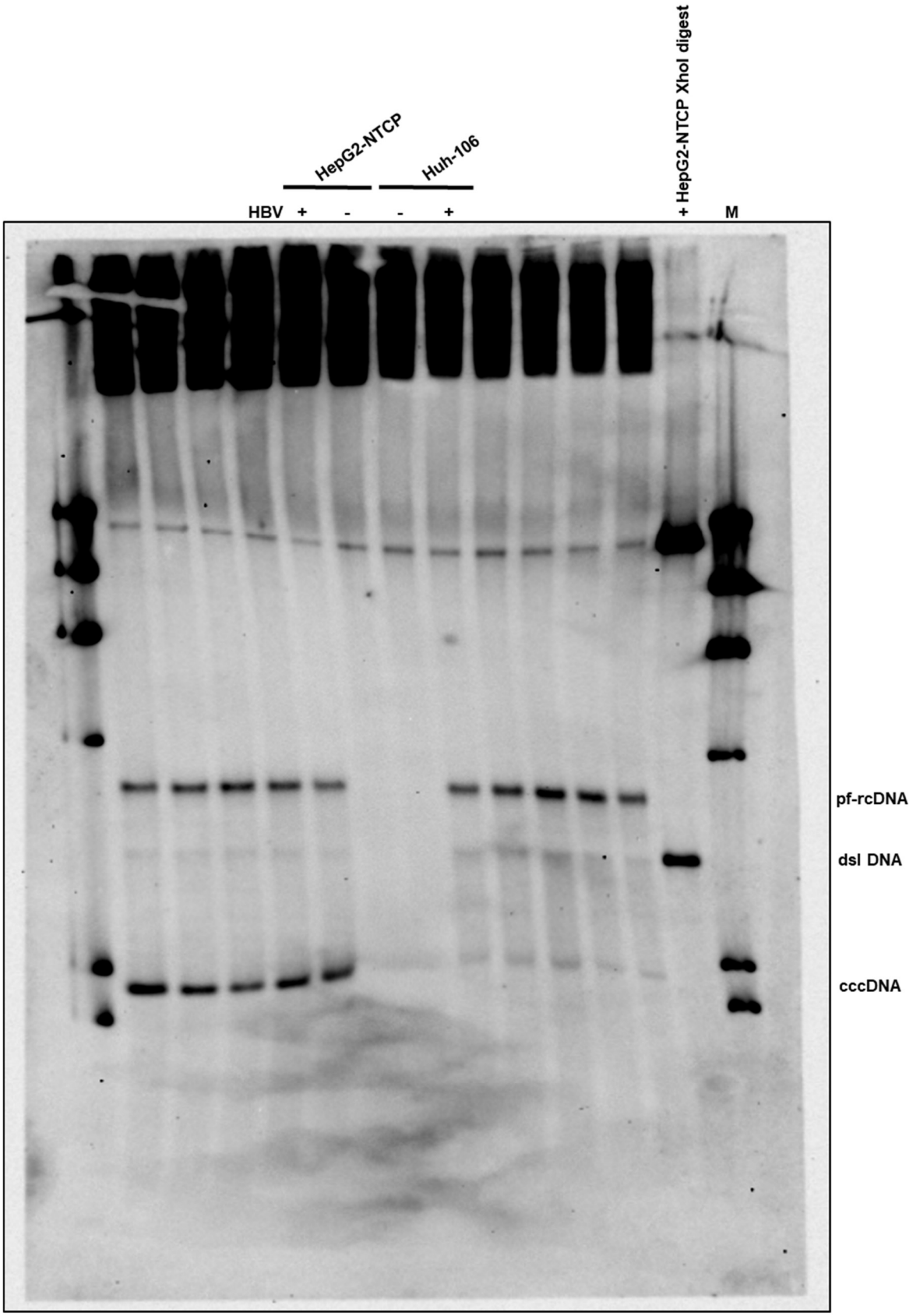



**Figure S3.7** (related to **Figures 2** and **7**)**. Flow cytometry gating strategy using Huh-106 cells.** The gating was performed using an FCS/SSC dot plot. The gain was adjusted when all cell populations were visible on the plot. The living cell population was gated to perform a "singlets" plot through FSC-H and FSC-A parameters. Histogram- and dot plots were obtained from the "singlets" gate using Count/APC-A or SSC-A/APC parameters.

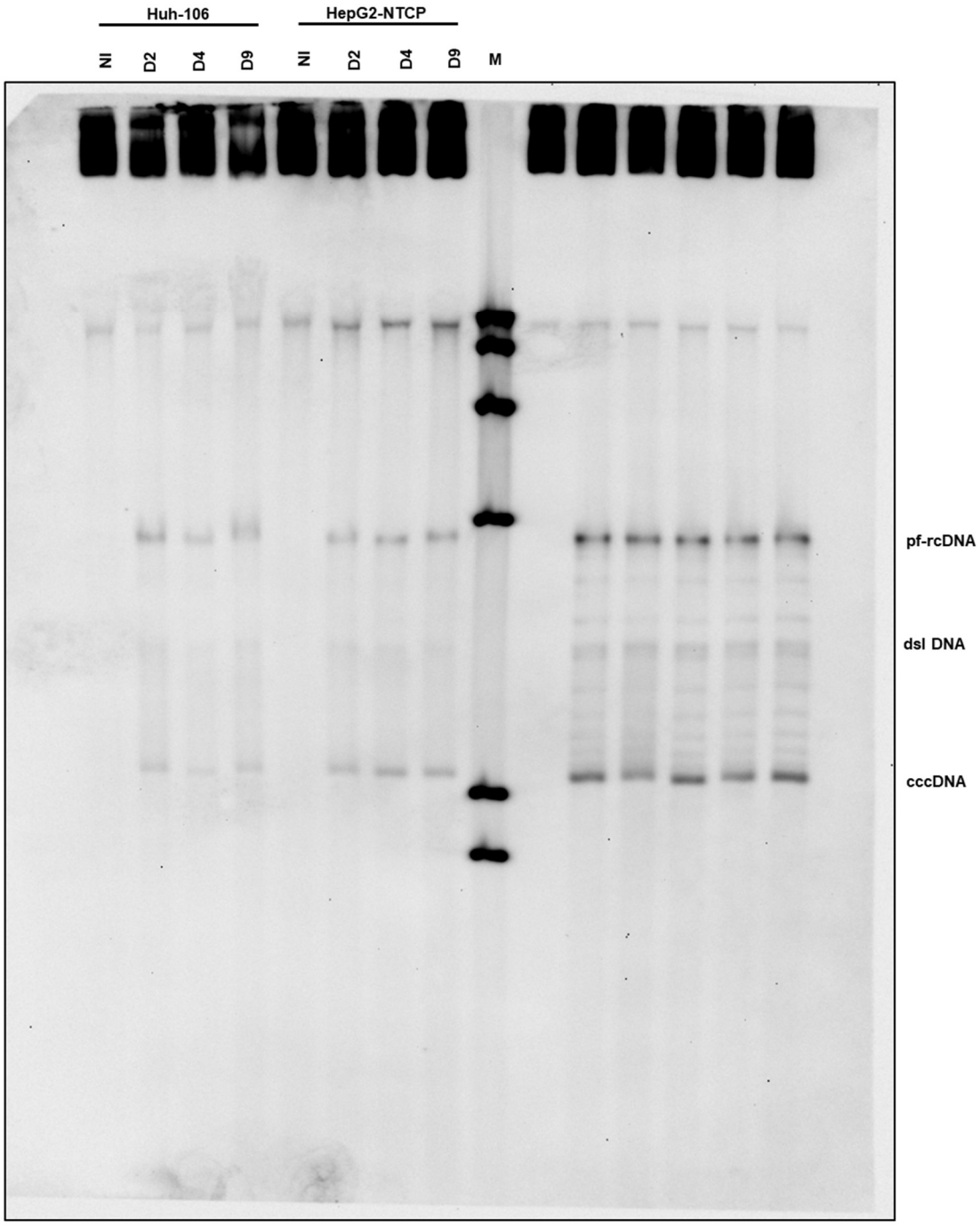
**Supplementary Figure 8. Full immunoblots**

**a**

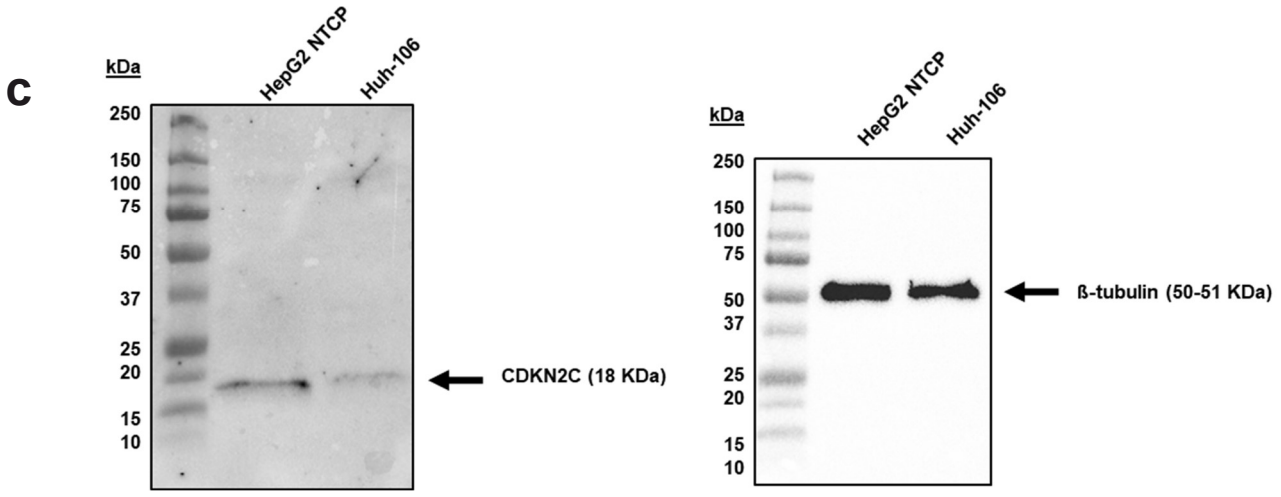



**(a) related to Figure 1c, d**

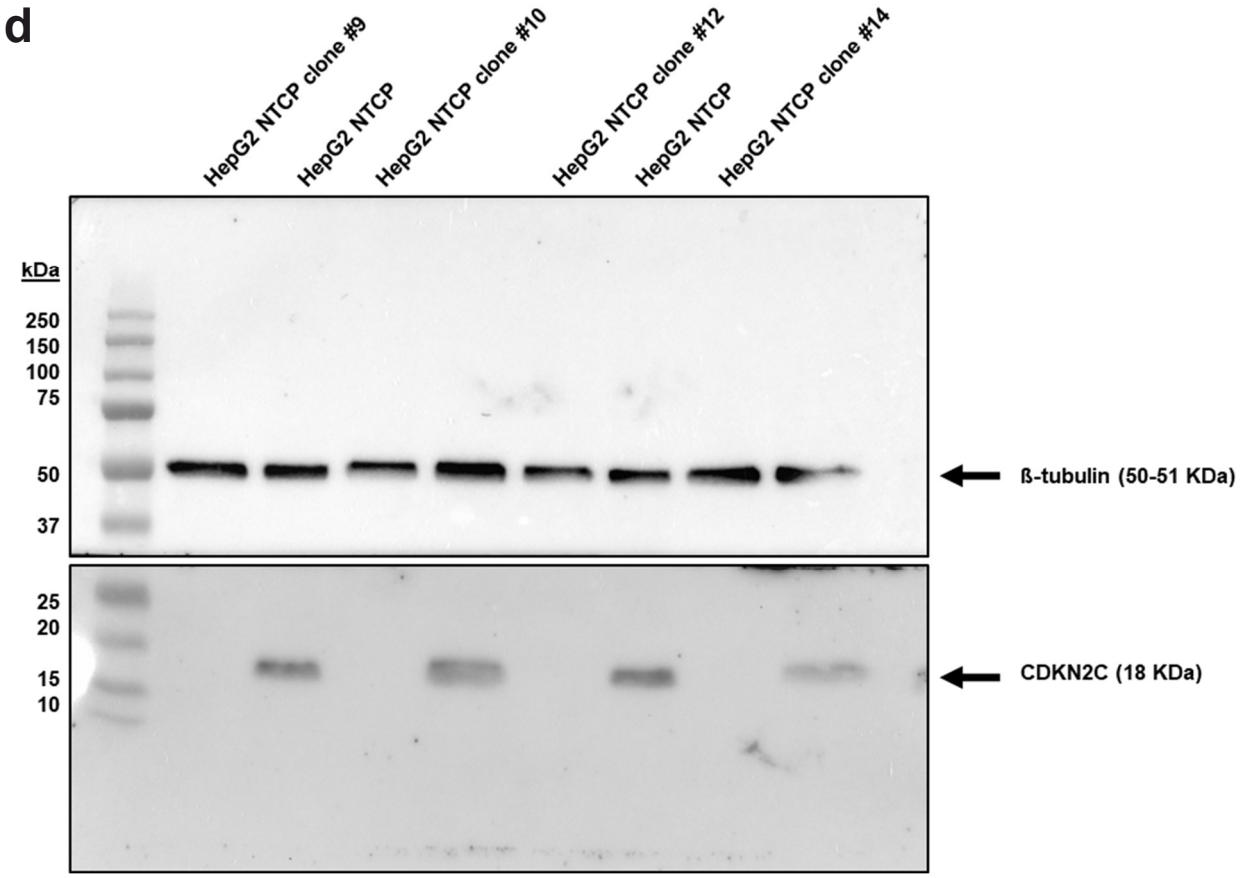
**b**




**(b) related to Figure 1e, S5a**

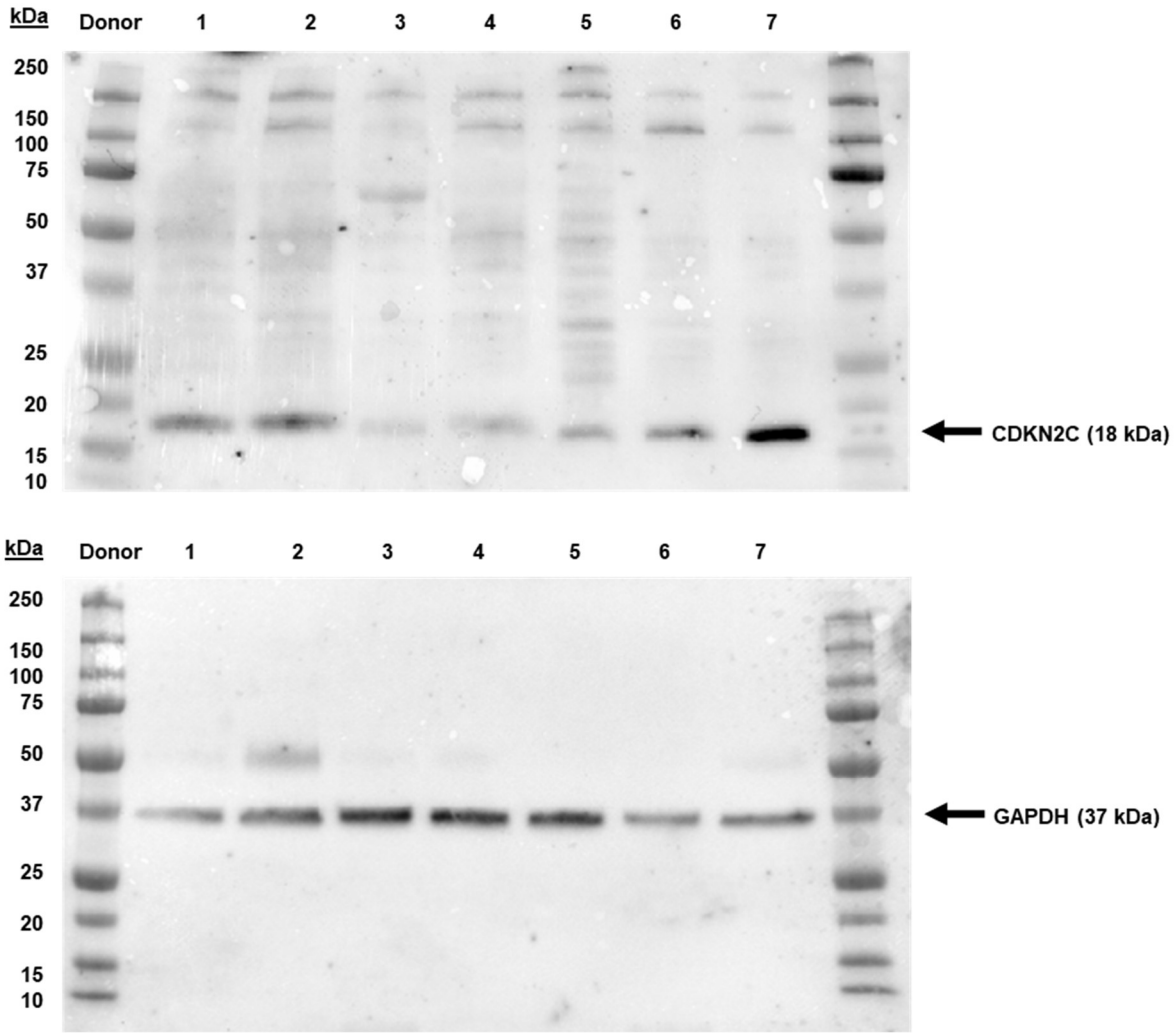



(c) related to Figure 3e



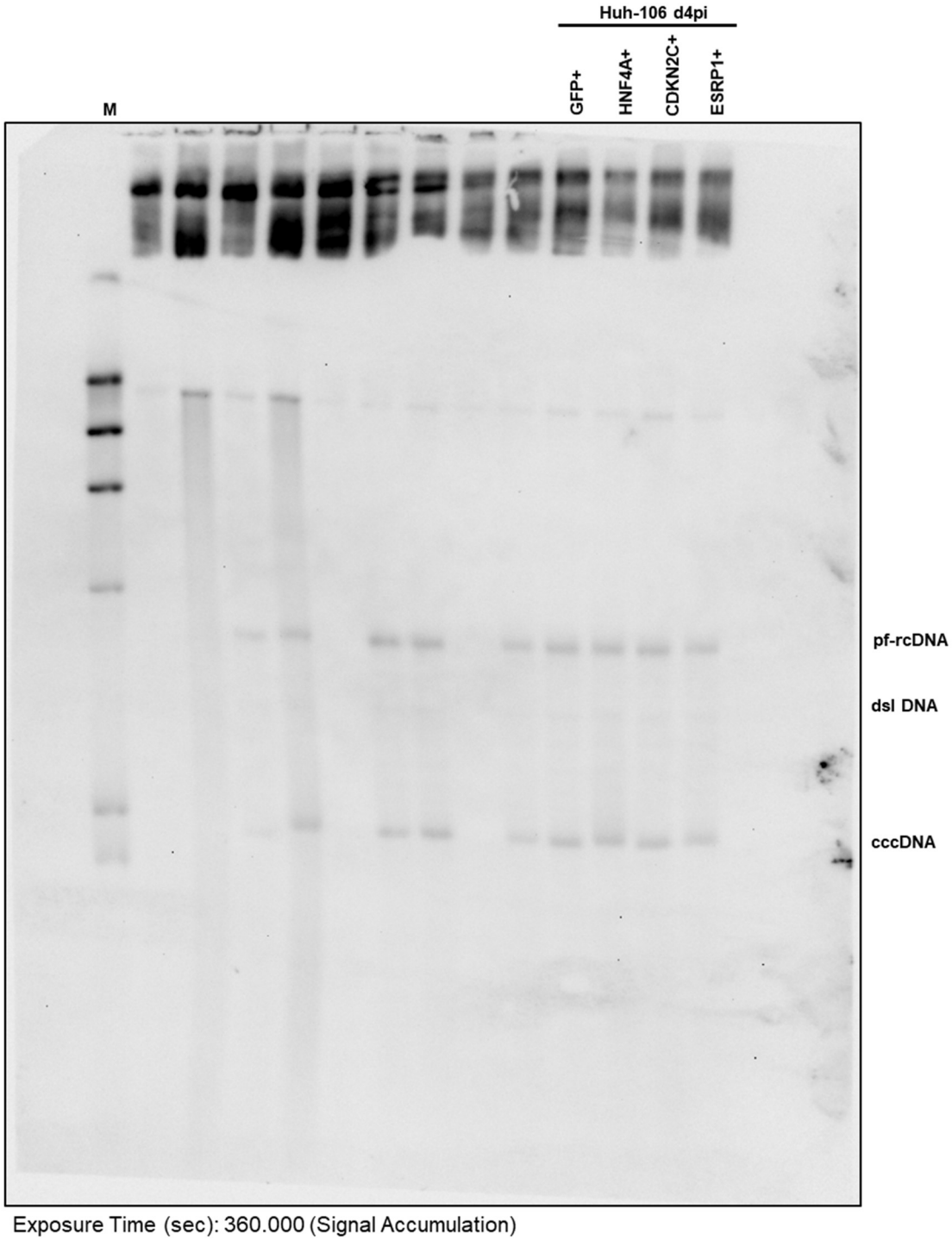

(d) related to Figure 4d

e



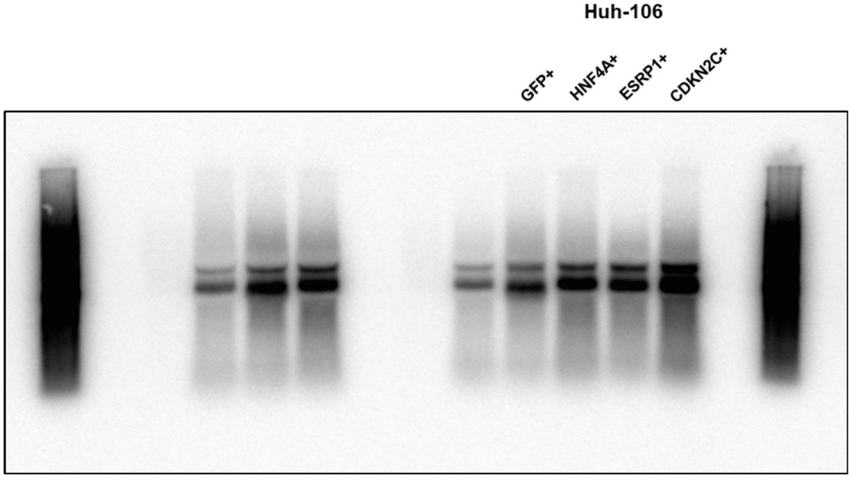

**(e) related to Figure 4f**

f



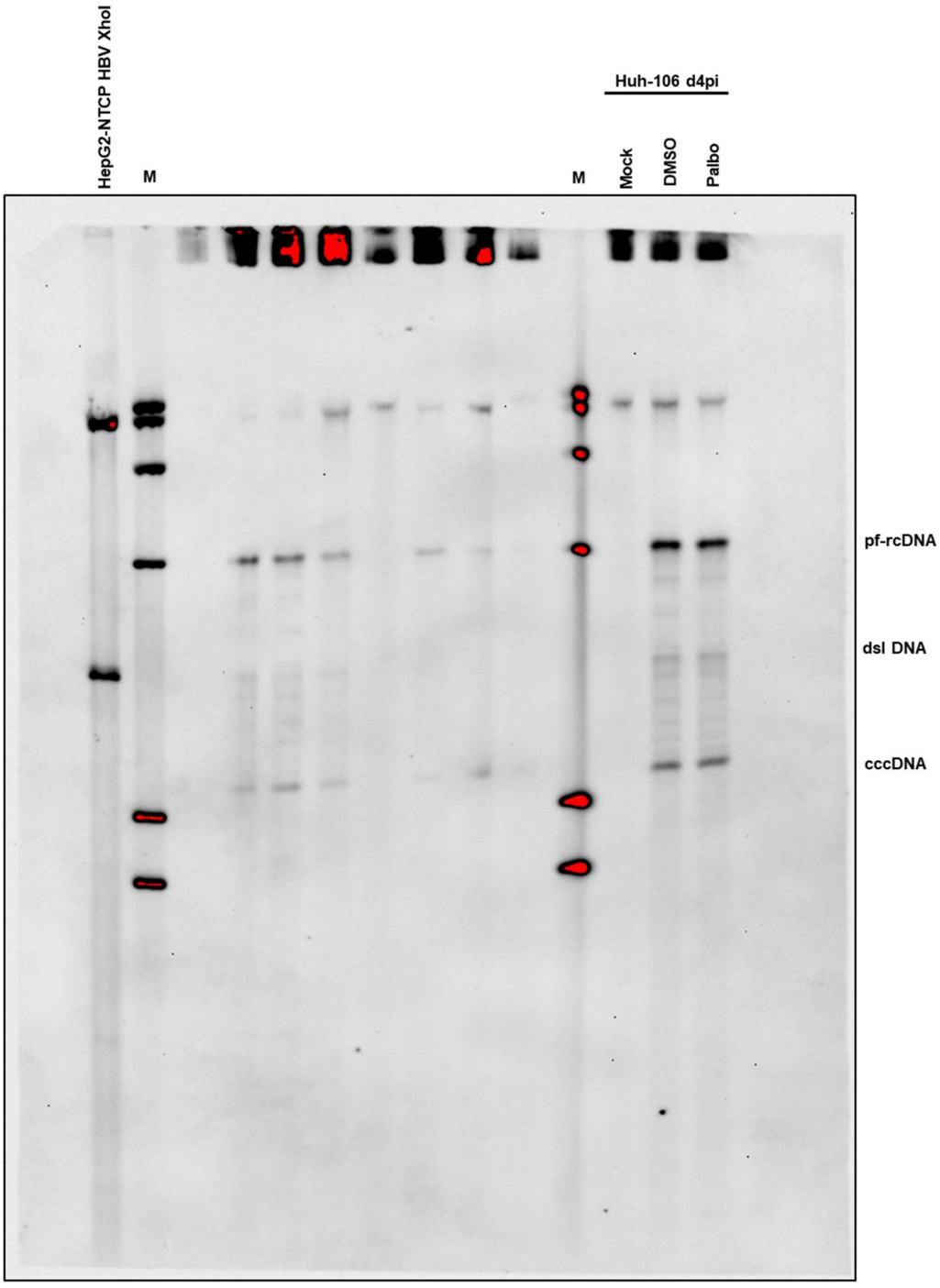

**(f) related to Figure 5d, e**

g




**(g) related to Figure 5f, g**

**h**




**(h) related to Figure 7f, S5b**

## SUPPLEMENTARY TABLES

**Table S3.1.** Candidates from 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 | 1.60 | X | 236.1 |
| SDC1 | 1.59 | X | 144.7 |
| TOB1 | 1.56 | X | 58.5 |
| HLA-DRB3 | 1.90 | X | 49.5 |
| USO1 | 1.93 | X | 40.1 |
| CLEC1B | 2.20 | 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 | 1.50 | 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 | 1.90 | 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 | 2.00 | 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 | 1.50 | 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 | 2.40 | | |
| MASTL | 1.89 | | |
| NAPSA | 1.60 | | |
| NEK8 | 2.01 | | |
| NME3 | 1.70 | | |
| 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 | 1.50 | | |
| 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

**Table S3.2.** Box plot details from Fig. 9b-d. T: Tumor. NT: Non-tumor

| | Fig. 9b | | | Fig. 9c | | | Fig. 9d | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | GSE83148 | | | GSE65359 | | | GSE14520 | | GSE65485 | |
| | Healthy | HBV DNA (-) | HBV DNA (+) | Tolerance | Clearance | Inactive | NT | T | NT | T |
| N. of values | 6 | 32 | 90 | 22 | 50 | 11 | 198 | 198 | 5 | 50 |
| Minimum | 13.66 | 14.97 | 12.37 | 6.84 | 6.21 | 6.37 | 16.74 | 18.45 | 1.99 | 0.72 |
| 25% Percentile (lower bound) | 14.34 | 25.33 | 32.53 | 7.32 | 7.62 | 6.89 | 22.20 | 32.09 | 2.06 | 5.16 |
| Median | 19.80 | 31.12 | 48.58 | 7.59 | 8.12 | 7.41 | 24.52 | 48.65 | 2.63 | 7.50 |
| 75% Percentile (upper bound) | 26.44 | 45.96 | 78.11 | 7.94 | 8.40 | 7.68 | 29.20 | 75.15 | 3.39 | 15.90 |
| Maximum | 30.11 | 100.20 | 186.10 | 8.32 | 9.15 | 8.22 | 47.18 | 213.30 | 3.49 | 42.56 |

**Table S3.2.** Box plot details from Fig. 9e and Supplementary Fig. 6b. T: Tumor. NT: Non-tumor

| | Fig. 9e | | | | | | | | Supplementary Fig. 6b | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Alc. | | HBV | | HCV | | NAFLD | | GSE84044 | | | |
| | T | NT | T | NT | T | NT | T | NT | F0 | F1 | F2 | F3 |
| Number of values | 70 | 8 | 76 | 7 | 34 | 5 | 11 | 2 | 37 | 33 | 34 | 15 |
| Minimum | 0.72 | 0.20 | 1.12 | 0.66 | 0.72 | 0.82 | 0.79 | 0.51 | 3.58 | 3.76 | 3.56 | 4.33 |
| 25% Percentile (lower bound) | 1.77 | 0.36 | 2.23 | 0.76 | 2.45 | 0.86 | 1.03 | 0.51 | 4.49 | 4.42 | 4.93 | 5.24 |
| Median | 3.28 | 0.78 | 4.09 | 0.87 | 4.93 | 0.98 | 2.30 | 0.53 | 4.93 | 4.85 | 5.51 | 5.71 |
| 75% Percentile (upper bound) | 8.37 | 0.91 | 7.89 | 0.98 | 9.37 | 1.12 | 5.18 | 0.56 | 5.44 | 5.40 | 5.93 | 6.22 |
| Maximum | 30.67 | 1.10 | 24.82 | 1.08 | 21.79 | 1.19 | 12.23 | 0.56 | 5.96 | 6.04 | 6.65 | 6.78 |

# DISCUSSION GENERALE

L'infection chronique par le HBV est un problème mondial de santé publique, conduisant à de graves maladies hépatiques progressives telles que la cirrhose et le cancer du foie (El-Serag, 2012). Alors que le génome et la structure du virus ont été étudiés depuis de nombreuses années, les interactions entre le HBV et l'hôte sont peu décrites (Summers *et al.*, 1982). Une meilleure compréhension de celles-ci permettrait le développement de nouvelles cibles thérapeutiques innovantes, permettant une guérison totale de l'infection chronique et ainsi éviter la carcinogénèse hépatique.

Le fil conducteur de mes travaux de thèse a été de caractériser de nouveaux facteurs hépatiques impliqués dans l'infection par le HBV grâce, en particulier, à une stratégie d'approche de génomique fonctionnelle. Ainsi, lors des différentes études présentées dans ce manuscrit, j'ai (i) établi et créé les outils de validation et utilisé les modèles cellulaires robustes et performants pour l'étude de l'infection par le HBV - afin (ii) d'identifier un nouveau mécanisme d'échappement du HBV à la détection par le senseur cellulaires cGAS (ii) et d'identifier de nouveaux facteurs cellulaires, comme CDKN2C, impliqués dans le cycle viral.

Le manque de modèles cellulaires d'infection efficaces a longtemps été un frein à la compréhension des interactions moléculaires entre le virus et son hôte. L'identification du transporteur NTCP comme premier récepteur d'entrée du HBV et du HDV a contribué à lever le flou sur les interactions virus-hôte et la biologie du cycle viral (Yan *et al.*, 2012 ; Ni *et al.*, 2014). En effet, la surexpression du transporteur NTCP à la surface de lignées cellulaires hépatocytaires a permis la mise au point des premiers modèles robustes d'étude du HBV et du HDV. Il est à noter que Kang *et al.* ont démontré que dans le CHC, la cycline D1 inhibe la transcription du gène *SLC10A1* codant pour NTCP (Kang *et al.*, 2017). Cette caractéristique pourrait expliquer l'absence d'expression du transporteur dans les lignées dérivées de CHC – telles que les HepG2 et les Huh7 - et donc la perte de sensibilité de ces cellules d'origine hépatocytaire à l'infection par le HBV et le HDV.

Une première lignée hépatocytaire surexprimant le récepteur NTCP, les Huh7-NTCP (aussi nommée Huh-106) a initialement été utilisée pour l'étude de l'entrée du HDV dans les hépatocytes. Différents travaux ont démontré le rôle des HSPG dans l'étape d'attachement du HBV et du HDV à la cellule hôte, sans jamais décrire les facteurs spécifiques impliqués dans cette étape du cycle viral (Schulze *et al.*, 2007 ; Lamas Longarela *et al.*, 2013 ; Somiya *et al.*, 2016). Ainsi, une approche de génomique fonctionnelle utilisant des siARN ciblant les membres de la famille des HSPG a permis l'identification du protéoglycane à héparane sulfate GPC5 comme facteur d'attachement du HDV. Le HBV et le HDV ont la particularité de partager les mêmes protéines d'enveloppe AgHBs et donc de partager le même récepteur d'entrée. C'est dans ce contexte que la « Partie I » de mes travaux de thèse a débuté par le développement d'une lignée cellulaire sensible au HBV par la surexpression du transporteur NTCP, les HepG2-NTCP (Verrier *et al.*, 2016a ; Verrier *et al.*, 2016b). En effet, les HepG2 ont été montrées comme mieux adaptées à l'infection par le HBV que les Huh7 (Yan *et al.*, 2012 ; Ni *et al.*, 2014). En parallèle, j'ai développé le système de production *in vitro* de HBV recombinant selon

la méthode de Ladner *et al.*, utilisant les HepAD38 (Ladner *et al.*, 1997). J'ai également activement participé à la mise au point les différentes techniques spécifiques de quantification de l'infection par le HBV, basées sur la détection des acides nucléiques viraux par qRT-PCR et des AgHBs et AgHBe par immunofluorescence et CLIA. La mise au point de tous ces outils cellulaires et moléculaires a été une étape clé dans l'objectif du laboratoire de découvrir de nouveaux facteurs d'hôte impliqués dans le cycle viral du HBV. Grâce à ces outils cellulaires, j'ai pu valider le rôle de GPC5 dans le cycle viral du HBV et spécifiquement dans l'étape d'attachement des virions à la cellule hôte (Verrier *et al.*, 2016a). Cette étude a confirmé la pertinence de l'utilisation de ces modèles cellulaires surexprimant NTCP pour l'identification de facteur d'hôte du HBV et du HDV. De plus, GPC5 est physiologiquement impliqué dans la régulation des voies de signalisation hedgehog et Wnt (Filmus *et al.*, 2008). Le HBV pourrait donc perturber cette voie de signalisation et ainsi induire une pathogénèse. Ainsi, ces modèles cellulaires ont également permis d'identifier un facteur pouvant être impliqué dans la carcinogénèse induite par le HBV. Dans une récente étude, Liu *et al.* ont démontré la pertinence de l'utilisation d'un peptide de liaison aux HSPG dérivé du HBV dans un système d'administration de médicaments (Liu *et al.*, 2018).

Dans le cadre de la découverte de NTCP comme récepteur du HBV et du HDV, les auteurs ont également démontré une compétition entre les virus et les ligands naturels du transporteur, les acides biliaires (Yan *et al.*, 2012). La fixation des ligands sur le transporteur inhibe donc la réplication du HBV et HDV. Au contraire, différentes études ont montré que les acides biliaires pouvaient favoriser la réplication d'un autre virus hépatique, le HCV (Chang *et al.*, 2007 ; Chhatwal *et al.*, 2012). Néanmoins, le mécanisme impliqué et le rôle du transporteur des acides biliaires NTCP dans cette régulation n'était alors pas connus. Le HBV et le HCV sont des virus présentant un cycle viral très différent mais qui partagent un hépatotropisme strict. Cette caractéristique de cellule hôte commune pourrait laisser supposer que des facteurs cellulaires spécifiques du foie interagissent avec ces deux virus. Ayant au laboratoire tous les outils d'étude du HCV et des lignées cellulaires hépatocytaires surexprimant NTCP, nous avons enquêté sur le rôle du transporteur dans l'infection par le HCV. Par des stratégies de gain et de perte de fonction ainsi que par une étude transcriptionnelle en micro-puce, nous avons déterminé que NTCP pouvait réguler l'infection par le HCV et la transmission cellule à cellule. Notre étude a montré que le transport des acides biliaires par NTCP favorisait l'infection par le HCV via l'inhibition de l'activité antivirale des ISG. Parmi les ISG régulés par NTCP, nous avons identifié IFITM3 (pour *interferon-induced transmembrane protein* 3), déjà connu pour son activité antivirale et en particulier son rôle dans l'inhibition de l'entrée du HCV (Narayana *et al.*, 2015 ; Savidis *et al.*, 2016).

Nous avons également démontré que l'inhibition du transport des acides biliaires par le peptide PréS1- mimant le site de liaison de l'AgHBs du HBV - augmentait l'expression d'ISG et donc l'immunité innée cellulaire contre le HCV. Cette observation pose la question de l'influence du HBV sur le cycle viral du HCV lors d'une co-infection (Rodriguez-Inigo *et al.*, 2005).

NTCP joue donc un rôle particulier dans l'infection des hépatocytes par trois virus hépatotropes et ce transporteur d'acides biliaires peut réguler la réponse immunitaire innée cellulaire (Verrier *et al.*, 2016b). Cette découverte confirme l'intérêt de la caractérisation des interactions entre les virus pour le développement de nouvelles stratégies antivirales et suggère que NTCP pourrait être une cible thérapeutique prometteuse pour les patients co-infectés HBV/HDV/HCV (Colpitts *et al.*, 2015).

Ainsi, l'utilisation des outils de biologie moléculaire et cellulaire dans des analyses de génomique fonctionnelle, et en particulier nos robustes modèles d'infection *in vitro*, ont été validée pour l'identification de nouveaux facteurs d'hôte du HBV (Verrier *et al.*, 2016a ; Verrier *et al.*, 2016b).

Notre précédente étude sur le rôle de NTCP dans l'infection par le HCV a montré que la fixation de PréS1 induit une levée de l'inhibition de l'expression d'ISG. Or, PréS1 est un déterminant de l'infectiosité de la protéine d'enveloppe du HBV. Dans le cas du HBV, les interactions entre le HBV et la réponse immunitaire innée est un processus complexe encore peu compris et restant controversé (Ferrari, 2015). L'hypothèse dominante, soutenue par de nombreuses données *in vivo* et *in vitro*, est que le HBV est un virus « silencieux » induisant peu ou pas de réponse immunitaire (Rehermann *et al.*, 2005).

La « Partie II » de mes travaux de thèse a porté sur la meilleure compréhension des interactions entre le HBV et le senseur d'ADN récemment décrit cGAS, connu notamment pour détecter l'ADN des rétrovirus (Gao *et al.*, 2013). En effet, cGAS est un senseur cytosolique de l'ADN simple brin et double brin mais également de l'ARN simple brin et des molécules hybrides ADN/ARN (Li *et al.*, 2013 ; Mankan *et al.*, 2014). En 2014, Schoggins *et al.* ont notamment décrit cGAS comme un acteur majeur de la réponse antivirale innée, possédant une activité antivirale contre un large spectre de virus à ADN et ARN (Schoggins *et al.*, 2014). Cependant, les interactions entre cGAS et le HBV restaient inconnues.

Dans notre étude publiée en 2018, nous avons (i) démontré que la forme encapsidée de l'ADN viral n'induisait pas de réponse interféron contrairement à l'ADN-rc nu qui est reconnu par cGAS et induit l'expression d'ISG, (ii) que la voie cGAS/STING possède une activité antivirale contre le HBV permettant une diminution de l'ADNccc intracellulaire et que (iii) le HBV induit une inhibition de l'expression et de la fonction de cGAS *in vitro* et *in vivo*. Ainsi, nos résultats suggèrent que la voie de signalisation cGAS/STING serait active contre le HBV sans détection de l'ADN viral mais par un ligand inconnu (Verrier *et al.*, 2018).

L'absence de réponse immunitaire innée lors de l'infection par le HBV soulève de nombreuses questions et reste très controversée. Quelques études suggèrent que le HBV est détecté par les senseurs cytosoliques. En effet, Sato et collègues démontrent une induction de la réponse IFN de type III dans les hépatocytes après infection par HBV. Cette réponse innée serait dépendante de la reconnaissance de la région ε de l'ARNpg par RIG-I (Sato *et al.*, 2015). De même, Luangsay *et al.* ont suggéré que les hépatocytes étaient capables de détecter le HBV et d'induire l'expression des ISG lors de leur exposition au virus. Ils proposent également que les réponses immunitaires innées

de l'hôte sont inhibées lors des interactions virus/hôte et cela potentiellement par un facteur exogène encore non connu, et qui pourrait expliquer le caractère furtif du HBV (Luangsay *et al.*, 2015).

A l'inverse, certaines observations tendent à confirmer l'hypothèse que tout au long de son cycle viral, le HBV n'est pas détecté par les PRR tels que cGAS via différents mécanismes d'échappement. En effet, lors de la réplication, les transcrits viraux sont synthétisés par la machinerie cellulaire et présentent une coiffe en 5' et une queue polyA en 3'. Ces caractéristiques leur permettent de ne pas être détecté par les senseurs cellulaires. De plus, la conversion de l'ARNpg en ADN s'effectue dans la nucléocapside et celle-ci semble présenter des pores trop petits pour le passage des PRR (Wynne *et al.*, 1999). Suslov *et al.* ont décrit qu'aucune réponse IFN n'était détectée dans des tissus de foie de patients chroniquement infectés par le HBV, suggérant ainsi que le virus n'induit pas de réponse innée. Ils démontrent également que le traitement de ces prélèvements au Poly I:C ou l'infection par le virus Sendai permet d'induire une réponse IFN et la production d'ISG, confortant ainsi l'hypothèse que le HBV est invisible aux PRR (Suslov *et al.*, 2018). De plus, Mutz *et al.* suggèrent que le HBV échappe à la réponse immunitaire innée en contournant les mécanismes de la réponse immunitaire cellulaire (Mutz *et al.*, 2018)

La disparité des observations sur la réponse immunitaire face à l'infection par le HBV peut être expliquée par des différences dans les conditions expérimentales. En effet, l'utilisation de particules virales purifiées à partir de sérum de patient chroniquement infectés ou de particules virales recombinantes peut expliquer ces écarts dans les résultats. De plus, certaines de ces études montrant que les protéines virales bloquent la réponse IFN ont été réalisées à partir de cellules surexprimant artificiellement les protéines du HBV (Wu *et al.*, 2007 ; Chen *et al.*, 2013). En parallèle, Sato *et al.*, ne démontrent l'interaction entre RIG-I et l'ARNpg uniquement avec un virus de génotype C (Sato *et al.*, 2015). De plus, l'utilisation de modèles cellulaires différents se rapprochant plus ou moins des conditions physiologiques - lignées cellulaires / HepaRG / PHH / souris chimériques – sont des critères à prendre en considération dans l'analyse critique des résultats. Nous pouvons noter que la majorité de ces études ont été réalisées sur les hépatocytes et qu'elles ne prennent pas en compte le microenvironnement. Or, il serait intéressant d'observer la réponse innée face à l'infection par le HBV dans des modèles plus physiologiques. Pour cela, de nouveaux modèles cellulaires, basés sur des co-cultures d'hépatocytes avec des cellules de l'immunité ou et des modèles 3D en sphéroïdes et organoïdes, permettraient d'observer les réponses IFN dans des modèles plus complets.

Le rôle de la voie de signalisation cGAS/STING dans la détection du HBV est également très controversé. Alors que STING est fortement exprimé dans certaines cellules comme les cellules de poumon, les hépatocytes et cellules dérivées ne semblent présenter que peu d'expression du gène *TMEM173* codant pour STING. Cette observation a été validée par Thomsen *et al.*, qui affirment que les hépatocytes n'expriment pas la protéine STING (Thomsen *et al.*, 2016).

Cependant, notre étude a démontré que nos modèles cellulaires expriment cGAS et STING et que l'activation de cette voie de signalisation induit l'expression d'IFN et d'ISG. L'expression de STING

dans les hépatocytes a également été démontré par Guo *et al* (Guo *et al.*, 2017). Dans cette même étude, les auteurs confirment notre hypothèse que le HBV n'induit pas l'activation de la voie de signalisation cGAS/STING et que cela peut s'expliquer par un mécanisme d'échappement du génome viral à la détection des senseurs intracellulaires. Une autre étude a également confirmé que la voie cGAS/STING possède une activité antivirale face au HBV et en particulier sur le niveau d'ADNccc dans les cellules infectées (He *et al.*, 2016). Enfin, Lauterbach-Rivière *et al.* ont récemment confirmé le caractère immunogène de l'ADN nu du HBV et son rôle dans la réponse IFN médiée par la voie cGAS/STING (Lauterbach-Riviere *et al.*, 2020). Enfin, toutes ces études sur les interactions entre cGAS et le HBV, dont la nôtre, suggèrent que le faible niveau d'expression de cGAS et STING dans les hépatocytes humains n'explique pas l'absence de réponse IFN lors de l'infection par le HBV.

Ces données mettent en évidence la pertinence de l'activation de la réponse antivirale via la voie cGAS/STING comme stratégie thérapeutique contre le HBV. De plus, notre étude suggère que l'ADN nu est détecté par les senseurs cellulaires. Cette observation suggère que la déstabilisation de la capside et ainsi la libération de l'ADN du HBV dans le cytoplasme pourrait être intéressante pour l'induction d'une activité antivirale médiée par la voie cGAS/STING. Or, les modulateurs de capside actuellement testés en phases cliniques n'induisent pas la présentation de l'ADN du HBV aux senseurs cytoplasmiques. En effet, les CAMs de type I agissent sur la cinétique de formation de la nucléocapside alors que les CAMs de type II forment des capsides aberrantes resserrées induisant leur destruction rapide (Cole, 2016).

Nos modèles cellulaires ainsi qu'une approche de génomique fonctionnelle ont permis l'identification d'un nouveau mécanisme d'échappement du HBV au système immunitaire cellulaire ainsi que l'interaction entre la voie cGAS/STING et le HBV. En effet, même si la détection du virus par cGAS n'est pas efficace pour induire une forte réponse IFN, celle-ci permet tout de même une activité antivirale basale. De plus, nous avons démontré que cette défaillance de la réponse innée n'était pas due à l'absence de détection. L'interaction entre le HBV et le système immunitaire innée est complexe et de nombreux paramètres restent encore inconnus.

Dans le cadre de mes travaux de thèse, j'ai donc mis au point les différents modèles cellulaires pour l'étude du cycle viral complet du HBV. La pertinence de ces modèles pour l'étude des interactions virus/hôte a été démontrée dans deux publications présentées en **annexe 2 et 3** (Verrier *et al.*, 2016a ; Verrier *et al.*, 2016b). Par la suite, ces modèles ont été utilisés pour la mise en évidence de l'interaction entre le HBV et la voie de signalisation cGAS/STING (Verrier *et al.*, 2018). Dans le cadre de la « Partie III » de mes travaux de thèse, nous avons réalisé une étude de génomique fonctionnelle toujours dans le but de mieux comprendre les interactions virus-hôte. La première étape de ce projet a été de caractériser les différences d'infection par le HBV de nos deux lignées cellulaires hépatocytaires surexprimant le transporteur NTCP. Basée sur l'observation d'une infection par le HBV moins efficace des Huh7-106 par rapport au HepG2-NTCP, j'ai réalisé un criblage en gain de fonction à l'échelle du génome sur la lignée Huh7-106 infectée par le HBV, en utilisant une banque lentivirale

permettant l'expression de plus de 16 000 gènes. Cette stratégie innovante a permis d'identifier 47 facteurs favorisant l'infection virale dont les facteurs de transcription HNF4A ou HLF, déjà décrits comme importants pour la réplication du HBV (Raney *et al.*, 1997 ; Ishida *et al.*, 2000).

Mes résultats de validation, combinés à la comparaison transcriptomique des deux lignées cellulaires ont mis en évidence le facteur de l'hôte CDKN2C comme étant fortement surexprimé dans les HepG2 en comparaison aux Huh7, et jouant un rôle clé dans la réplication virale de HBV. Ainsi, grâce à des études fonctionnelles, nous avons (1) observé l'effet du gain et de la perte de fonction de CDKN2C sur l'infection par le HBV, (2) corrélé l'expression et la fonction de CDKN2C avec la réplication du HBV en utilisant des inhibiteurs mimant sa fonction, (3) déterminé le mécanisme d'action de l'arrêt en phase G1 sur la réplication du HBV et (4) corrélé l'expression de CDKN2C avec la progression de l'infection chronique et des maladies hépatiques.

CDKN2C est un inhibiteur des *« cyclines dépendantes kinases »* 4 et 6 (CDK4/6) (Guan *et al.*, 1994). Dans notre étude, nous avons démontré que l'inhibition de CDK4/6 à l'aide de molécules thérapeutiques induisait une augmentation de la réplication virale. Un de ces inhibiteurs de kinase est le Palbociblib, actuellement utilisé dans le cadre du traitement du cancer du sein localement avancé ou métastatique (Turner *et al.*, 2015). Nos observations soulèvent la question de l'impact du traitement au Palbociclib sur des patients atteints d'un cancer du sein et ayant des antécédents d'infection au HBV.

L'inhibition des CDK4/6 par CDKN2C induit l'arrêt du cycle cellulaire en phase G1 qui semble donc impacter favorablement l'infection par le HBV. Le rôle du cycle cellulaire et donc l'impact de l'état physiologique de la cellule sur la réplication du HBV reste controversé. Wang *et al.* ont démontré que le HBV induit l'arrêt du cycle cellulaire en phase G1 en régulant l'expression des gènes impliqués dans la prolifération cellulaire (Wang *et al.*, 2011a). L'arrêt du cycle en phase G1 empêche donc la prolifération des cellules et bloque la cellule dans un état de quiescence. Allweiss *et al.* ont récemment démontré la corrélation entre le niveau de réplication du HBV et la quiescence des hépatocytes dans un modèle de xénogreffe de souris avec des PHH (Allweiss *et al.*, 2016). Plusieurs études montrent que la réplication du HBV et l'expression des protéines virales sont facilitées dans les hépatocytes quiescents (Aden *et al.*, 1979 ; Sureau *et al.*, 1986). Les protéines virales elles-mêmes peuvent également jouer sur la réplication cellulaire. En effet, HBx a été identifié comme étant en partie responsable de l'arrêt du cycle cellulaire en phase G1 (Gearhart *et al.*, 2010). Or, HBx favorise la carcinogénèse et la prolifération accrue et non contrôlée des cellules (Li *et al.*, 2019a). Ainsi, l'effet de HBx sur le cycle cellulaire reste contradictoire. Une autre étude a affirmé que le virus induit le passage de la cellule en phase G2/M via la diminution du TGF-β, ce qui pourrait également expliquer la carcinogénèse induite par le HBV (Xia *et al.*, 2018). De manière intéressante, un autre virus comme le virus Epstein-Barr, pourtant également oncogène, induit un arrêt en phase G1 du cycle cellulaire via la modulation de l'expression d'inhibiteur de cycline (Cayrol et Flemington, 1996).

Afin de déterminer l'importance clinique de CDKN2C, nous avons analysé l'expression de l'inhibiteur de kinase dans des hépatocytes primaires humains infectés et, à partir de base de données, dans des tissus de foie de patients infectés par le HBV. De manière intéressante,

l'expression de CDKN2C est augmentée par l'infection virale. De plus, il existe une corrélation entre la progression des maladies hépatiques et l'expression de l'inhibiteur de kinase chez les patients chroniquement infectés. Ces résultats suggèrent un rôle fonctionnel de CDKN2C dans le développement des maladies du foie induites par le HBV.

Le mécanisme d'action de CDKN2C sur l'infection par le HBV a pu être déterminé par des expériences basées sur la détection des ARN néo-transcrits après modulation de l'activité des CDK4/6. Elles ont permis de montrer que l'arrêt du cycle en phase G1, induit par la surexpression de CDKN2C ou le traitement au Palbociclib, est corrélé avec l'induction de l'expression des facteurs favorisant la transcription du HBV, dont HNF4A et HLF. Un mécanisme similaire de modulation de l'expression de facteurs de transcription par des virus a également été démontré dans le cas du VIH (Clark *et al.*, 2017). CDKN2C a été décrit comme étant impliqué dans les interactions avec d'autres virus tels que le virus T-lymphotropique humain (HTLV-1) ou le papilloma virus (HPV) (Neuveut *et al.*, 1998 ; Wang *et al.*, 2011b).

Le rôle précis de l'état physiologique de la cellule sur l'infection par le HBV et en particulier la phase du cycle cellulaire favorisant la réplication virale reste encore très flou. Cependant, il a clairement été décrit que lors de l'infection, le HBV détourne la machinerie cellulaire afin de créer un environnement favorable à sa réplication (Fan *et al.*, 2018).

Les différents travaux réalisés dans le cadre de ma thèse suivent le même objectif : développer des stratégies innovantes pour l'identification de nouveaux facteurs de l'hôte impliqués dans le cycle viral du HBV. Les nouvelles connaissances obtenues par la caractérisation des interactions virus-hôte permettent une meilleure compréhension du cycle viral du HBV. Elles peuvent également être la base du développement de nouveaux modèles *in vitro* et *in vivo* se rapprochant des conditions physiologiques de l'infection virale. Les nouveaux modèles cellulaires d'infection du HBV tels que les HepG2-NTCP, ne permettent toujours pas l'étude du virus dans des conditions physiologiques. Si moins de 20 particules virales sont suffisantes pour infecter un foie de patient, les modèles cellulaires nécessitent un inoculum fortement concentré en particules virales (Verrier *et al.*, 2016a ; Witt-Kehati *et al.*, 2016 ; Candotti *et al.*, 2019). L'utilisation de PEG 8000 est également préférable pour augmenter le niveau d'infection en culture cellulaire. Ces polyéthers linéaires permettent une meilleure adsorption et une concentration des particules virales à la surface des hépatocytes (Gripon *et al.*, 1993 ; Yan *et al.*, 2012 ; Ni *et al.*, 2014 ; Verrier *et al.*, 2016a). Enfin, l'infection efficace des cellules hépatocytaires *in vitro* nécessite l'ajout de DMSO dans le milieu de culture. Ce solvant aurait la propriété de différencier les cellules hépatocytaires mais également d'induire une quiescence cellulaire (de Abreu Costa *et al.*, 2017).

Même si les modèles cellulaires nécessitent des conditions particulières de culture et d'infection, ils permettent l'étude des interactions HBV/hôte. De plus, la découverte de NTCP a permis d'améliorer les modèles *in vitro* et *in vitro* existants et ainsi l'étude du cycle viral complet. Ils sont maintenant suffisamment robustes pour permettent la réalisation d'approches à haut débit telles que des criblages

en génomique fonctionnelle. Ils permettent des analyses à grandes échelles et favorisent la découverte de nouveaux facteurs d'hôte du HBV.

L'étude portant sur la découverte de NTCP a mis en évidence une infection moins efficace du HBV dans la lignée hépatocytaire Huh7 par rapport aux HepG2 (Yan *et al.*, 2012). Pourtant le niveau d'infection du HDV, qui partage les mêmes protéines d'enveloppe et donc logiquement la même étape d'entrée, est identique entre les deux lignées. Cette observation suppose donc la restriction de l'infection par le HBV lors des étapes post-entrée. Dans le cadre de la « partie III », nous avons mis en évidence une plus faible expression de *CDKN2C* dans les Huh7 qui s'est avérée délétère pour l'infection virale. Cependant, notre étude a montré l'impact de CDKN2C sur la transcription des ARN viraux. Or, la différence de niveau de l'ADNccc observée entre les lignées tend à supposer une restriction au niveau de la formation de la matrice transcriptionnelle du HBV. Notre approche de criblage à haut débit n'a pas permis de mettre en évidence des facteurs impliqués dans la biologie de l'ADNccc. Nous avons cependant identifié d'autres facteurs précédemment décrits comme favorables à l'infection virale comme HNF4a et HLF, confirmant l'intérêt de notre approche pour l'identification de facteur de l'hôte important pour l'infection virale. Notre stratégie de génomique fonctionnelle utilisant une banque gain de fonction à l'échelle du génome présente pourtant la limite de ne pas pouvoir détecter des complexes ou des synergies de facteurs pouvant être importants pour le cycle viral. De plus, il est également possible que la restriction de l'infection dans les Huh7 soit causée non pas par le manque de facteur proviral mais par la forte expression d'un facteur de restriction du cycle viral. Cette hypothèse pourrait être vérifiée par la réalisation d'un criblage à haut débit basé sur la technologie de KO par CRISPR/Cas9. J'ai déjà utilisé cette technique dans la partie III, afin de valider le rôle de CDKN2C dans l'infection virale. En parallèle, afin d'aborder les différences entre cellules Huh7 et HepG2 sous un autre angle, j'ai réalisé un criblage en perte de fonction par KO basé sur une librairie de sgARN ciblant le génome entier, suivi d'une infection par le HBV afin d'identifier de potentiels facteurs de restriction du virus. L'analyse des données de ce criblage permettra de découvrir de nouveaux facteurs cellulaires impliqués dans le cycle viral du HBV. Une autre approche pouvant permettre de mieux comprendre la restriction de l'infection dans les Huh7 serait de comparer les transcriptomes des lignées cellulaires permissives au HBV comme les HepaRG mais également avec des PHH.

La question de la sensibilité des lignées cellulaires à l'infection HBV après surexpression de NTCP n'est pas restreinte aux cellules Huh7. En effet, les cellules non hépatiques comme les HeLa ou les lignées cellulaires murines telles que les Hepa1-6 ou les Hep56.1D sont permissives à une infection du HDV mais pas du HBV (Lempp *et al.*, 2016). Dans leur étude, Lempp *et al.*, suggèrent que la restriction d'infection dans les cellules de souris serait liée à l'absence d'un facteur proviral impliqué en amont de la formation de l'ADNccc. En effet, la fusion des cellules de souris avec les cellules humaines HepG2 semble permettre la réplication du HBV. Ces observations suggèrent la pertinence d'une approche de criblage à haut débit en gain de fonction dans le modèle cellulaire de

souris Hep56.1D. En effet, la découverte de facteur(s) permettant l'infection robuste des hépatocytes de souris serait une étape majeure dans le développement de modèle *in vivo* murin immunocompétent de l'infection par le HBV, non disponible actuellement. Afin de tirer parti de la non-permissivité des hépatocytes de souris, nous souhaiterions réaliser notre approche de criblage en gain de fonction à l'échelle du génome humain dans une lignée hépatocytaire de souris. Pour cela, j'ai dans un premier temps surexprimé le récepteur NTCP humain dans la lignée cellulaire Hep56.1D **(Figure 15 A-B)** et caractérisé l'infection des cellules avec le HDV et le HBV. De même que les Huh-106, les Hep56.1D-hNTCP sont permissive au HDV mais pas au HBV **(Figure 15 C-D)**. Afin de mettre au point le criblage, j'ai réalisé des expériences préliminaires basées en bas débit. Nous avons alors observé que la surexpression de NTCP humain dans les Hep56.1D et la transduction de la librairie d'ORF ne permettent pas de détecter d'infection virale par cytométrie en flux **(Figure 15 E)**.

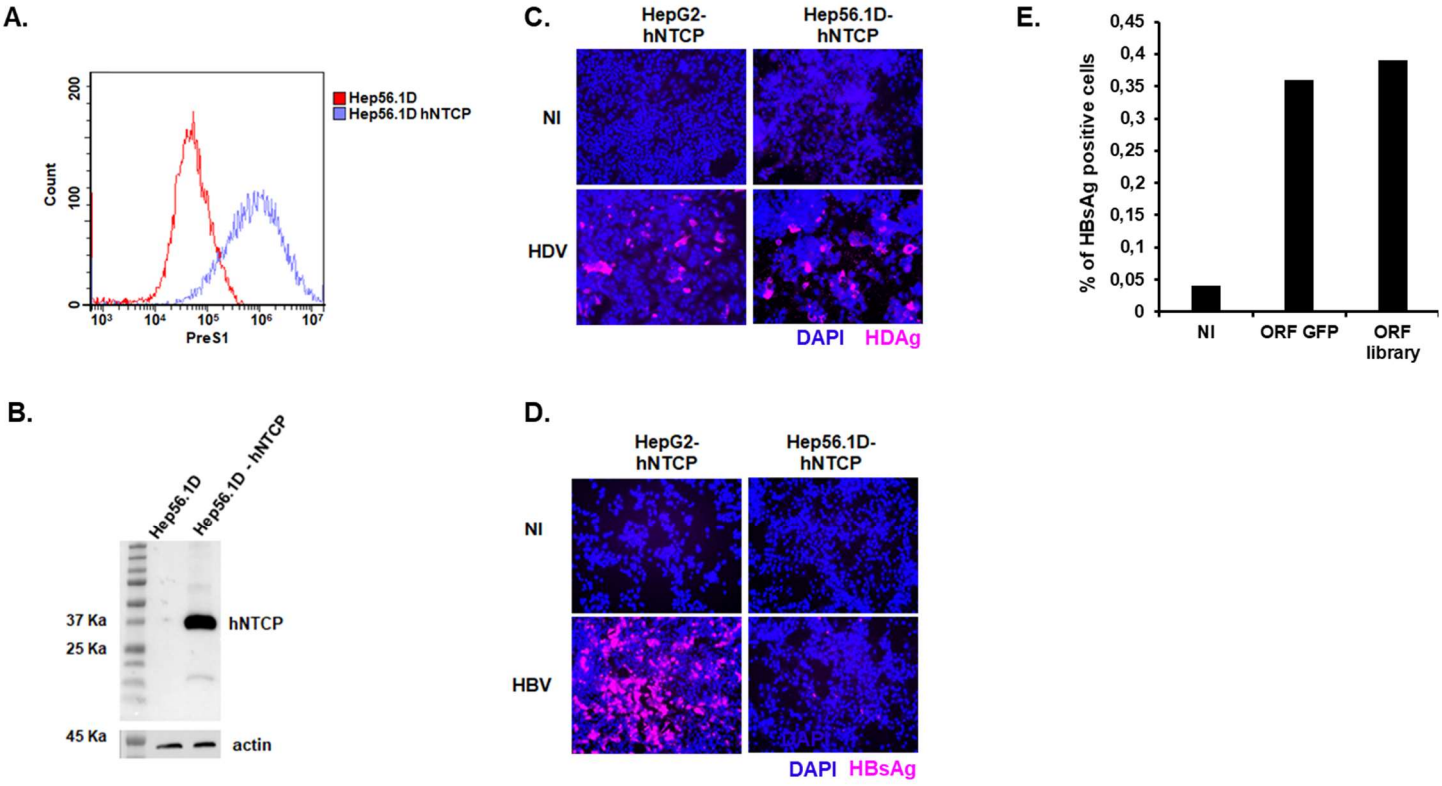



**Figure 15 : Production du modèle cellulaire de souris Hep56.1D surexprimant hNTCP et permissif au HDV.** (A) Quantification de hNTCP à la surface des Hep56.1D par cytométrie en flux par utilisation d'un peptide dérivé de PréS1 marqué. Les cellules ont été traitées 1 heure avec un peptide dérivé de PréS1 marqué Alexa Fluor 647 (200 nM) et fixées avec de le PFA à 2%. L'expression de hNTCP a été déterminée par cytométrie en flux en quantifiant la liaison du peptide. Une expérience représentative est montrée. (B) Détection de la protéine hNTCP par western blot. Les lysats cellulaires ont été traités avec une peptide-N-glycosidase puis détectée en immunoblot par un anticorps monoclonal anti-NTCP de souris (Sigma). Une expérience représentative est montrée. (C-D) Infection HDV et HBV des Hep56.1D-hNTCP et des HepG2-NTCP et détection des cellules infectées par IF. Le noyau des cellules est détecté en bleu par du DAPI. (C) Les cellules positives au HDV sont visualisées par IF en rose après immunomarquage des antigènes AgHD avec un anticorps anti-AgHD. (D) Infection HBV des Hep56.1D-hNTCP et des HepG2-NTCP. Les cellules positives au HBV sont visualisées par IF en rose après immunomarquage des antigènes AgHBs avec un anticorps monoclonal de souris anti-AgHBs (R&D).

(E) Détection des cellules AgHBs positives par cytométrie en flux après transduction de la librairie d'ORFéome. Les Hep56.1D-hNTCP ont été transduites avec la librairie d'ORFéome pendant 3 jours (Broad Institut). Après 3 jours de sélection à la puromycine, les cellules ont été infectées avec du HBV. 10 jours post-infection, les cellules ont été fixées à la PFA 2% et marquées avec un anticorps monoclonal de souris anti-AgHBs conjugué à l'Alexa Fluor 647. Les cellules positives au HBV ont été quantifiées par cytométrie en flux.

Ces résultats préliminaires semblent montrer une restriction de l'infection au HBV malgré la transduction des cellules avec la librairie d'ORFéome. Une récente étude ayant pour objectif de reconstituer le cycle viral du HBV dans la lignée cellulaire non hépatique HEK 293T montre que l'infection HBV nécessite la présente de facteurs proviraux indispensables à la réplication virale (Yang *et al.*, 2020). En effet, l'ajout des facteurs proviraux NTCP, HNF4α, RXRα et PPARα suffit à permettre l'infection d'une lignée cellulaire non susceptible au HBV. Cette étude suggère l'hypothèse d'un manque de base de ces facteurs proviraux dans notre modèle Hep56.1D. Il serait donc intéressant de réaliser cette approche de surexpression de ces facteurs du HBV dans la lignée murine Hep56.1D-hNTCP avant la transfection de la librairie.

Nos recherches ainsi que celles d'autres équipes ont notamment identifié les étapes en amont de la production de l'ADNccc comme limitante pour l'infection par le HBV dans les cellules Huh7 et dans les lignées cellulaires de souris (Lempp *et al.*, 2016 ; Eller *et al.*, 2018). Les découvertes des facteurs impliqués dans la biologie de l'ADNccc sont d'autant plus importantes que ce facteur de chronicité n'est pas ciblé par les traitements actuels chez les patients chroniquement infectés (Levrero *et al.*, 2016 ; Zoulim *et al.*, 2016). Or, les techniques de détection de l'ADNccc disponibles sont encore très limitées par la quantité de particules d'ADNccc par cellule, ce qui empêche la réalisation de criblage à haut débit (Werle-Lapostolle *et al.*, 2004 ; Li *et al.*, 2017). Alors que la technique de détection du minichromosome viral la plus fiable et la plus sensible reste le Southern blot, elle n'est absolument pas adaptée à une utilisation en criblage génomique (Cai *et al.*, 2013). Une autre technique décrite est la détection de l'ADNccc par PCR quantitative (Qu *et al.*, 2018). Or cette technique est actuellement très controversée car peu fiable dans la discrimination de l'ADNccc face aux autres formes de l'ADN viral (Lucifora *et al.*, 2016). Cette controverse a amené la création d'un consortium international afin de standardiser la méthode quantitative de détection de l'ADNccc. Récemment, une étude a proposé une nouvelle méthode de quantification de l'ADNccc basée sur une PCR quantitative après traitement enzymatique et par chaleur de l'ADN du HBV et nommée cinqPCR (Tu *et al.*, 2020). Cependant, cette technique n'a été démontrée que sur le génotype D pour le moment.

Une autre approche d'intérêt serait la détection indirecte de l'effet de la modulation de l'expression de gènes sur l'ADNccc. En effet, *in vitro*, la production de l'AgHBe peut être utilisée comme marqueur de substitution de la formation de l'ADNccc. Dans leur étude, Cai *et al.*, ont développé une lignée cellulaire stable inductible du HBV exprimant des AgHBe taggés et permettant le criblage à haut débit des modulateurs de l'ADNccc (Cai *et al.*, 2016). Également dans le but de pallier la faible proportion d'ADNccc dans les cellules infectées par le HBV, une approche similaire

pourrait être réalisée en utilisant le génome du DHBV, présentant près de 20 fois plus de minichromosome par cellules (Zhang *et al.*, 2003).

Basé sur ces nouvelles technologies, nous avons mis en place un projet au laboratoire auquel je participe activement. Nous avons utilisé une lignée cellulaire stable inductible du DHBV exprimant des AgHBe taggés HA (pour *human influenza hemagglutinin*) (Schreiner et Nassal, 2017). Dans ce modèle cellulaire, l'AgHBe n'est produit qu'après formation de l'ADNccc et peut être détecté par utilisation d'un anticorps anti-HA. Ainsi, l'AgHBe est utilisé comme marqueur indirect de l'ADNccc. Afin de mieux comprendre la formation de l'ADNccc dans la cellule infectée, nous avons réalisé un criblage en perte de fonction utilisant des shARN ciblant les gènes de la machinerie cellulaire et basé sur la détection de l'AgHBe par ELISA ciblant le HA. Ce criblage a permis l'identification du facteur YBX1 comme nouveau facteur d'hôte du HBV et ayant un rôle dans la biologie de l'ADNccc. Cette étude est en finalisation.

Mes travaux de thèse, et les publications y étant associées, ont permis de démontrer que nos modèles cellulaires d'étude *in vitro* de l'infection du HBV sont pertinents pour l'identification et la caractérisation de nouveaux facteurs d'hôte du HBV. En effet, ces modèles nous ont permis de mettre en évidence, par des approches de génomique fonctionnelle, le rôle de GPC5, cGAS et CDKN2C dans le cycle viral du HBV. Ces études démontrent le lien étroit qu'il existe entre le virus et la cellule hôte et la nécessité de mieux comprendre ces interactions pour permettre le développement de systèmes infectieux physiologiques pour la découverte de nouveaux traitements.

# CONCLUSION

Le HBV est le premier facteur de risque des maladies progressives hépatiques telles que la cirrhose et le carcinome hépatocellulaire (El-Serag, 2012). Alors que le cycle viral du HBV est assez bien caractérisé, les interactions virus-hôtes restent peu connues (Summers *et al.*, 1982). Lors de cette thèse, j'ai (i) mis au point et utilisé les modèles d'études robustes d'infection par le HBV - afin (ii) d'identifier le mécanisme d'échappement du HBV à sa détection par le senseur cellulaire cGAS (ii) et d'identifier de nouveaux facteurs cellulaires, comme CDKN2C, impliqués dans le cycle viral.

Pour conclure ce manuscrit, je tenais à préciser le contexte dans lequel cette thèse a été réalisée. Travaillant au sein de l'institut de recherche sur les maladies virales et hépatiques depuis 2010 en tant qu'assistante ingénieure de l'Université de Strasbourg, j'ai souhaité poursuivre mon cursus universitaire en parallèle de mes fonctions. Débutant par un Master Physiopathologie : de la Molécule à l'Homme, j'ai entrepris cette aventure de Doctorat en 2017. Forte de mes compétences et mon expertise sur les virus hépatiques - HCV, HBV et HDV – et les approches de génomique fonctionnelle, j'ai souhaité élargir mes domaines de compétence dans ce monde passionnant qu'est la recherche fondamentale.

Mes travaux de thèse ont permis de contribuer à l'assemblage des pièces du puzzle que sont les interactions entre le HBV et les hépatocytes. Les modèles cellulaires *in vitro* de l'infection que j'ai mis au point permettront, je l'espère, de découvrir de nombreux autres facteurs impliqués dans le cycle du HBV et potentiellement d'être au cœur du développement de nouvelles stratégies thérapeutiques.

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# ANNEXES

# ANNEXE 1

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

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REVIEW

# The functional role of sodium taurocholate cotransporting polypeptide NTCP in the life cycle of hepatitis B, C and D viruses

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## Abstract

Chronic hepatitis B, C and D virus (HBV, HCV and HDV) 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 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]. 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 target host cell factors required for viral replication. HTAs have been shown to be promising candidates for the prevention

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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 Fig. 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 cholestasis 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 Huh7 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 Fig. 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

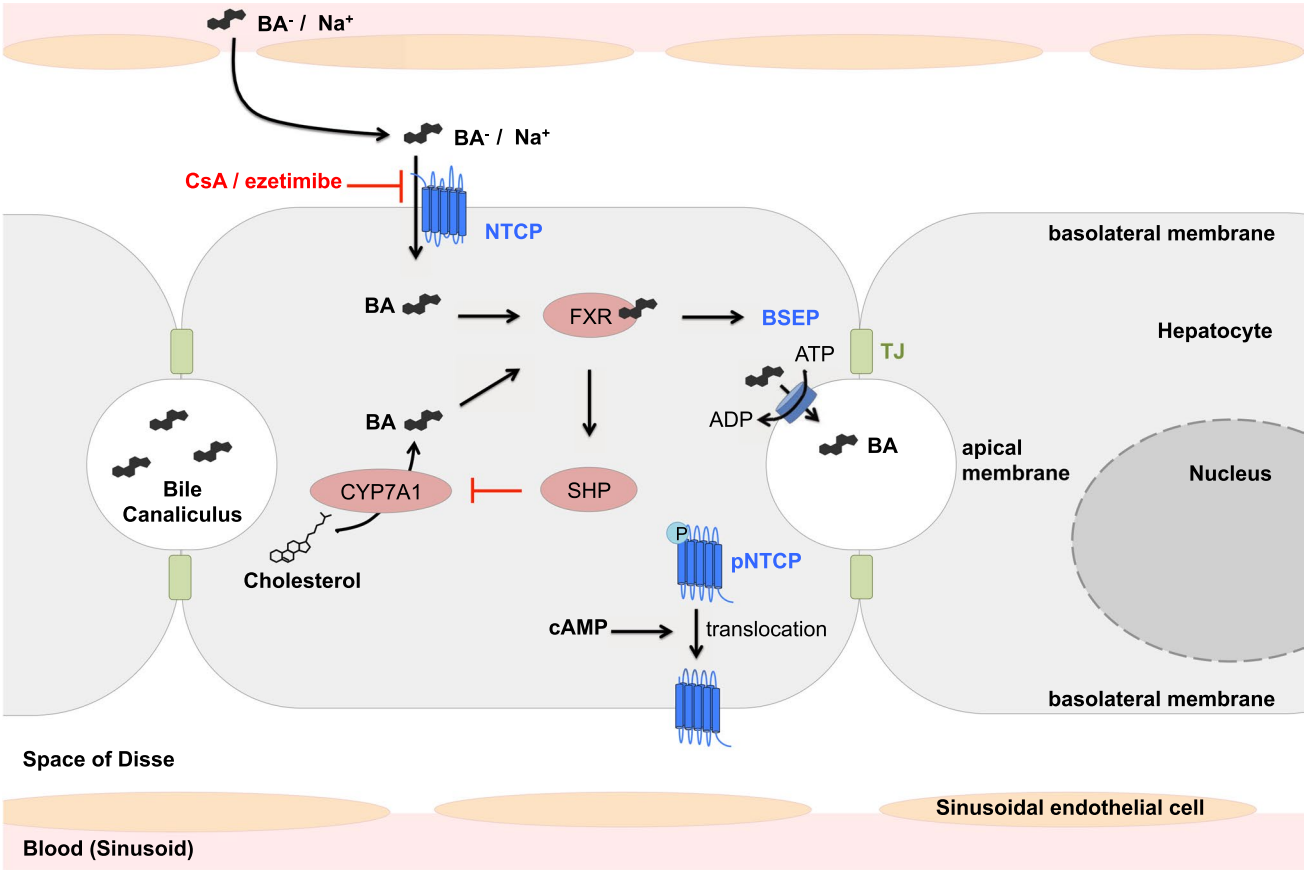


**Fig. 1** Model of the functional role of NTCP in hepatic bile acid transport and metabolism. Transport of bile acids from portal blood into hepatocytes via NTCP depends on a sodium gradient and is inhibited by CsA or ezetimibe. Secretion into the bile canaliculus via bile salt export pump (BSEP) in 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

L envelope protein is known to bind to the hepatocyte cell surface and is required for HBV and HDV entry [55]. The HBV capsid is comprised of HBV core protein (HBcAg) and encapsidates a partially double-stranded relaxed circular DNA (rcDNA) genome of 3.2 kilobases. Upon infection of hepatocytes, genomic rcDNA is converted into covalently closed circular DNA (cccDNA), a minichromosome-like structure that persists in the nucleus as a 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.

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 belangeri*)

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 2–78 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 Fig. 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 that did not affect 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, it was shown that

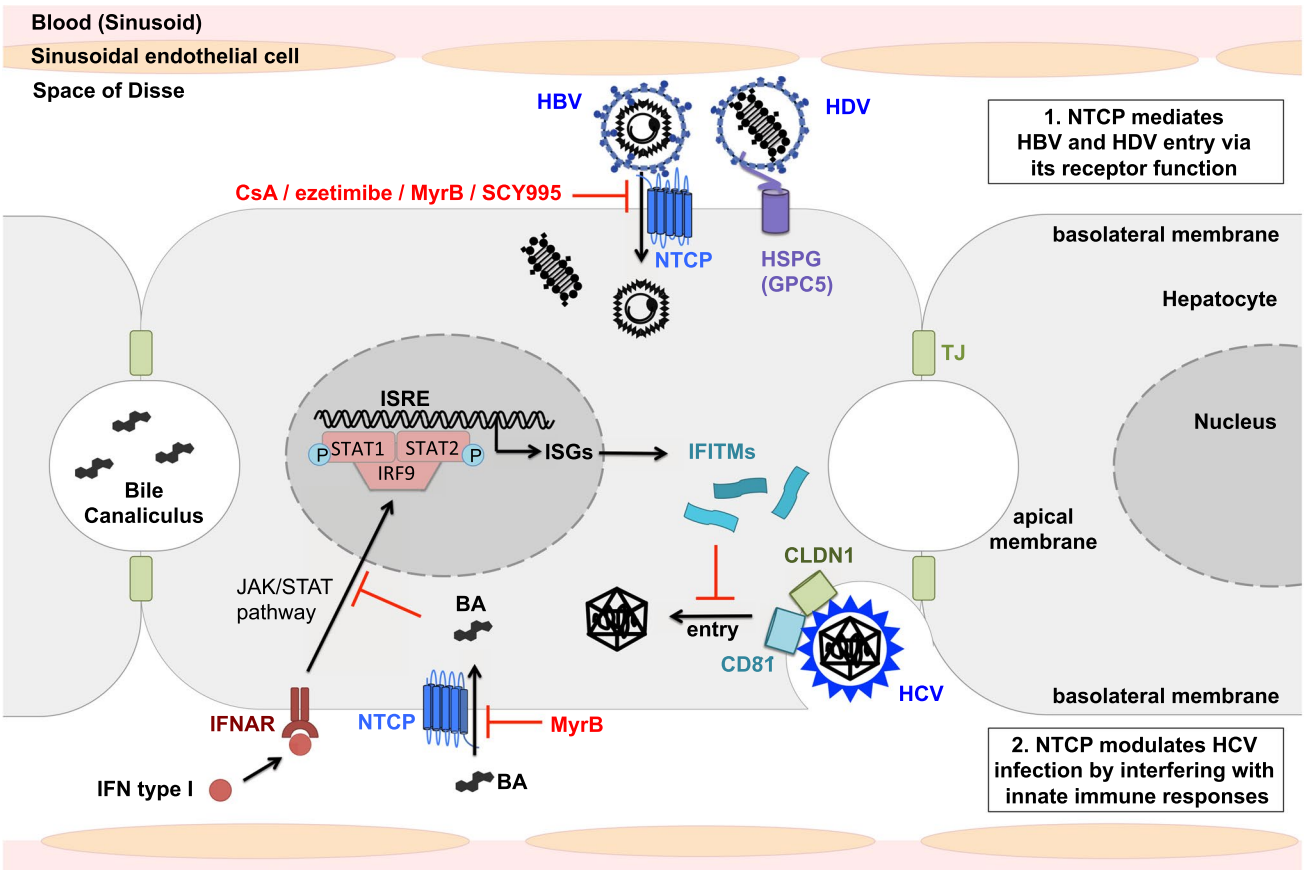


**Fig. 2** Model of interactions between NTCP and the entry of HBV, HDV, and HCV in hepatocytes. After initial attachment to HSPG including GPC5, HBV and HDV virions bind to the receptor NTCP through the preS1-domain of the large envelope protein. NTCP inhibitors CsA and ezetimibe block viral entry like 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 *IFITM*s. *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

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 inhibits 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].

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 chimpanzees and tree shrews can experimentally support HBV and HDV infections [87]. The state-of-the-art mouse model for the study of HBV/HDV 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 due to 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 Fig. 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 impairs 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 Fig. 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 identified a role for NTCP in HCV infection (see Fig. 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.

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## Compliance with ethical standards

**Conflict of interest** The authors have no conflicting interests to disclose.

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## ANNEXE 2

# *A targeted functional RNA interference screen uncovers glypican 5 as an entry factor for hepatitis B and D viruses*

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# A Targeted Functional RNA Interference Screen Uncovers Glypican 5 as an Entry Factor for Hepatitis B and D Viruses

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**Chronic hepatitis B and D infections are major causes of liver disease and hepatocellular carcinoma worldwide. Efficient therapeutic approaches for cure are absent. Sharing the same envelope proteins, hepatitis B virus and hepatitis delta virus use the sodium/taurocholate cotransporting polypeptide (a bile acid transporter) as a receptor to enter hepatocytes. However, the detailed mechanisms of the viral entry process are still poorly understood. Here, we established a high-throughput infectious cell culture model enabling functional genomics of hepatitis delta virus entry and infection. Using a targeted RNA interference entry screen, we identified glypican 5 as a common host cell entry factor for hepatitis B and delta viruses. *Conclusion*: These findings advance our understanding of virus cell entry and open new avenues for curative therapies. As glypicans have been shown to play a role in the control of cell division and growth regulation, virus–glypican 5 interactions may also play a role in the pathogenesis of virus-induced liver disease and cancer. (HEPATOLOGY 2016;36:35-48)**

See Editorial on Page 11

Hepatitis B virus (HBV) is a small, enveloped DNA virus[1] and a leading cause of liver cirrhosis and hepatocellular carcinoma (HCC) worldwide. More than 350 million individuals are chronically infected with HBV.[2] Among these, 5%-10% are likely coinfected with hepatitis delta virus (HDV) and exhibit an increased HCC risk.[3] HDV is a small RNA satellite virus of HBV that uses the HBV envelope proteins to assemble into infectious particles and enter its target cell.[4] Nucleos(t)ide analogues and interferon-based treatment can control HBV infection, but virus eradication and cure remain largely unattainable.[5] While HDV can partially respond to interferon-based treatment,[6] long-term response is marginal.[6,7]

*Abbreviations: CsA, cyclosporin A; DMSO, dimethylsulfoxide; dpi, day postinoculation; ELISA, enzyme-linked immunosorbent assay; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GPC, glypican; HBeAg, hepatitis B e antigen; HBsAg, HBV surface antigen; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HDAg, hepatitis delta antigen; HDV, hepatitis delta virus; HSPG, heparan sulfate proteoglycan; IF, immunofluorescence; IgG, immunoglobulin G; mRNA, messenger RNA; NTCP, sodium/taurocholate cotransporting polypeptide; PBS, phosphate-buffered saline; PEG, polyethylene glycol; PFA, paraformaldehyde; pgRNA, pregenomic RNA; PHH, primary human hepatocyte; pp, pseudoparticle; qRT-PCR, quantitative real-time polymerase chain reaction; RNAi, RNA interference; SDC, syndecan; siRNA, small interfering RNA.*

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Although many aspects of the HBV and HDV life cycles have been characterized in great detail, others, including viral entry, are still unexplored due to the lack of robust infectious tissue culture systems. Recently, the human sodium/taurocholate cotransporting polypeptide (hNTCP), a bile acid transporter expressed at the basolateral membrane of human hepatocytes, has been identified as a functional receptor of HBV and HDV.[8,9] Exogenous expression of hNTCP in permissive, nonsusceptible human hepatoma cells, such as HepG2 or Huh7, confers susceptibility to HBV and/or HDV infection, thereby constituting cell culture models for HBV/HDV entry.[8-10] However, their limited robustness and the requirement of dimethylsulfoxide (DMSO) and polyethylene glycol (PEG) preclude their use in a high-throughput format and for functional genomics.

Heparan sulfate proteoglycans (HSPGs), composed of a protein core carrying heparan sulfate glycosaminoglycan chains, are widely expressed at the cell surface of mammalian cells in a cell-specific and tissue-specific manner.[11] Many viruses use HSPGs for cell surface attachment and entry.[11,12] HSPGs contribute to HBV/HDV entry, as treatment of target cells or virions with heparinase or soluble heparin (a homologue of a highly sulfated prototype member of the glycosaminoglycan family), respectively, inhibits viral infection.[13,14] HBV is believed to first attach to HSPGs at the hepatocyte surface, through the antigenic loop of the HBV envelope protein[15] and possibly a contribution of the preS1 domain of the large HBV envelope protein.[13] Subsequently, HBV binding to NTCP is engaged by the preS1 domain, which interacts with a discrete domain of NTCP.[8,9] However, the detailed HBV/HDV entry mechanisms are still poorly understood.[10,16,17] Particularly, the HSPG core protein responsible for HBV/HDV attachment remains to be identified.

Viral entry is an important antiviral target complementing direct-acting antivirals targeting the viral polymerase. Indeed, a myristoylated HBV preS1-derived peptide (Myrcludex B) and cyclosporin A (CsA)[18,19] potently inhibit HBV and HDV entry,[17] and Myrcludex B has shown antiviral efficacy in humans.[20] Unraveling the HBV/HDV entry process may thus uncover further antiviral targets.

In this study, we established a robust HDV infection assay based on a novel NTCP-expressing Huh7 cell line that does not require the use of infection-enhancing treatments with DMSO and PEG. Using an RNA interference (RNAi)–based loss-of-function screen, we showed that this model system is amenable to functional genomics and identified glypican 5 (GPC5) as a previously undiscovered HBV and HDV entry factor and antiviral target.

## Materials and Methods

***Patient Samples.*** Human material including serum from patients with chronic HBV/HDV infection followed at the Strasbourg University Hospitals, Strasbourg, France, was obtained with informed consent. Primary human hepatocytes (PHHs) 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 10-17).

***Cell Lines and Human Hepatocytes.*** Huh7[21] and HepG2[22] cells have been described. PHHs were isolated and cultured as described.[22] The HepAD38 cell line is an inducible human hepatoblastoma cell line harboring an integrated tetracycline-responsive 1.2-fold HBV genome (serotype ayw, genotype D) and has been described.[23]

***Reagents.*** DMSO and PEG 8000 were from Sigma-Aldrich; CsA was from Sandimmun (Novartis, Switzerland). HBV preS1-derived peptide, HBV preS1-derived peptide labeled with Alexa Fluor 647 fluorophore, and scrambled peptide control were synthesized by Bachem (Switzerland). The enhanced chemiluminescence reagent and Hyperfilms were from GE Healthcare.

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***Small Interfering RNAs Used for Functional Studies.*** ON-TARGETplus small interfering RNA (siRNA) pools (Dharmacon) targeting the transcripts of SLC10A1 (NTCP), syndecan (SDC) family genes, GPC family genes, HSPG2 (perlecan), and agrin were reverse-transfected into cells with Lipofectamine RNAiMAX (Invitrogen) as described.[22] Subsequently, an individual siRNA of the pool (Dharmacon) targeting GPC5 was used: GPC5 no. 3, targeted sequence CUUCAAAC GUCCAGCUCUA.

***Establishment of Stable NTCP-Overexpressing Hepatoma Cell Lines.*** The NTCP expression vector was generated containing the full open reading frame of human NTCP complementary DNA (SLC10A1; GenBank L21893.1) inserted between the *Hind*III and *Xba*I restriction sites of the pRc-CMV plasmid (Invitrogen). Huh7 cells were cultured in William's medium E supplemented with 10% fetal bovine serum (Fetaclone-II; Thermo Scientific) and gentamicin. After transfection of Huh7 cells with pRc-CMV-NTCP plasmid DNA, transfected cells were selected for neomycin (G418) resistance, and isolated clones were tested for susceptibility to HDV infection as described.[24] One clone (referred to here as Huh-106) was selected based on the high level of intracellular HDV RNA that accumulated 9 days postinoculation (dpi) with HDV virions. Huh-106 cells were maintained at a concentration of 250 μg/mL G418. HepG2 cells were seeded in six-well plates at 50% confluence 1 day prior to transduction with human NTCP-expressing vesicular stomatitis virus pseudoparticles (pp) (GeneCopoeia). After 3 days, cells were expanded and selected for NTCP expression with 0.9 μg/mL puromycin. HepG2-NTCP cells were maintained at a concentration of 0.9 μg/mL puromycin.

***Analysis of Messenger RNA Expression by Quantitative Real-Time Polymerase Chain Reaction.*** Total RNA was extracted from cells using the RNeasy Mini Kit (Qiagen). Gene expression in the total RNA extracts was assessed using quantitative real-time polymerase chain reaction (qRT-PCR). Reverse-transcription of total RNA extracts was performed using MAXIMA reverse-transcriptase (Thermo Scientific). Quantitative PCR was performed using a Corbett rotor gene 6000 (Qiagen) following the manufacturer's instructions. Primers and TaqMan probes for NTCP, GPC5, SDC4, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) messenger RNA (mRNA) detection were from Applied Biosystems. All values were normalized to GAPDH expression.

***Protein Expression Analysis.*** Immunoblots of cell lysates using protein–specific antibodies (rabbit polyclonal anti-NTCP antibody [Sigma; HPA042727], rabbit anti-GPC5 monoclonal antibody [Abcam; EPR6756(B)], and mouse anti-$\beta$-actin monoclonal antibody [Sigma; A5441]) were performed following GE Healthcare protocols using Hybond-P membranes and visualized using enhanced chemiluminescence western blotting detection reagents following the manufacturer's instructions. For western blot detection of NTCP, cell lysates were pretreated with peptide-N-glycosidase (New England Biolabs, Evry, France) following the manufacturer's instructions. For fluorescent detection of NTCP protein in cells, an HBV preS1-derived peptide labeled with Alexa Fluor 647 fluorophore (Bachem, Switzerland) was used as described.[8,9] Cells were treated with the peptide for 1 hour at 37°C and then fixed with 4% paraformaldehyde (PFA). Fluorescent imaging was performed using an Axio Observer Z1 microscope (Carl Zeiss, Germany). NTCP expression at the cell surface was quantified by flow cytometry as described.[8,22]

***HDV Production and Infection.*** The HDV recombinant plasmid pSVLD3 and the HBV expression vector pT7HB2.7 were used for production of HDV ribonucleoprotein and of L, M, and S HBV surface antigen (HBsAg) proteins.[25] Huh7.5.1 cells were transfected with the two plasmids using FuGENE HD Transfection Reagent. HDV-containing supernatants were harvested 9 days after transfection and subsequently used for infection experiments. For HDV infection, Huh-106 cells were plated in 96-well or 384-well plates and maintained in culture for 1 day without DMSO. HBV preS1-derived peptide or scrambled peptide control (control), CsA, rabbit anti-GPC5 antibody, rabbit anti-SDC4 antibody (Life Technologies; 36-3100), and rabbit control immunoglobulin G (IgG; Invitrogen) were added to the cell medium 1 hour before infection with recombinant HDV with or without PEG at the indicated concentrations. Cells were cultured in primary hepatocyte maintenance medium[8] containing 2% DMSO following infection to slow cell growth. HDV infection was assessed 7 dpi by immunofluorescence (IF) or qRT-PCR.

***Detection of Hepatitis Delta Antigen and HDV RNA in Infected Cells.*** Cells were washed with phosphate-buffered saline (PBS) and fixed with 4% PFA. Nonspecific binding sites were saturated using 0.5% bovine serum albumin, and cells were permeabilized using 0.05% saponin. Cells were stained with an antibody targeting the hepatitis delta antigen (HDAg) purified from serum of an HBV/HDV coinfected patient[26] and Alexa Fluor 647-labeled secondary antibody targeting human IgGs (Jackson Research). Cell nuclei were stained with 4′,6-diamidino-2-phenylindole. Fluorescent imaging was performed using an Axio Observer Z1 microscope (Carl Zeiss, Germany). Total RNA extraction and reverse-

transcription were performed as described above. Quantitative PCR was performed using the SensiFAST Probe No-ROX Kit (Bioline) and Corbett rotor gene 6000 (Qiagen) following the manufacturer's instructions. The following specific primers for HDV RNA quantification were used: forward primer 5′-GCATGGTCCCAGCCT CC-3′, reverse primer 5′-CTTCGGGTCGGCATGG-3′; TaqMan probe 5′-[fluorescein amidite]-ATGCCCAGGT CGGAC-[Black Hole Quencher-1]-3′. All values were normalized to GAPDH (Applied Biosystems) expression.

***HBV Production and Infection.*** HBV infectious particles from sera of HBV carriers (genotype D) were concentrated with a 30% sucrose cushion and purified using a 10%-45% iodixanol density gradient. Recombinant HBV (strain ayw, genotype D) was obtained by 100-fold concentration of supernatant of HepAD38 cells using 8% PEG.[23] HepG2-NTCP cells were plated in 96-well plates and maintained in culture for 1 day without DMSO prior to HBV infection in the presence of 4% PEG. Cells were cultured in 2% DMSO primary hepatocyte maintenance medium following infection to slow cell growth. HBV infection was assessed 10 dpi by immunodetection of HBsAg using an HBsAg-specific monoclonal antibody (NCL-HBsAg-2, clone 1044/341; Leica Biosystems) and by qRT-PCR quantification of HBV pregenomic RNA (pgRNA) using the following primers and probe[27]: forward primer 5′-GGTCCCCT AGAAGAAGAACTCCCT-3′, reverse primer 5′-CATT GAGATTCCCGAGAT TGAGAT-3′; TaqMan probe 5′-[6-fluorescein amidite]-TCTCAATCGCCGCGTC GCAGA-[carboxytetramethylrhodamine]-3′. All values were normalized to GAPDH (Applied Biosystems) expression.

***Analysis of HBV and HDV Binding to Liver Cells.*** Huh-106 and HepG2-NTCP cells were incubated for 1 hour at 37°C with a GPC5-specific or control antibody at various time points before and after virus inoculation. Recombinant HBV was pretreated with PBS, heparin (30 μg/mL), rGPC5 (5 μg/mL and 30 μg/mL; R&D Systems), or rSDC4 (30 μg/mL; R&D Systems) for 30 minutes at 37°C. Cells were washed twice with cold PBS and then incubated with pretreated HBV or HDV in the presence of 4% PEG for 24 hours at 16°C. Unbound virions were removed by three washes with PBS, and cells and bound virions were lysed. A 24-hour incubation period was chosen based on previous observations showing the requirement of a >16-hour virus/cell exposure[28,29] and given that transcription from incoming viral DNA and *de novo* production of virus are not detectable prior to 2 and 3 dpi, respectively.[29] Therefore, the HBV DNA that is associated with cells at 24 hours postinoculation represents HBV particles that have attached to the cells during this time period and potentially virions that have been internalized following attachment, but does not include progeny virions. DNA was extracted from HepG2-NTCP cells according to the QiaAMP DNA MiniKit protocol (Qiagen). HBV total DNA was detected by qPCR using the following primers and probe[27]: forward primer 5′-CA CCTCGCCTAATCATC-3′, reverse primer 5′-GGAAA GAAGTCAGAAGGCA-3′; TaqMan probe 5′-[fluorescein amidite]-TGGAGGCTTCAACAGTAGGACATGAA C-[carboxytetramethylrhodamine]-3′. A standard curve generated from known HBV genome copies was used for quantification. Values were normalized to initial DNA concentration. Total RNA was extracted from Huh-106 cells and HDV RNA was quantified as described above.

***Analysis of HBV Replication in Huh-106 Cells With Silenced GPC5 Expression.*** Huh-106 cells were reverse-cotransfected with the replication-competent HBV expression plasmid adwR9[30] and GPC5-targeting or control siRNAs using Lipofectamine RNAiMAX (Invitrogen). Three days after transfection, HBV DNA was purified and quantified from lysates by qPCR as described above. Hepatitis B e antigen (HBeAg) and HBsAg in cell culture supernatants were quantified using enzyme-linked immunosorbent assays (ELISAs) N0140 and N0019 (Diasorin). Cytoplasmic HBV core antigen was quantified using a sandwich ELISA as described.[31]

***Statistical Analysis.*** All experiments were performed at least three times in an independent manner. Statistical analyses were performed using a Student *t* test or Mann-Whitney test; $P < 0.01$ was considered statistically significant. Significant *P* values are indicated by asterisks in the individual figures.

## Results

***A Novel hNTCP-Overexpressing Huh7 Cell Line Readily Susceptible for HDV Infection.*** Aiming to develop a high-throughput system for functional genomics, we established a Huh7-NTCP cell line in which constitutive hNTCP expression is under the control of a cytomegalovirus promoter. Out of 120 single-cell clones, one, termed Huh-106, was found reproducibly susceptible to HDV infection irrespective of the culture conditions. Huh-106 cells express significantly higher levels of NTCP (mRNA and protein) than the parental Huh7 cells (Fig. 1A,B). Moreover, an AF647-labeled peptide derived from the HBV preS1 domain known to bind NTCP,[8,9] specifically bound to Huh-106 cells, demonstrating high levels of cell surface NTCP expression (Fig. 1C,D). To confirm NTCP function, Huh-106 cells were inoculated with recombinant HDV. Indeed, Huh-106 cells were susceptible to HDV entry,

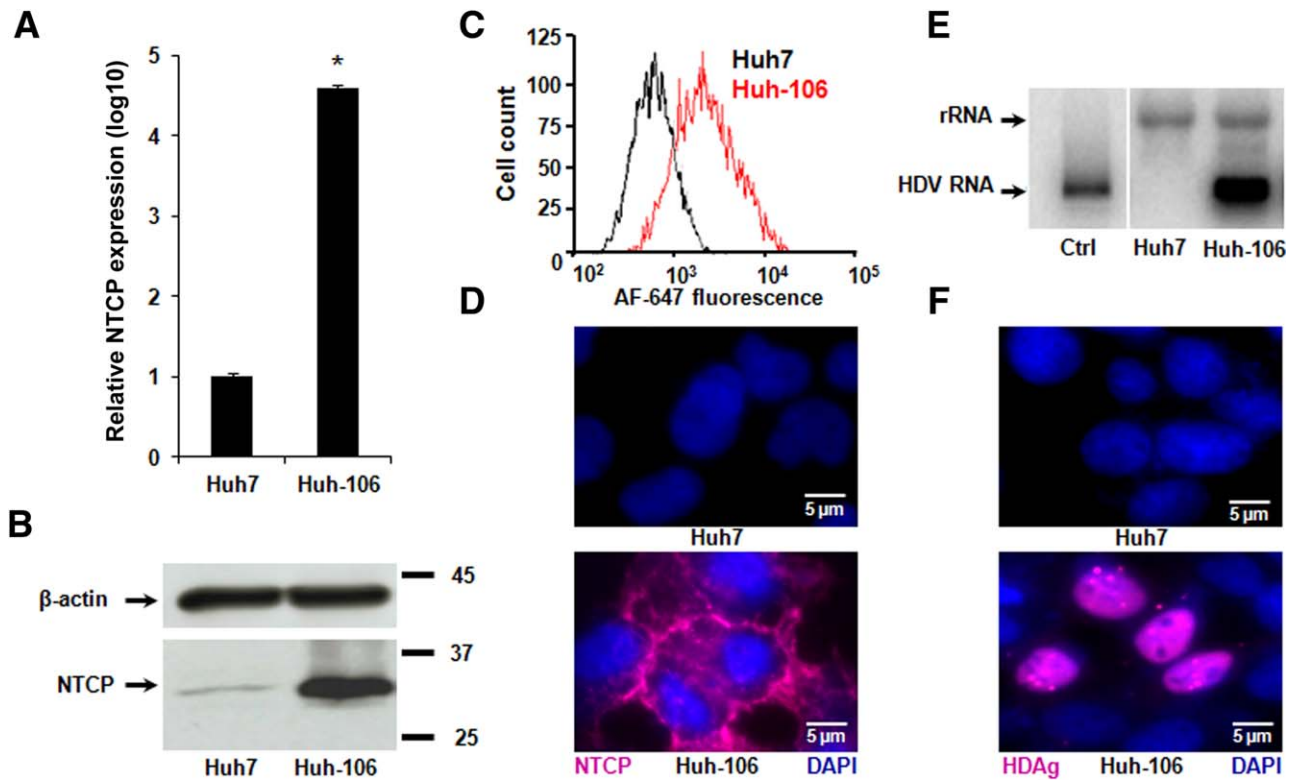



Fig. 1. Production of HDV permissive cell lines overexpressing human NTCP. (A) Relative NTCP expression in Huh-106 cells compared to parental Huh7 cells using qRT-PCR. Results are expressed as means ± standard deviation showing NTCP mRNA expression normalized to GAPDH mRNA expression from three experiments performed in duplicate. (B) Detection of NTCP protein by western blot. Cell lysates were treated with peptide-N-glycosidase and subsequently subjected to immunoblotting using a mouse monoclonal anti-NTCP antibody (Sigma). One representative experiment is shown. (C) Quantification of NTCP at the cell surface by flow cytometry using a labeled HBV preS1-derived peptide. Cells were treated for 1 hour with Alexa Fluor 674–labeled HBV preS1-derived peptide (200 nM) and fixed with 4% PFA. NTCP expression was assessed by flow-cytometric quantification of bound preS1 peptide. One representative experiment is shown. (D) Fluorescence microscopic analysis of NTCP expression in Huh7 and Huh-106 cells. Cells were treated with Alexa Fluor 674–labeled HBV preS1-derived peptide (200 nM) and fixed with 4% PFA before visualization using fluorescence microscopy. HBV preS1-derived peptide binding is shown in red. One representative experiment is shown. (E,F) Functional evaluation of NTCP cell lines using HDV infection. Huh-106 and Huh7 cells were infected with recombinant HDV for 7 days. (E) Total RNA was purified and HDV RNA was detected by northern blot. Control corresponds to approximately $2 \times 10^7$ HDV RNA genome equivalents extracted from HDV particles produced in Huh7 cells. One representative experiment is shown. (F) HDV-positive cells were visualized in red after HDAg immunodetection with an anti-HDAg antibody. Cell nuclei were stained with 4′,6-diamidino-2-phenylindole (blue). One representative experiment is shown. Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole; rRNA, ribosomal RNA.

as demonstrated by detectable intracellular HDAg and HDV RNA (Fig. 1E,F) at 7 dpi.

Most current *in vitro* HBV/HDV infection systems require the addition of PEG to the inoculum to increase infection.[8,9,29] PEG may modulate viral entry by bypassing virus–host factor interactions. To assess whether PEG increases viral entry in our model, we inoculated Huh-106 cells with HDV in the presence of increasing concentrations of PEG. Approximately 15%-25% of Huh-106 cells were infected in the absence of PEG treatment compared to 30% of HDAg-positive cells after 6% PEG treatment (Fig. 2A,B). Notably, a two-fold increase of HDV-positive cells is observed in the presence of 8% PEG but with significant toxicity (Fig. 2B). Silencing of NTCP expression with siRNA prior to inoculation (Fig. 2C) led to a significant decrease (from 22% to 7%) of HDV-positive cells when cells were inoculated in the absence of PEG. In contrast, in the presence of 6% PEG, only a slight but insignificant decrease of infection was observed (Fig. 2D), suggesting that HDV infection in the absence of PEG is more susceptible to RNAi-based perturbation studies. These data indicate that our model efficiently supports HDV infection in the absence of PEG.

Treatment of cell cultures with DMSO prior to inoculation has also been shown to improve HBV and HDV infection, possibly by modifying NTCP trafficking.[8,9,32] When Huh-106 cells were treated for 2 days with 2% DMSO prior to inoculation (Fig. 2E), no change in NTCP localization at the cell surface or in HDV infection was observed. Taken together, our data show that the Huh-106 cells are readily susceptible to HDV infection in the absence of PEG and DMSO and, hence, in conditions amenable to functional genomics and RNAi-based loss of function studies.

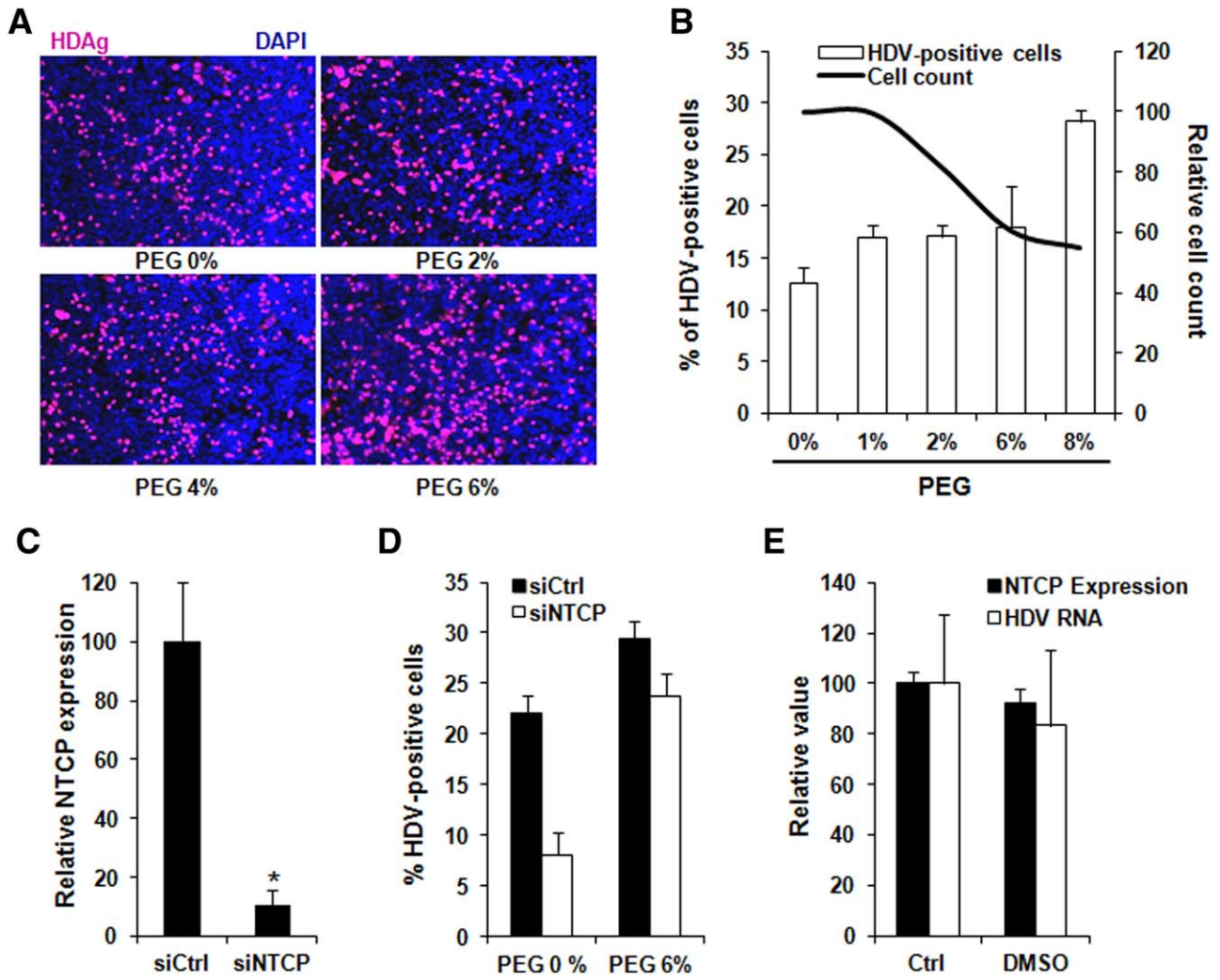



Fig. 2. HDV infection in Huh-106 cells is PEG-independent and DMSO-independent. (A) The effect of PEG on HDV infection. Huh-106 cells were infected with HDV in the presence of increasing concentrations of PEG (0%, 2%, 4%, 6%). Infection was assessed 7 dpi by IF. (B) Effect of PEG on cell viability. Huh-106 cells were infected with HDV in the presence of increasing concentrations of PEG. Infection was measured 7 dpi by quantification of HDV-positive cells. Results are expressed as mean ± standard deviation percentage of HDV-infected cells from one representative experiment performed in triplicate. The number of cells 7 dpi was evaluated by quantification of viable 4′,6-diamidino-2-phenylindole–stained nuclei. Results are expressed as means ± standard deviation percentage of cells relative to cells infected in the absence of PEG from one representative experiment performed in triplicate. (C,D) Effect of PEG treatment on silencing of NTCP expression. NTCP expression was silenced in Huh-106 cells. Two days posttransfection silencing efficacy was assessed by qRT-PCR (C), and cells were infected with HDV in the presence or absence of 6% PEG. Infection was measured 7 dpi by quantification of HDV-positive cells (D). Results are expressed as means ± standard deviation percentage of infected cells from three independent experiments performed in triplicate. (E) Effect of DMSO treatment on NTCP expression and HDV infection. Huh-106 cells were treated for 2 days with 2% DMSO. NTCP expression at the cell surface was assessed by flow cytometry as described in Fig. 1. Cells were then infected with HDV, and infection was assessed 7 dpi by quantification of HDV RNA. Results are expressed as means ± standard deviation percentage NTCP expression at the cell surface or percentage HDV infection relative to control from three independent experiments performed in triplicate. Abbreviation: DAPI, 4′,6-diamidino-2-phenylindole.

***Development of a High-Throughput System for Functional Perturbations of the HDV Life Cycle.*** To establish a high-throughput model enabling perturbation studies of the HDV life cycle, we applied an automated imaging system for detection of viral infection using a 96-well or a 384-well plate format and immunodetection of intracellular HDAg. As HDAg localizes in Huh7 cells predominantly to the nucleus (Fig. 1F), a simple 4′,6-diamidino-2-phenylindole–HDAg costaining allows for discrimination of infected versus noninfected cells. To determine the best conditions for infection assay, HDAg detection was performed at 5, 7, and 9 dpi. While about 5% of cells were HDAg-positive at 5 dpi, 20% were positive at 7 dpi (Fig. 3A-D), and no further increase was observed at 9 dpi (Fig. 3A). Furthermore, the level of intracellular HDV RNA was measured at 5, 7, and 9 dpi. A significant increase was observed between 5 and 7 dpi, and no further increase was observed at 9 dpi (Fig. 3C).

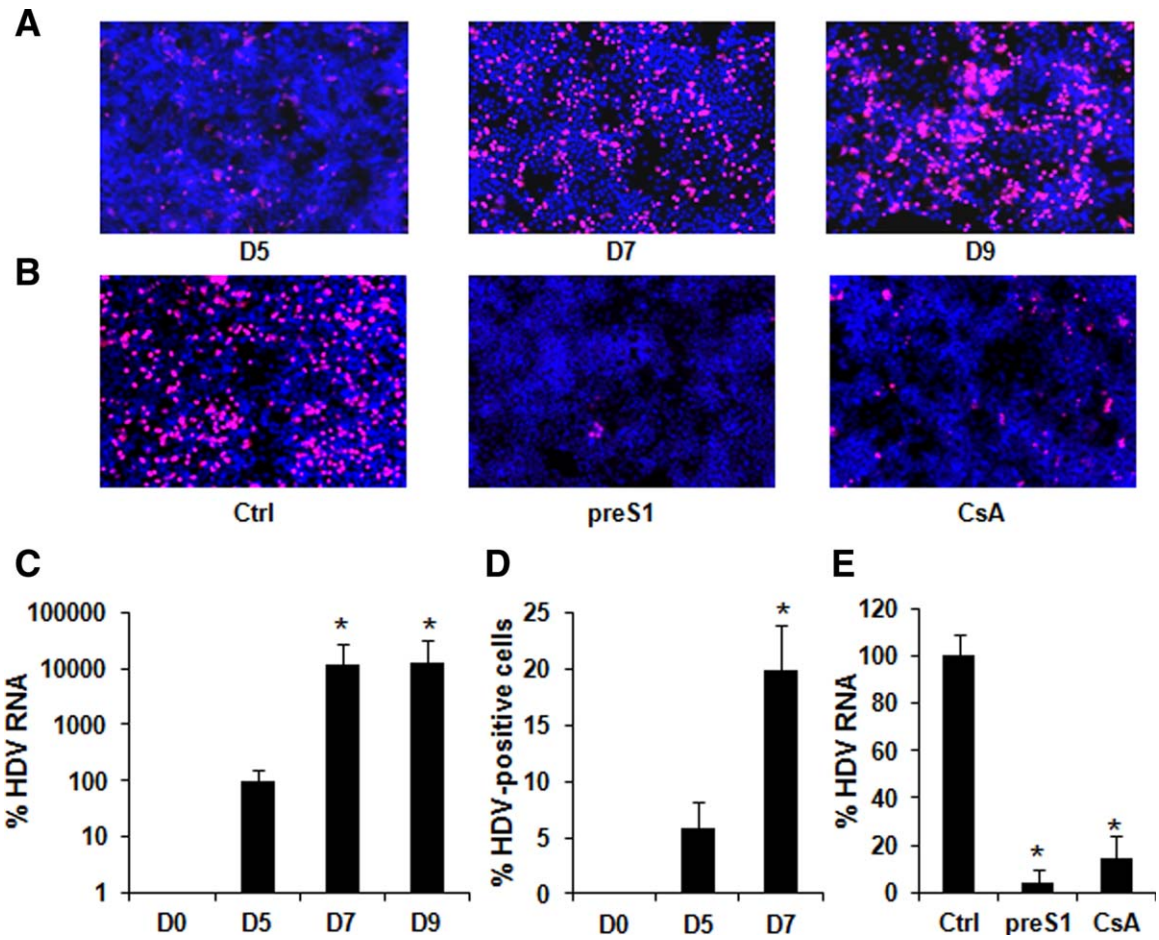
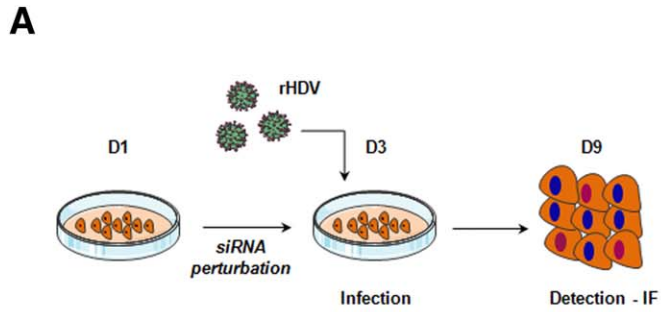



Fig. 3. A high-throughput assay for drug screening and functional genomics of HDV infection using Huh-106 cells. (A,C,D) HDV time-course of infection. Huh-106 cells were infected in 96-well (A,C) and 384-well (D) plates with HDV for 5, 7, and 9 days. (A,D) Infection was assessed by quantification of HDAg-positive cells after HDAg immunodetection. One representative experiment (A) and means ± standard deviation percentage HDV-positive cells from three independent experiments performed in triplicate (D) are shown. (C) Total RNA was extracted after 5, 7, and 9 dpi; and HDV infection was assessed by qRT-PCR quantification of HDV RNA normalized to GAPDH mRNA. Results are expressed as means ± standard deviation relative to the HDV RNA level at 5 dpi, set at 100% from three experiments performed in triplicate. (B,E) Inhibition of HDV infection using NTCP inhibitors. Cells were treated for 1 hour with HBV-derived preS1 peptide (200 nM) or with CsA (8 μM) and subsequently infected with HDV for 7 days. Infection was assessed by HDAg immunodetection (B) or qRT-PCR quantification of HDV RNA (E). Results are expressed as means ± standard deviation percentage HDV infection from three independent experiments performed in triplicate.

According to these data, we developed a functional high-throughput HDV infection protocol by infecting Huh-106 cells in 96-well or 384-well plates with HDV (without PEG and DMSO preincubation) for 7 days before quantification of HDAg-positive cells using IF. To ascertain the robustness of this model to monitor inhibition of HDV infection, we silenced NTCP expression using siRNA and incubated Huh-106 cells with NTCP inhibitors. As shown by a marked decrease in the number of HDAg-positive cells (Fig. 3B) and HDV RNA levels (Fig. 3E), HDV infection was impaired by preincubation with an HBV preS1-derived peptide or CsA, which are known to block NTCP function,[8,9,18,33] and following silencing of NTCP expression (Fig. 2D). Collectively, these data demonstrate that our model is suitable for high-throughput perturbation screens.

***A Targeted RNAi Entry Screen Identifies GPC5 as a Host Cell Entry Factor.*** We next applied this cell-based model for a targeted RNAi HDV entry screen (Fig. 4A). Given the importance of HSPGs for HDV/HBV attachment and entry, we investigated their role in HDV infection by silencing the expression of all genes belonging to the HSPG families (Fig. 4B). Huh-106 cells were transfected with pools of four siRNAs 2 days before exposure to HDV for 24 hours (Fig. 4A). Only silencing of GPC5 expression inhibited HDV infection, leading to a 45% decrease in the number of HDAg-positive cells at 7 dpi (Fig. 4B). Nonspecific toxic effects were excluded by two independent cell viability assays (Supporting Fig. S1). To validate the role of GPC5 in HDV entry, we performed additional silencing studies using individual GPC5-specific siRNAs. A 50%




B

| Gene symbol | Gene - family | HDV-positive cells (% relative to Ctrl) | Cell count (% relative to Ctrl) |
|---|---|---|---|
| Ctrl | | 100.0 ± 1.1 | 100.0 ± 1.4 |
| SDC1 | Syndecan | 91.3 ± 3.3 | 105.6 ± 1.9 |
| SDC2 | | 75.4 ± 0.9 | 118.0 ± 3.9 |
| SDC3 | | 81.2 ± 2.1 | 93.0 ± 11.0 |
| SDC4 | | 87.0 ± 0.4 | 118.2 ± 4.1 |
| GPC1 | Glypican | 83.3 ± 1.5 | 111.0 ± 1.4 |
| GPC2 | | 80.2 ± 1.4 | 115.4 ± 5.4 |
| GPC3 | | 88.6 ± 1.6 | 99.7 ± 0.4 |
| GPC4 | | 83.4 ± 1.5 | 115.6 ± 8.4 |
| **GPC5** | | **55.7 ± 3.3** | **98.7 ± 5.9** |
| GPC6 | | 89.0 ± 1.6 | 110.9± 4.0 |
| HSPG2 | Perlecan | 117.5 ± 2.1 | 81.7 ± 6.9 |
| AGRN | Agrin | 95.6 ± 1.5 | 108.8 ± 9.5 |

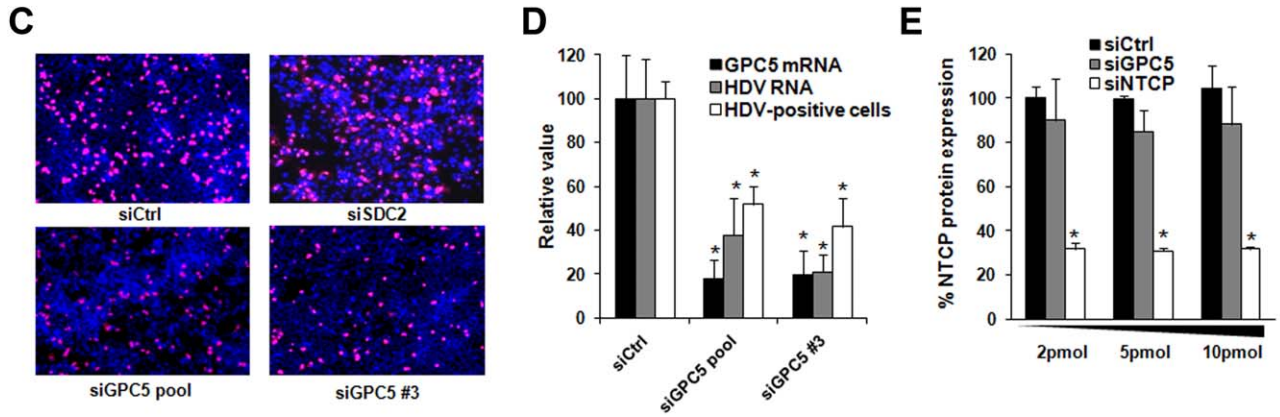



Fig. 4. A targeted functional RNAi HDV entry screen uncovers GPC5 as an HDV entry factor. (A) Flowchart of the siRNA screen. Huh-106 cells were reverse-transfected with siRNAs 48 hours prior to infection with HDV for 24 hours. HDV infection was measured 7 dpi by IF and quantification of HDV-positive cells. (B) Effect of HSPG gene silencing on HDV infection. Cells were transfected with siRNAs targeting the transcripts of genes belonging to members of different HSPG families and then infected as described in Fig. 3. Results are expressed as means ± standard deviation percentage HDV-positive cells or percentage cell count relative to control siRNA transfected cells from one experiment performed in triplicate. (C,D) Confirmation of GPC5 involvement in HDV infection using an individual siRNA. Huh-106 cells were silenced using an SDC2-specific siRNA pool, a GPC5-specific siRNA pool, or the individual GPC5-specific siRNA #3 for 48 hours and then infected with HDV. Infection was revealed 7 dpi by IF (C), quantification of HDV-positive cells, and qRT-PCR quantification of HDV RNA (D). Silencing efficacy was assessed by qRT-PCR (D). Results are expressed as means ± standard deviation percentage mRNA expression, HDV-positive cells, or HDV RNA from three independent experiments performed in triplicate. (E) Effect of GPC5 silencing on NTCP expression at the cell surface. Huh-106 cells were silenced for NTCP of GPC5 expression for 2 days using increasing quantities of siRNA. NTCP expression at the cell surface was assessed by flow cytometry. Results are expressed as means ± standard deviation percentage NTCP expression from three independent experiments performed in triplicate.

decrease in the number of HDAg-positive cells and a strong reduction of HDV RNA were observed after transfection of Huh-106 cells with GPC5 siRNA #3, similar to the siRNA pool (Fig. 4C,D). In contrast, only a minimal effect on HDV infection was observed after silencing of SDC2, an HSPG that was not identified in the RNAi screen. To verify that GPC5 silencing did not simply affect NTCP expression, we evaluated NTCP expression in GPC5-silenced Huh-106 cells. NTCP expression at the cell surface was not affected by GPC5 silencing (Fig. 4E). These data identify GPC5 as an HDV entry factor candidate.

***GPC5 Mediates HDV Entry Into Human Hepatocytes.*** To validate the role of GPC5 as an HDV entry factor, we performed mechanistic studies using an antibody targeting GPC5. The GPC5-specific antibody inhibited HDV infection of Huh-106 cells in a dose-dependent manner, contrary to an antibody targeting SDC4, another HSPG expressed in the liver and involved in hepatitis C virus (HCV) attachment[34] but not identified in our RNAi screen (Fig. 5B). Finally, we validated the functional role of GPC5 as an HDV entry factor in PHHs, the natural target cells of HDV, which express high levels of GPC5 protein (Fig. 5A). PHH cultures were silenced for GPC5 expression using siRNAs (Fig. 5C), inoculated with HDV before assessing viral infection using IF. In contrast to cell lines, HDAg was detected predominantly in the cytoplasm of PHHs. Silencing of NTCP and GPC5 similarly resulted in a marked decrease of HDAg-positive cells (Fig. 5D),

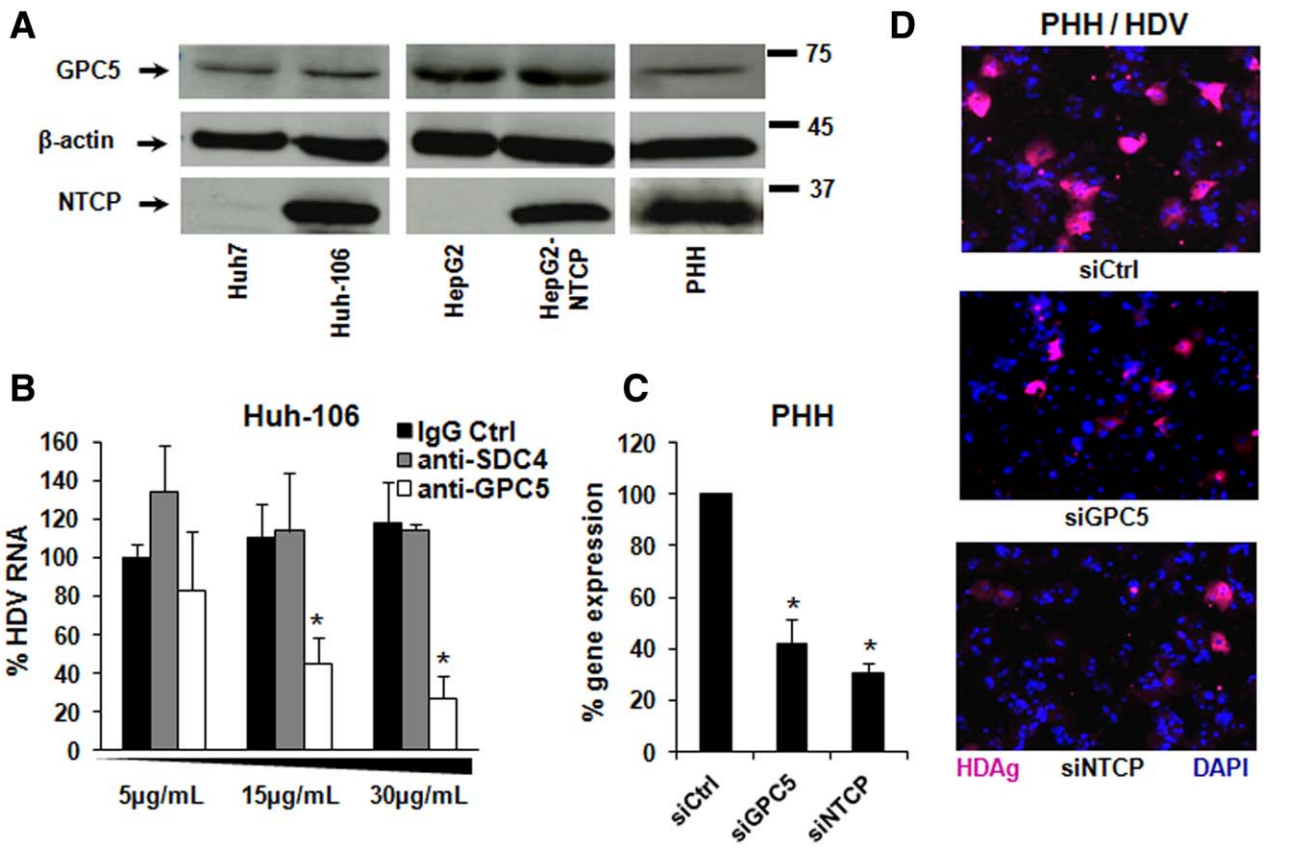



Fig. 5. GPC5 is a specific HDV entry factor in hepatoma cells and human hepatocytes. (A) GPC5 and NTCP protein expression in different cell culture models assessed by western blot. (B) Inhibition of HDV infection using anti-GPC5 antibody. Huh-106 cells were treated for 1 hour with an antibody targeting GPC5, an antibody targeting SDC4, or a control IgG at different concentrations. Cells were then infected with HDV in the presence of the antibody. Infection was assessed 7 dpi by qRT-PCR quantification of HDV RNA. Results are expressed as means ± standard deviation percentage HDV infection from three independent experiments performed in triplicate. (C,D) GPC5 silencing inhibits HDV infection in PHHs. PHHs were transfected with siRNAs targeting GPC5 or NTCP 2 days prior to infection with HDV for 7 days. Silencing efficacy was assessed by qRT-PCR of GPC5 and NTCP mRNA (C), and HDV infection was assessed by IF as described in Fig. 1 (D). One representative experiment is shown. Abbreviation: DAPI, 4′,6-diamidino-2-phenylindole.

in agreement with a functional role of GPC5 as an entry factor for HDV infection of human hepatocytes.

***GPC5 Mediates HBV, but not HCV, Entry.*** Because HBV and HDV are believed to use the same receptors and early steps of viral entry, we next evaluated whether GPC5 is also an entry factor for HBV. Because HepG2 cells were shown to be better adapted to HBV infection assays than Huh7 cells,[8,9] we established an HepG2-NTCP cell line for productive HBV infection, as confirmed by immunodetection of HBsAg and HBV-specific pgRNA postinoculation (Supporting Fig. S2). The specificity of RT-PCR for pgRNA, but not viral DNA, was confirmed by the lack of signal upon prior DNAse treatment (Supporting Fig. S2). Inhibition of HBV infection using a preS1-derived peptide (Supporting Fig. S2) and time-course experiments demonstrating *de novo* synthesis of HBeAg (assessed by ELISA), HBsAg (assessed by IF), covalently closed circular DNA (assessed by qPCR), and HBV RNA (assessed by qRT-PCR of HBV pgRNA or total HBV RNA using RNA-seq) confirmed that the signals were from *de novo* synthesis and not from incoming virions (Supporting Fig. S3).

To determine whether GPC5 is also required for HBV infection, GPC5 expression was silenced in HepG2-NTCP cells. Silencing of GPC5 expression decreased HBV infection, as measured by the number of HBsAg-positive cells and the level of intracellular HBV pgRNA at 10 dpi, similar to observations following NTCP silencing (Fig. 6A-D). To assess whether GPC5 is a specific entry factor for HBV and HDV or may also contribute to HCV entry, we studied its functional role for HCV entry using HCVpp. The silencing of GPC5 expression does not affect HCVpp entry (Fig. 6E,F), indicating that GPC5 specifically mediates HBV and HDV entry into hepatocytes.

***GPC5 Mediates Attachment of the Virion to the Target Cell Surface.*** To investigate the steps of the HBV entry process involving GPC5, we tested its role in

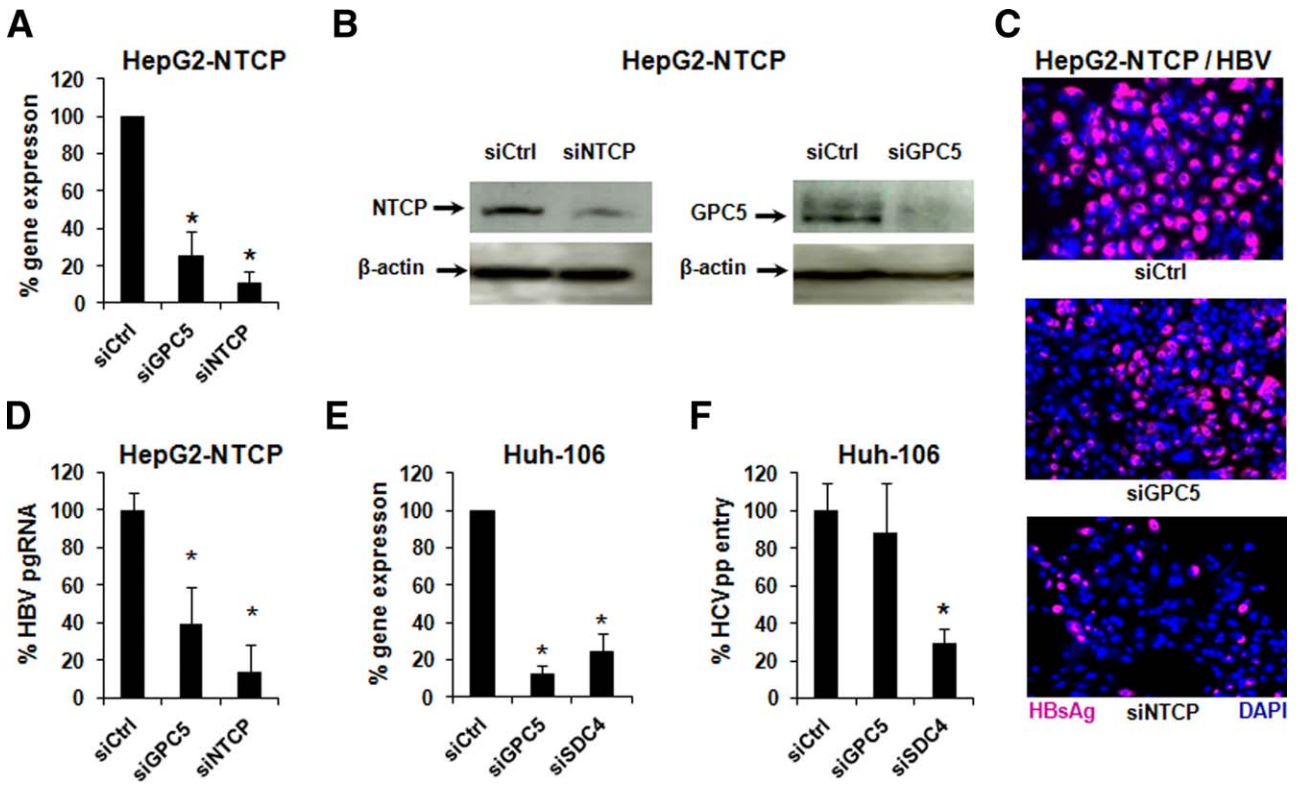



Fig. 6. GPC5 is an HBV host entry factor. (A-D) GPC5 silencing inhibits HBV infection in HepG2-NTCP cells. HepG2-NTCP cells were transfected with siRNAs targeting GPC5 and NTCP. Silencing efficacy was assessed after 2 days by qRT-PCR (A, means ± standard deviation percentage mRNA expression normalized to GAPDH mRNA expression from three independent experiments performed in duplicate) and western blot (B, one representative experiment is shown). Cells were then infected by HBV for 10 days, and infection was detected by IF (C, one representative experiment is shown) and qRT-PCR quantification of HBV pgRNA (D). Results are expressed as means ± standard deviation percentage HBV pgRNA levels from three independent experiments performed in triplicate. (E,F) GPC5 silencing does not affect HCVpp entry. Huh-106 cells were transfected with siRNAs targeting GPC5 and SDC4 48 hours prior to infection with HCVpp genotype 1b for 3 days. Silencing efficacy was assessed by qRT-PCR (E). HCVpp entry was measured by quantification of luciferase activity (F). Results are expressed as means ± standard deviation percentage HCVpp entry relative to control siRNA transfected cells from three independent experiments performed in triplicate. Abbreviation: DAPI, 4′,6-diamidino-2-phenylindole.

HBV binding to HepG2-NTCP cells. Similar to pretreatment of virions with heparin, which is known to inhibit HBV-HSPG binding, silencing of GPC5 expression significantly decreased HBV binding (Fig. 7A), strongly suggesting that GPC5 is a major player in HBV/HDV attachment at the cell surface of hepatocytes. To ascertain the ability of GPC5 to interact with the HBV envelope, we performed a competition experiment in which HBV was pretreated with soluble recombinant GPC5 protein (rGPC5). Recombinant GPC5 neutralized HBV at concentrations similar to heparin, unlike soluble recombinant SDC4 (rSDC4) (Fig. 7B). Moreover, pretreatment of Huh-106 and HepG2-NTCP cells with an anti-GPC5 antibody inhibited HDV and HBV binding, respectively (Fig. 7C). However, HBV binding was no longer inhibited when the anti-GPC5 antibody or recombinant protein was added 12 hours after virus inoculation (Fig. 7D; Supporting Fig. S4), confirming that GPC5 plays a role in the early steps of virus entry. Furthermore, to rule out that GPC5 inhibits HBV infection by interfering with other steps of the HBV life cycle, we studied the effect of GPC5 silencing on HBV replication in stably HBV-replicating HepAD38 cells.[23] Neither HBsAg nor HBV pgRNA was affected by silencing of GPC5 expression, suggesting that GPC5 has no functional role in HBV replication (Fig. 8A,B). To confirm that GPC5 has no effect on HBV replication when HBV DNA and GPC5 siRNA are present in the same cells, Huh-106 cells were cotransfected with siRNA and a plasmid encoding the HBV genome prior to measurement of soluble HBsAg and HBeAg in cell culture supernatants and quantification of viral capsids (by HBV core antigen ELISA) and viral DNA (by qPCR) in cytoplasmic lysates. Using this approach, HBV DNA and protein levels were not affected by GPC5 silencing (Fig. 8C,D,E). Collectively, these data demonstrate that GPC5 contributes to viral entry by mediating the initial attachment of HBV to its target cell.

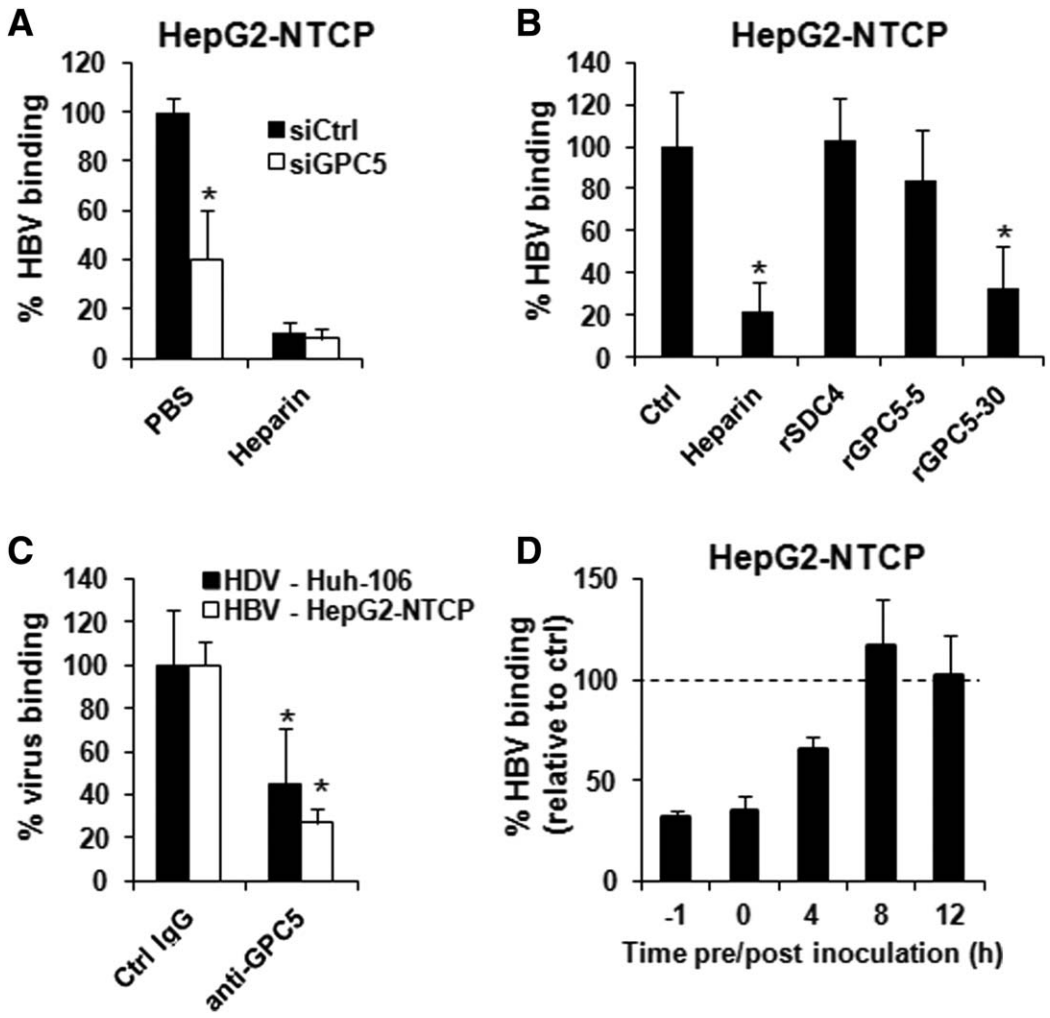



Fig. 7. GPC5 mediates binding of HBV and HDV virions to the liver cell surface. (A) GPC5 silencing inhibits HBV binding. HepG2-NTCP cells silenced for GPC5 expression were treated for 24 hours at 16°C with HBV particles pretreated or not (PBS) with heparin (30 μg/mL). HBV binding was measured by qPCR quantification of total HBV DNA bound to cells after 24 hours. Results are expressed as means ± standard deviation percentage HBV binding relative to control siRNA transfected cells treated with PBS from three independent experiments performed in duplicate. (B) Neutralization of HBV infectious particles using soluble recombinant GPC5 protein (rGPC5). HBV particles were pretreated for 30 minutes at 37°C with heparin (30 μg/mL), with soluble recombinant SDC4 (rSDC4, 30 μg/mL), or with rGPC5 at the indicated concentrations. HBV binding was measured by qPCR quantification of total HBV DNA bound to cells after 24 hours. Results are expressed as means ± standard deviation percentage HBV binding relative to control cells from three independent experiments performed at least in duplicate. (C) A specific anti-GPC5 antibody inhibits HDV and HBV binding at the cell surface. Huh-106 and HepG2-NTCP cells were incubated for 1 hour at 37°C with an anti-GPC5 antibody or a rabbit control IgG and then incubated for 24 hours at 16°C with HDV and HBV infectious particles, respectively. HBV and HDV binding was measured by qPCR quantification of total HBV DNA and HDV RNA bound to cells after 24 hours. Results are expressed as means ± standard deviation percentage HBV or HDV binding relative to control cells treated with a control IgG from three independent experiments performed at least in duplicate. (D) Time course of antibody-mediated inhibition of HBV binding. HepG2-NTCP cells were incubated with an anti-GPC5 antibody at various time points before and after incubation with HBV particles as indicated. HBV binding was measured by qPCR quantification of total HBV DNA bound to cells after 24 hours. Results are expressed as means ± standard deviation percentage HBV binding relative to cells incubated with control antibody from two independent experiments performed in triplicate.

## Discussion

Here, we report a novel cell-based infection model system that allows the screening of host factors involved in HDV infection in functional assays (Fig. 4). Using this model and a loss-of-function approach, we identified GPC5 as an HDV/HBV entry factor. The functional role of GPC5 as an entry factor for these two viruses was confirmed by several lines of evidence and complementary approaches: (1) silencing of GPC5 expression decreased HDV and HBV infection in NTCP-expressing hepatoma cells and PHHs (Figs. 4–6), (2) an anti-GPC5 antibody impaired viral infection (Fig. 5), and (3) HBV binding to the cell surface decreased after silencing GPC5 expression or pretreatment of HBV with rGPC5 (Fig. 7), confirming a direct interaction between the virus and GPC5. Notably, the HBV–GPC5

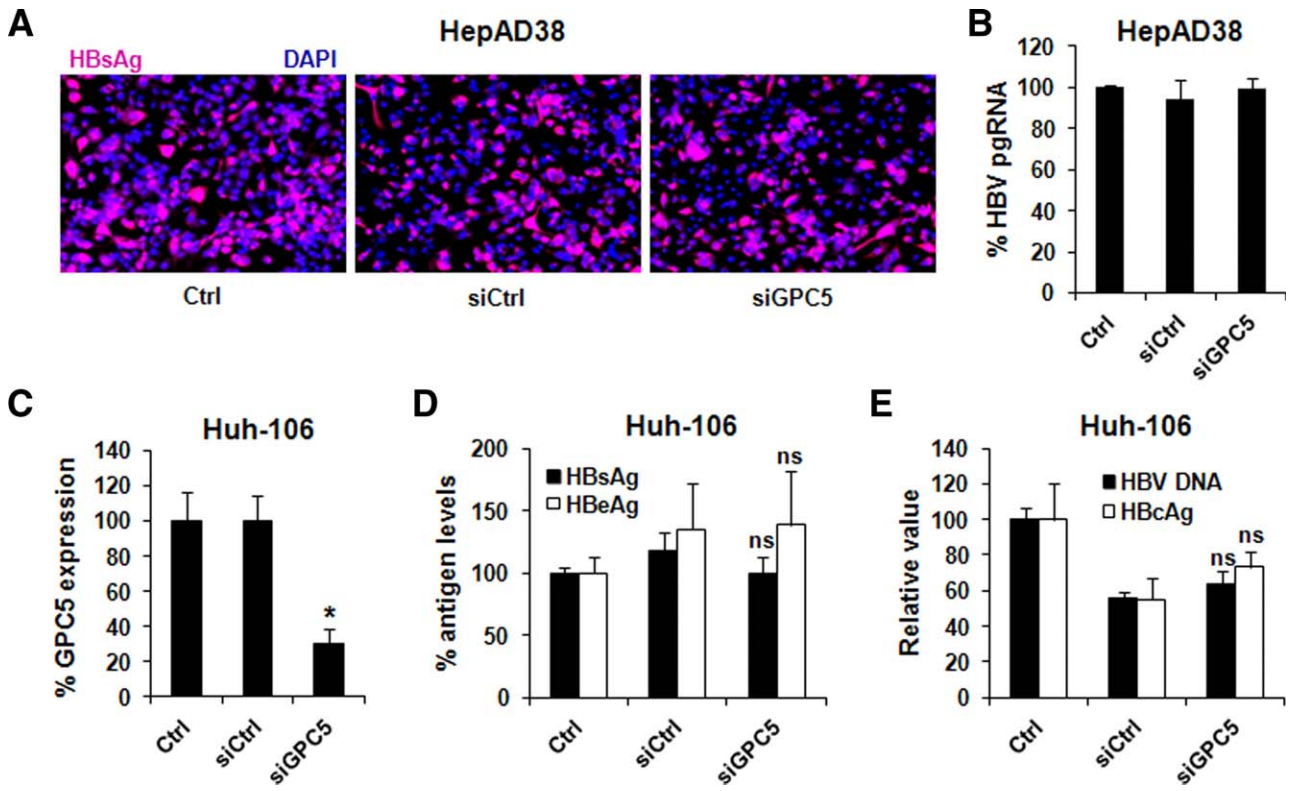



Fig. 8. GPC5 is not involved in HBV replication and production. (A,B) Absent effect of GPC5 silencing on HBV production in HepAD38 cells. HepAD38 cells stably replicating HBV were transfected with siRNAs targeting GPC5. Three days after transfection, the HBsAg level was evaluated by IF (A, one representative experiment is shown) and HBV pgRNA level was assessed by qRT-PCR quantification (B). Results are expressed as means ± standard deviation percentage HBV pgRNA levels relative to control nontransfected cells from three independent experiments performed in triplicate. (C-E) Absent effect of GPC5 silencing on HBV replication in Huh-106 cells. Huh-106 cells were reverse-cotransfected with a plasmid encoding the HBV genome (adwR9) or control plasmid and siGPC5 or control siRNA. Silencing efficacy was assessed by qRT-PCR 3 days after transfection (C). Three days after transfection, HBV replication was assessed by quantification of HBeAg and HBsAg in the supernatants (D) and total HBV DNA and HBV core antigen in cell lysates (E). Results are expressed as means ± standard deviation percentage GPC5 expression, HBV antigen, and DNA levels normalized to the HBV plasmid-only control (control) set as 100% from three independent experiments performed in triplicate. Abbreviation: DAPI, 4′,6-diamidino-2-phenylindole; ns, nonsignificant difference between nontargeting control siRNA and siGPC5.

interaction was independent of NTCP expression (Figs. 4 and 5).

PEG has been shown to favor HBV infection in PHH cultures,[35] and it is used, as well as DMSO, in most of the *in vitro* HBV and HDV infection systems based on NTCP-expressing hepatoma cells.[8,9,18] In our study, we observed that the use of PEG and DMSO was not necessary for conducting high-throughput *in vitro* HDV infection assays that are adapted to functional siRNA and small molecule screening. We thus made the choice of using these PEG/DMSO-free conditions for practical reasons in conducting the assays but also for clarity in the interpretation of the data. Although DMSO is known to induce polarization and differentiation of hepatocytes in culture, it may also affect transcriptomic patterns of cells[36] and modulate the expression of factors involved in viral entry independently of differentiation.

Using this model system and an RNAi approach to uncover which HSPG(s) mediate(s) HBV/HDV entry, we identified GPC5 as a host factor involved in the initiation of HDV and HBV infection. GPC5 is a member of the glypican family, a group of six HSPGs (GPC1-6) that are attached to the cell membrane by a glycosylphosphatidylinositol anchor.[37] Glypicans are coreceptors for numerous heparin-binding factors, and they regulate the signaling activity of many growth factors, including Wnts and hedgehogs.[37] GPC5 is highly expressed during development in a tissue-specific manner, suggesting a major role in morphogenesis.[38] In adult tissues, GPC5 is mainly expressed in the brain, kidney, and liver.[39] Modulation of GPC5 expression is observed in various cancers and other disorders, including nephrotic syndrome,[39] alveolar and embryonal rhabdomyosarcoma,[40] and lung cancer in nonsmoker patients.[41] However, a comprehensive understanding of the GPC5 functions has yet to be established. Here, we report that GPC5 acts as an entry factor for both HBV and HDV through its interactions with the HBV envelope proteins during viral attachment. Given its tissue distribution and high expression level in

the liver, GPC5 may contribute to the hepatotropism of HBV and HDV. Unlike other glypican members, GPC5 carries chondroitin sulfate chains in addition to heparan sulfate chains.[37] Chondroitin sulfate chains can function as receptors for porcine circovirus 2 and herpes simplex virus,[42,43] suggesting a role for these polysaccharides in microbial adherence. The presence of chondroitin sulfate, in addition to heparan sulfate, could explain the specificity of GPC5 as an HBV/HDV attachment factor compared to other glypican members. In line with a recent study,[44] we observed that silencing of GPC5 expression had no effect on HCV entry. Similar to HBV, HCV attachment to hepatocytes is mediated by HSPGs[45]; and two recent studies identified SDC1 and SDC4 as mediators of HCV attachment.[34,44] Our data demonstrate that neither of these two HSPGs is involved in HBV entry, highlighting high specificity of virus–HSPG interactions at the surface of hepatocytes. Our binding data indicate that GPC5 is involved in the initial attachment of the virus at the hepatocyte surface, likely at a step prior to HBV preS1 binding to NTCP, which would subsequently trigger viral entry. The interaction between the HBV envelope and GPC5 may involve both glycosaminoglycans and the GPC5 core protein.

Because glypicans have been shown to play a role in the control of cell division and growth regulation, virus–GPC5 interactions may also play a role for pathogenesis of virus-induced liver disease and cancer. Indeed, HBV[46] and HCV[47] can transmit signals during hepatocyte binding and cell entry. In turn, glypicans are involved in the modulation of several signal transduction pathways. In particular, GPC3, a closely related member in the glypican family, may serve as a biomarker and target of HCC therapy.[48] GPC3 modulates fibroblast growth factor 2 and hedgehog signaling pathways in HCC (reviewed in Ho and Kim[48]). Similarly, GPC5 modulates fibroblast growth factor 2 signaling and stimulates hedgehog signaling in rhabdomyosarcoma cells.[49,50] However, its potential role in liver disease has not yet been studied. Collectively, the interaction between GPC5 and HBV envelope proteins may modify glypican-specific signaling pathways with potential implications for liver disease progression and hepatocarcinogenesis.

Furthermore, GPC5 may represent a previously undiscovered target for urgently needed antiviral therapies. Targeting viral entry using NTCP inhibitors is a promising approach for HBV treatment.[16] Myrcludex B, an HBV preS1 NTCP-targeting peptide, strongly inhibits HBV infection *in vivo*[19] and is currently being evaluated in a phase 2 clinical trial.[17] Thus, inhibiting HBV–GPC5 interactions using small molecules or antibodies (Fig. 5) opens new perspectives for control or cure of HBV and HDV infections.

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## Supporting Information

Additional Supporting Information may be found at onlinelibrary.wiley.com/doi/10.1002/hep.28013/suppinfo.

# ANNEXE 3

## *Solute carrier NTCP regulates innate antiviral immune responses targeting hepatitis C virus infection of hepatocytes*

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* ont contribué à part égale à ce travail comme premiers auteurs

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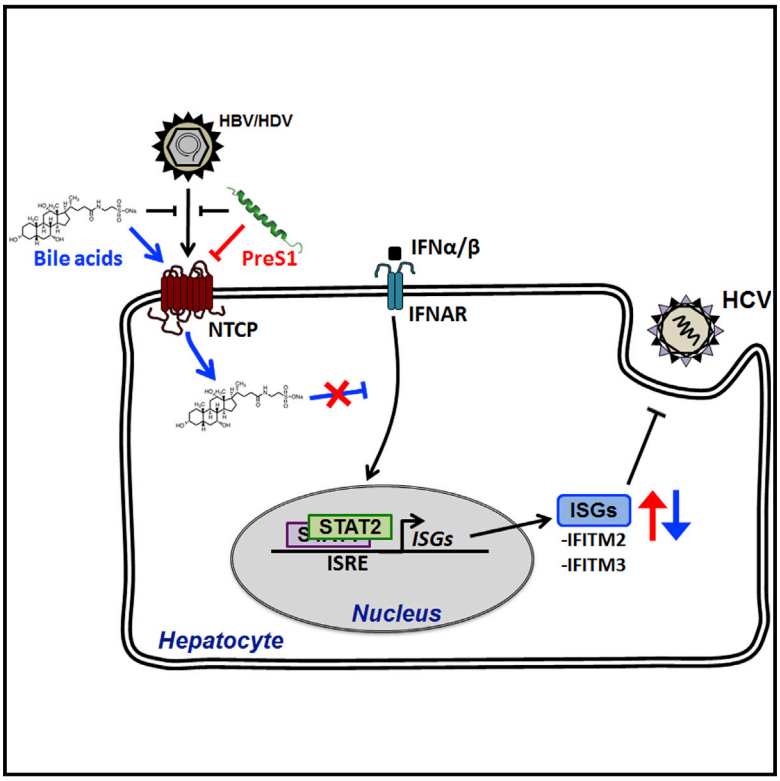
Article

Cell Reports

# Solute Carrier NTCP Regulates Innate Antiviral Immune Responses Targeting Hepatitis C Virus Infection of Hepatocytes

## Graphical Abstract

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## In Brief

Verrier et al. identify the sodium taurocholate co-transporter NTCP as a host factor regulating HCV infection. NTCP-mediated bile acid transport regulates innate responses targeting HCV infection. NTCP is a mediator of innate immunity and plays a role in the infection of multiple hepatotropic viruses.

## Highlights

- NTCP is involved in hepatocyte infection by multiple viruses via distinct mechanisms
- NTCP facilitates HCV infection by modulating innate antiviral responses
- Solute carrier NTCP is a regulator of antiviral immune responses in the liver
- This function is relevant for infection and therapies for hepatotropic viruses

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# Solute Carrier NTCP Regulates Innate Antiviral Immune Responses Targeting Hepatitis C Virus Infection of Hepatocytes

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## SUMMARY

**Chronic hepatitis B, C, and D virus (HBV, HCV, and HDV) infections are the leading causes of liver disease and cancer worldwide. Recently, the solute carrier and sodium taurocholate co-transporter NTCP has been identified as a receptor for HBV and HDV. Here, we uncover NTCP as a host factor regulating HCV infection. Using gain- and loss-of-function studies, we show that NTCP mediates HCV infection of hepatocytes and is relevant for cell-to-cell transmission. NTCP regulates HCV infection by augmenting the bile-acid-mediated repression of interferon-stimulated genes (ISGs), including *IFITM3*. In conclusion, our results uncover NTCP as a mediator of innate antiviral immune responses in the liver, and they establish a role for NTCP in the infection process of multiple viruses via distinct mechanisms. Collectively, our findings suggest a role for solute carriers in the regulation of innate antiviral responses, and they have potential implications for virus-host interactions and antiviral therapies.**

## INTRODUCTION

Hepatitis B and C viruses (HBV and HCV) are the leading causes of chronic liver disease worldwide (El-Serag, 2012). With ~400 million individuals chronically infected with HBV or HCV and at risk for severe liver disease, these viruses are a major global health burden (El-Serag, 2012; Wedemeyer et al., 2015). Although HBV and HCV differ in their genomic organization and life cycles, these viruses exclusively infect human hepatocytes (Baumert et al., 2014), suggesting that liver-specific factors are important for the life cycle of both viruses.

Viral entry pathways contribute to HBV and HCV liver tropism. HCV requires a number of host proteins, including cluster of differentiation 81 (CD81), scavenger receptor BI (SR-BI), claudin-1 (CLDN1), occludin (OCLN), and accessory factors, such as epidermal growth factor receptor (EGFR), to enter hepatocytes (Baumert et al., 2014; Lupberger et al., 2011; Martin and Uprichard, 2013; Zeisel et al., 2013b; Zona et al., 2013). Some of these host factors interact directly with HCV glycoproteins, whereas others contribute to HCV internalization by promoting co-receptor associations and inducing intracellular signaling pathways (Kim et al., 2013; Lupberger et al., 2011). While key host factors mediating HCV entry are well characterized (Zeisel et al., 2013a), it is only partially understood how cell entry is regulated. Furthermore, the role of innate immune responses targeting HCV entry is poorly understood.

The sodium taurocholate co-transporting polypeptide (NTCP), a bile acid transporter expressed at the hepatocyte basolateral membrane (Claro da Silva et al., 2013), was identified previously as a receptor for HBV and hepatitis D virus (HDV) (Ni et al., 2014; Yan et al., 2012). HDV can use HBV envelope proteins to assemble infectious particles and is widely used as a surrogate model to study HBV entry (Sureau, 2010). Exogenous NTCP

expression in NTCP-lacking human hepatoma cell lines (such as Huh7 and HepG2) renders these cells susceptible to HBV/HDV entry (Ni et al., 2014; Yan et al., 2012). The natural ligands of NTCP (i.e., conjugated and hydrophilic bile acids) compete with HBV/HDV for NTCP binding and inhibit viral infection (Yan et al., 2014). In contrast, bile acids have been shown to enhance HCV replication (Chang and George, 2007; Chhatwal et al., 2012), although the mechanisms are not yet defined. Furthermore, whether bile acid transporters such as NTCP play a role in infection of alternative hepatotropic viruses, such as HCV, is unclear. In this study, we tested the role of NTCP in HCV infection, and we identified the mechanisms by which a solute carrier affects infectivity of three major hepatotropic viruses.

## RESULTS

### NTCP Overexpression Enhances HCV Infection

To investigate the effect of NTCP expression on HCV infection, we transduced the Huh7.5.1 cell line (Zhong et al., 2005) to express human NTCP. Huh7.5.1-NTCP cells expressed significantly higher NTCP mRNA and protein levels (Figure 1A) and surface levels of NTCP (Figure S1A) than parental Huh7.5.1 cells. To confirm that NTCP is functional as a viral host factor in these cells, Huh7.5.1-NTCP cells were infected with recombinant HDV. Huh7.5.1-NTCP cells supported HDV infection, as demonstrated by the presence of HDV RNA and delta antigen (HDAg) 7 days post-inoculation (Figure 1B). Moreover, HDV infection was inhibited by the HBV preS1-derived peptide (Figure 1B), which binds to NTCP and prevents HBV/HDV entry (Schieck et al., 2013).

To assess if NTCP expression affects HCV infection, we used cell culture-derived HCV (HCVcc) and lentiviral particles pseudotyped with HCV E1E2 glycoproteins (HCVpp) of different genotypes. Expression of NTCP significantly enhanced HCVcc (Jc1 and JcR2A) infection and HCVpp (genotypes 1b, 2a, 3a, and 4) entry compared to the parental cells (normalized as 100%) (Figures 1C and 1D). Interestingly, entry of vesicular stomatitis virus pseudoparticles (VSVpp) and murine leukemia virus pseudoparticles (MLVpp) was not significantly affected by NTCP expression under these conditions (Figure 1D). The growth of Huh7.5.1 and Huh7.5.1-NTCP cells after seeding was similar, indicating that our observations were unrelated to effects on cell proliferation (Figure S1B). Furthermore, the effect of NTCP on HCV entry/infection was independent of the infectivity levels of different HCVpp and HCVcc strains (Figures S1C and S1D).

Since HCV can infect cells by direct cell-to-cell transmission (Meredith et al., 2013; Timpe et al., 2008), we evaluated the role of NTCP in this mode of viral dissemination. HCV-replicating Huh7.5.1 cells were co-cultured with GFP-expressing Huh7.5 cells (Lupberger et al., 2011; Xiao et al., 2014) transduced or not to express NTCP (Figure 1E). In this assay, the presence of an anti-envelope E2 antibody inhibits cell-free infection, allowing the specific assessment of cell-to-cell transmission. NTCP expression significantly increased the percentage of GFP-positive HCV-infected cells, suggesting that NTCP also plays a role in cell-to-cell spread (Figures 1F and 1G).

### Silencing NTCP Expression Inhibits HCV Infection

We confirmed the role of NTCP in HCV infection by silencing NTCP expression in Huh7.5.1-NTCP cells using a small interfering RNA (siRNA) targeting NTCP, prior to infection with HCVcc. Following NTCP silencing in Huh7.5.1-NTCP cells, total protein expression was decreased by ~80%, which was reflected by decreased surface expression (Figure 2A). This corresponded to a significant decrease in HCVcc infection (Figure 2B). To rule out potential off-target effects, we tested multiple NTCP-targeting small hairpin RNA (shRNA) sequences in Huh7.5.1-NTCP cells. We confirmed that a decrease in NTCP expression (by shNTCP2 and shNTCP3) (Figure 2C) correlated with a decrease in HCVcc infection (Figure 2D). Furthermore, silencing NTCP expression with shNTCP2 decreased protein expression by >80% (Figure 2E), which significantly decreased entry of HCVpp into Huh7.5.1-NTCP cells (Figure 2F), confirming the relevance of NTCP for HCV entry.

### NTCP Modulates HBV/HDV and HCV Infection via Distinct Mechanisms

To assess whether NTCP interacts directly with HCV glycoproteins, we measured binding of recombinant soluble HCV E2 (sE2) to cells. We observed no significant difference in sE2 binding to NTCP-expressing or parental cells (Figure 3A). However, sE2 treatment of Huh7.5.1 cells inhibited HCVcc infection (Figure 3B), confirming the functionality of the sE2 protein. These findings indicate that NTCP is not involved in E2 binding. Furthermore, NTCP expression did not affect the expression of canonical HCV entry factors in Huh7.5.1-NTCP cells compared to the parental cells (Figure 3C).

We next tested the effect of the HBV preS1-derived peptide, which is known to bind to NTCP and inhibit HBV/HDV entry (Ni et al., 2014). Although preS1 inhibited HDV infection of Huh7.5.1-NTCP cells after 1 hr pre-treatment, it had no effect on HCVpp entry under these conditions (Figure 3D), indicating that the HBV preS1-binding domain of NTCP is not required to promote HCV entry. We next evaluated whether the bile acid transporter function of NTCP is important for HCV entry. Huh7.5.1-NTCP cells were treated with the HBV preS1-derived peptide (König et al., 2014; Slijepcevic et al., 2015), which inhibits bile acid uptake in vitro ($IC_{50}$, 4 nM) (Ni et al., 2014), for 24, 48, and 72 hr prior to the addition of HDV, HCVpp, or VSVpp (Figures 3E–3G). PreS1 treatment (200 nM) significantly inhibited HCVpp entry in a time-dependent manner, with a maximal inhibition of ~70% after 72 hr (Figure 3E). Under these conditions, VSVpp entry also was reduced by preS1, although to a lesser extent (Figure 3F), suggesting a general effect on viral infection. In contrast, HDV infection was impaired by preS1 regardless of the treatment duration (Figure 3G). HCVcc infection also was decreased by 72-hr preS1 treatment (Figure 3H), confirming that our observations are relevant for the infectious virus and the full HCV life cycle. Taken together, our data suggest that long-term preS1 inhibition of NTCP-mediated bile acid transport perturbs cellular physiology to modulate HCV entry.

### PreS1-Mediated Inhibition of NTCP Induces Interferon-Stimulated Gene Expression

Since preS1 only affected HCV entry after long-term treatment (Figure 3), we hypothesized that the bile acid transport activity

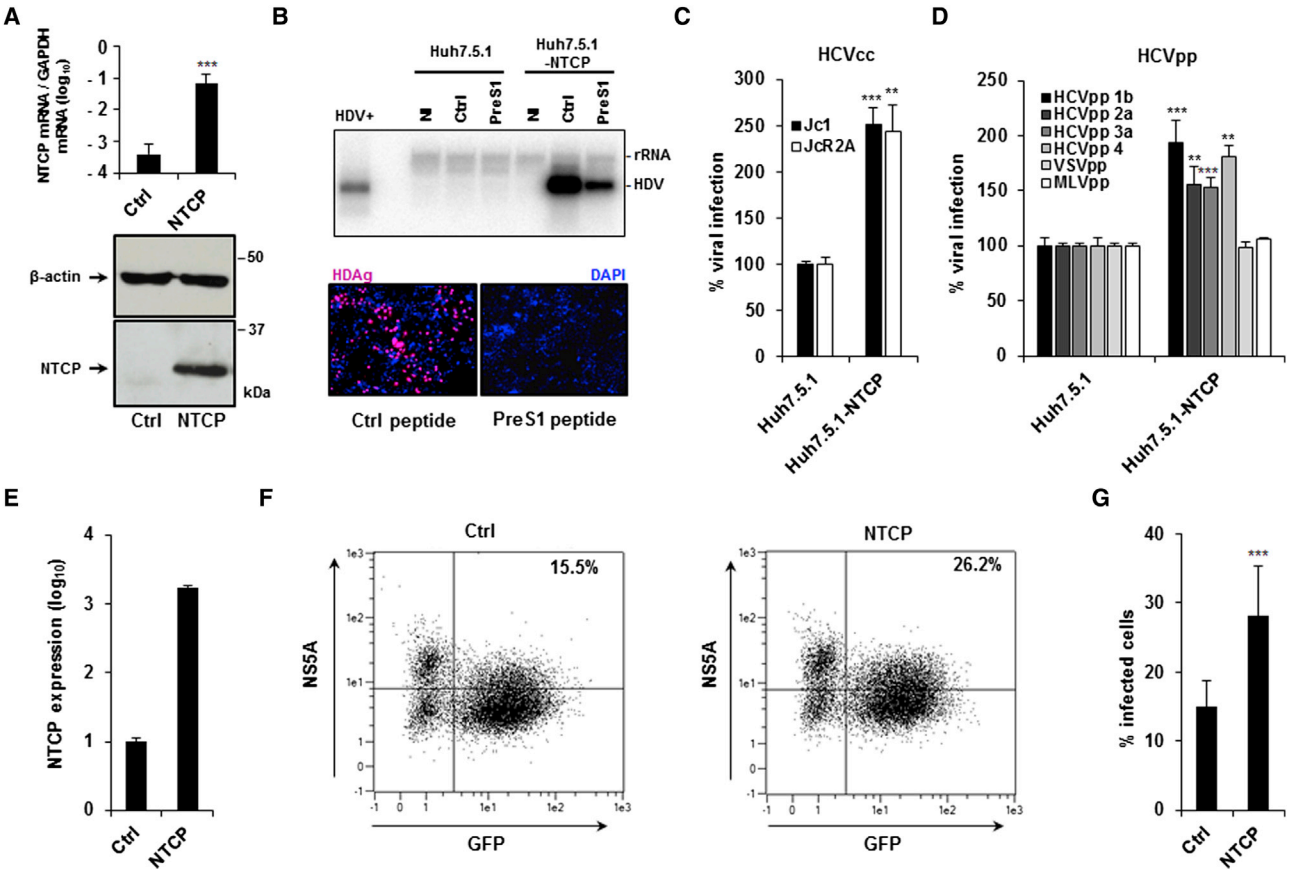


**Figure 1. NTCP Expression Modulates HCV Infection**

(A) NTCP expression in Huh7.5.1-NTCP cells compared to parental Huh7.5.1 cells evaluated by qRT-PCR and western blot. qRT-PCR results are expressed as means ± SD. NTCP expression was normalized by GAPDH expression (log) from three independent experiments performed in triplicate (n = 9).

(B) Functional evaluation of HDV infection in Huh7.5.1-NTCP cells using northern blot and immunofluorescence (IF). Huh7.5.1 and Huh7.5.1-NTCP cells were treated with an HBV-derived preS1 peptide (PreS1) or with a control peptide (Ctrl) (200 nM) and infected with recombinant HDV for 7 days. HDV RNA was detected by northern blot. HDV+ corresponds to ~$2 \times 10^7$ HDV RNA genome equivalents extracted from HDV particles produced in Huh7 cells. NI, non-infected cells. One experiment is shown. For IF analysis, cells were fixed 7 days after infection and stained with HDAg-specific antibodies purified from HBV/HDV co-infected patients. HDV-positive cells are visualized in red. Cell nuclei were stained with DAPI (blue). One experiment is shown.

(C and D) HCVcc and HCVpp infection of NTCP-overexpressing cells. Huh7.5.1 and Huh7.5.1-NTCP cells were infected with HCVcc (C, Luc-Jc1 and Luc-JcR2A; D, HCVpp (genotypes 1b, 2a, 3a, and 4), VSVpp, and MLVpp). Infection was assessed after 72 hr by measuring luciferase activity. Results are expressed as means ± SEM percentage virus infection relative to parental Huh7.5.1 cells from three independent experiments (one performed in triplicate, one in quintuplicate, and one in hextuplicate; n = 14) (C) or three independent experiments performed in triplicate (n = 9, except MLVpp: two independent experiments performed in triplicate, n = 6).

(E–G) NTCP overexpression increases HCV cell-to-cell transmission. HCV-electroporated Huh7.5.1 were transduced or not (Ctrl) with lentiviruses expressing NTCP. (E) NTCP expression was assessed by qRT-PCR. Results are expressed as means ± SD relative NTCP expression (log) compared to NTCP expression in non-transduced cells (set at 1) from one experiment performed in triplicate (n = 3). Cells were then co-cultured with GFP-expressing Huh7.5.1 cells in the presence of neutralizing antibody AP33. (F and G) After NS5A staining, HCV cell-to-cell transmission was assessed by flow cytometry as the percentage of GFP- and NS5A-positive cells. One experiment is shown (F) and results are expressed as means ± SD percentage infected cells from two independent experiments (one performed in hextuplicate and one in nonuplicate; n = 15) (G).

of NTCP induces metabolic or transcriptional changes that regulate virus infection. To test this hypothesis, we evaluated the effect of preS1-NTCP binding on gene expression in hepatoma cells. We performed genome-wide microarray analyses of Huh7.5.1-NTCP cells that were treated with preS1 or a scrambled peptide (200 nM) for 48 hr. Gene set enrichment analysis (GSEA) indicated that preS1 treatment induces the expression of genes involved in bile acid and fatty acid metabolism, as previously described (Oehler et al., 2014). Unexpectedly, preS1 treatment also induced the expression of genes involved in innate immunity, such as IFNα responses and the Jak/Stat-signaling pathway (Table 1; Figure 4A). Interestingly, *IFITM2* and *IFITM3*, which encode two restriction factors targeting HCV entry (Narayana et al., 2015), were among the preS1-induced genes (Figure 4B).

To confirm that IFITMs function as restriction factors in Huh7.5.1-NTCP cells, we exogenously overexpressed IFITM2 and IFITM3 (Figure 4C). Indeed, IFITM2 and IFITM3 overexpression in Huh7.5.1-NTCP cells inhibited HCVcc infection (Figure 4D). However, as endogenous *IFITM2* expression in Huh7.5.1 and

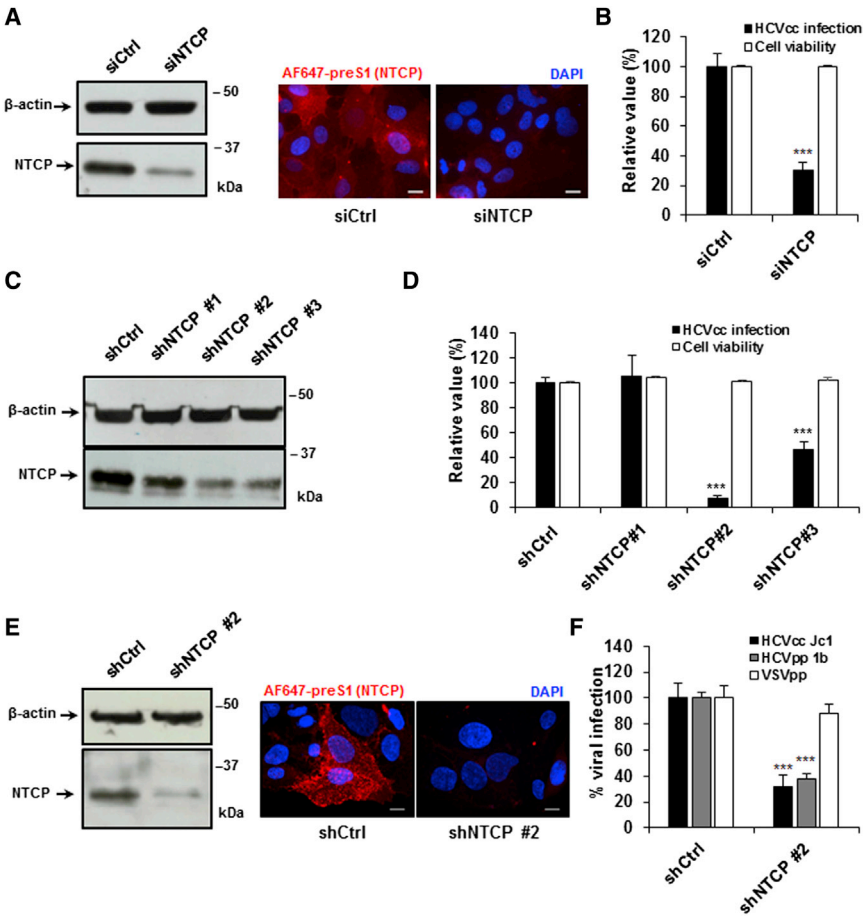


**Figure 2. Silencing NTCP Expression Inhibits HCV Infection in Human Hepatocytes**

(A and B) NTCP silencing in hepatoma cell lines. Huh7.5.1-NTCP cells were transfected with siRNA control (siCtrl) or siRNA targeting NTCP (siNTCP). siRNA efficacy was assessed 72 hr after transfection by western blot and IF (scale bar, 10 μm) (A), and cell viability was assessed using PrestoBlue reagent (B). 3 days after NTCP silencing, Huh7.5.1-NTCP cells were infected with HCVcc (Luc-Jc1). Infection was assessed after 72 hr by measuring luciferase activity. Results are expressed as means ± SEM percentage cell viability compared to cells treated with siCtrl from three independent experiments (one performed in sextuplicate and two performed in octuplicate; n = 22) and means ± SEM percentage HCVcc infection from four independent experiments performed in quadruplicate (n = 16) (B).

(C–F) Effect of shNTCP silencing in hepatoma cells. Huh7.5.1-NTCP cells were transduced with lentiviruses expressing shRNAs targeting NTCP (shNTCP1, shNTCP2, or shNTCP3) or control shRNA (shCtrl). shRNA efficacy was assessed 72 hr after transduction by western blot (C) or by western blot and IF (scale bar, 10 μm) (E). Cell viability was assessed using PrestoBlue reagent (D). Results are expressed as means ± SEM percentage cell viability from three independent experiments performed in octuplicate (n = 24). 3 days after NTCP silencing, Huh7.5.1-NTCP cells were infected with HCVcc (Luc-Jc1) (D and F), HCVpp (genotype 1b) (F), or VSVpp (F). Infection was assessed after 72 hr by measuring luciferase activity. Results are expressed as means ± SEM percentage HCVcc infection from three independent experiments (two performed in quadruplicate and one in sextuplicate; n = 14) (D), or they are expressed as means ± SEM percentage pseudoparticle entry from three independent experiments performed in triplicate (n = 9) (F) and as means ± SEM percentage HCVcc infection from three independent experiments performed in quadruplicate (n = 12) (F).

Huh7.5.1-NTCP cells was low and at the limit of detection, we focused on *IFITM3* in further functional studies. Notably, IFITM3 expression was decreased at the protein level by ~60% in Huh7.5.1-NTCP cells compared to parental cells (Figure 4E), suggesting that NTCP modulates *IFITM3* expression. To confirm that the changes in gene expression were directly related to NTCP and not to off-target effects of preS1, we selected *IFITM3* and two other genes involved in IFNα responses (*PARP9* and *CXCL10*) which were induced by preS1 treatment (Figure 4B), and we compared their expression levels in Huh7.5.1 and Huh7.5.1-NTCP cells. As expected, Huh7.5.1-NTCP cells had decreased mRNA expression of *IFITM3*, *PARP9*, and *CXCL10* compared to parental cells (Figure 4F), confirming the specific role of NTCP in the suppression of these genes. These data support previous findings that preS1 binds with high specificity to NTCP without off-target effects (Bogomolov et al., 2016).

## Bile Acid Transport through NTCP Modulates the Expression of Interferon-Stimulated Genes to Affect HCV Infection

The gene expression analyses implied that NTCP facilitates HCV entry by altering the expression of interferon-stimulated genes (ISGs). Given that the physiological function of NTCP is to transport bile acids, and bile acids are known to affect ISG expression in hepatocytes (Graf et al., 2010; Podevin et al., 1999), we hypothesized that bile acid transport by NTCP regulates the expression of ISGs and viral infection. Since IFITM3 functions as an HCV restriction factor in our model system (Figure 4D), we selected *IFITM3* as a representative ISG for functional studies to probe the link between NTCP and the IFN response. To ensure that IFN responses are indeed functional in Huh7.5.1-NTCP cells, we treated cells with Poly(I:C) and IFNα2, and we evaluated ISG induction by the expression of *IFITM3*. Poly(I:C) stimulation of Huh7.5.1-NTCP cells significantly increased *IFITM3* expression (Figure S2A). Moreover, STAT1 phosphorylation (Figure S2B) and *IFITM3* mRNA expression (Figure S2C) were markedly induced after IFNα2 treatment. This induction was repressed by a specific antibody targeting the type I IFN receptor (IFNAR) (Figures S2B and S2C).

We then evaluated the effect of NTCP on IFN responses in these cells. The mere presence of NTCP decreased mRNA expression of *IFITM3* by ~50% compared to parental cells (Figure 5A). The addition of bile acid (100 μM sodium taurocholate) to Huh7.5.1-NTCP cells decreased *IFITM3* mRNA expression even

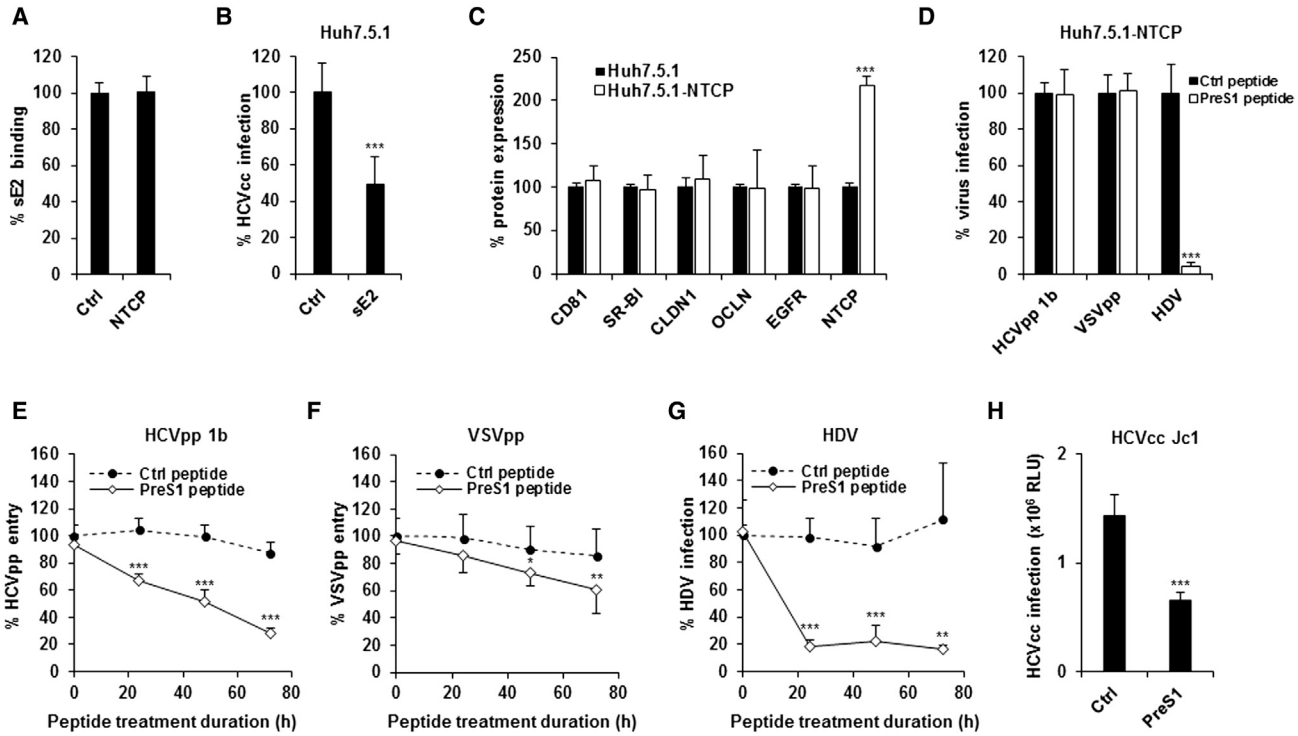


**Figure 3. Distinct Roles of NTCP in HDV/HBV and HCV Infections**

(A) Flow cytometry analysis of HCV glycoprotein sE2 binding to Huh7.5.1 (Ctrl) and Huh7.5.1-NTCP (NTCP). Results are expressed as means ± SD percentage sE2 binding compared to control cells from three independent experiments (two performed in triplicate and one in quadruplicate; n = 10).

(B) sE2 binding inhibits HCVcc infection. Huh7.5.1 cells were treated with soluble sE2 and then infected with HCVcc (Luc-Jc1). Infection was assessed after 72 hr by measuring luciferase activity. Results are expressed as means ± SD percentage HCVcc infection from three independent experiments performed in triplicate (n = 9).

(C) Flow cytometry analysis of HCV entry factor expression in Huh7.5.1-NTCP cells. Results are expressed as percentage protein expression compared to parental Huh7.5.1 cells (set at 100%) from four independent experiments performed in duplicate (n = 8).

(D) Effect of HBV preS1-derived peptide on HDV, HCVpp (genotype 1b), and VSVpp entry into Huh7.5.1-NTCP cells. Huh7.5.1-NTCP cells were treated for 1 hr with preS1 or Ctrl peptide (200 nM). For HDV, cells were then infected with recombinant HDV for 7 days. Total RNA was purified and HDV RNA was detected by qRT-PCR. Results are expressed as percentage HDV RNA level compared to non-treated Huh7.5.1-NTCP cells from three independent experiments performed in triplicate (n = 9). For HCVpp (genotype 1b) and VSVpp, cells were infected with pseudoparticles and infection was assessed after 72 hr by luciferase activity. Results are expressed as means ± SD percentage viral entry from three independent experiments performed in quintuplicate (n = 15).

(E–G) Time-dependent inhibition of viral entry by preS1 treatment. Cells were treated with preS1 or Ctrl peptide for 24, 48, and 72 hr prior to infection with HCVpp (genotype 1b), VSVpp, or HDV. Infection was assessed as described above. (E) Results are expressed as means ± SD percentage HCVpp entry from five independent experiments performed in triplicate (n = 15). (F) Results are expressed as means ± SD percentage VSVpp entry from four independent experiments performed in triplicate (n = 12). (G) Results are expressed as means ± SD percentage HDV infection from three independent experiments performed in duplicate (n = 6).

(H) Treatment with preS1 inhibits HCVcc infection. Cells were treated with preS1 or Ctrl peptide for 72 hr prior to infection with HCVcc (Luc-Jc1). Infection was assessed after 72 hr by measuring luciferase activity. Results are expressed as means ± SD HCVcc infection (relative light unit, RLU) from three independent experiments performed in triplicate (n = 9).

further, whereas blocking bile acid transport with the preS1 peptide restored *IFITM3* mRNA expression to the levels observed in Huh7.5.1 cells (Figure 5A). These findings suggest that the effect of NTCP on ISG expression and HCV infection is dependent on bile acid. Indeed, the addition of supplementary bile acid in the cell culture medium dose-dependently increased HCVcc infection in Huh7.5.1-NTCP cells, but not in parental cells (Figure 5B).

We next evaluated the effect of preS1 treatment on IFITM3 protein expression and HCV infection in the presence of bile acids. Reflecting our observations of the mRNA level (Figure 5A), IFITM3 protein expression was decreased in Huh7.5.1-NTCP cells compared to Huh7.5.1 cells in the presence of bile acid (Figure 5C). However, treatment of Huh7.5.1-NTCP cells with preS1 (to block bile acid uptake) under these conditions induced a 2-fold increase in IFITM3 protein expression, effectively restoring it to the level observed in Huh7.5.1 cells (Figure 5C). Furthermore, treatment of Huh7.5.1-NTCP cells with the preS1 peptide in the presence of bile acid inhibited HCVcc infection (Figure 5D). We did not observe these effects in Huh7.5.1 cells (Figures 5C and 5D), confirming that the bile acid-mediated effect is dependent on NTCP.

Interestingly, when we silenced *IFITM3* expression (Figure 5E) in the presence of bile acid, we observed an increase in HCV infection (Figure 5F). However, this effect was no longer

**Table 1. Hallmark Gene Sets Significantly Induced, Positive NES[a], or Repressed, Negative NES, after preS1 Treatment in Huh7.5.1-NTCP Cells Shown in Figure 4**

| Gene Set | NES[a] | p value | FDR[b] |
|---|---|---|---|
| Bile acid metabolism | 2.32 | <0.001 | <0.001 |
| Coagulation | 2.25 | <0.001 | <0.001 |
| Xenobiotic metabolism | 2.20 | <0.001 | <0.001 |
| Fatty acid metabolism | 2.20 | <0.001 | <0.001 |
| Heme metabolism | 2.04 | <0.001 | <0.001 |
| Adipogenesis | 1.91 | <0.001 | <0.001 |
| Peroxisome | 1.90 | <0.001 | <0.001 |
| Interferon alpha response | 1.90 | <0.001 | <0.001 |
| Oxidative phosphorylation | 1.90 | <0.001 | <0.001 |
| Estrogen response late | 1.89 | <0.001 | <0.001 |
| Angiogenesis | 1.72 | 0.002 | 0.003 |
| Cholesterol homeostasis | 1.68 | 0.004 | 0.005 |
| Hypoxia | 1.61 | <0.001 | 0.010 |
| Interferon gamma response | 1.60 | <0.001 | 0.009 |
| Reactive oxygen species pathway | 1.59 | 0.013 | 0.010 |
| Estrogen response early | 1.58 | <0.001 | 0.011 |
| Myogenesis | 1.57 | 0.002 | 0.012 |
| IL2 STAT5 signaling | 1.54 | <0.001 | 0.013 |
| IL6 JAK STAT3 signaling | 1.54 | 0.008 | 0.014 |
| Complement | 1.49 | 0.003 | 0.021 |
| MTORC1 signaling | −1.50 | 0.005 | 0.012 |
| Unfolded protein response | −1.82 | <0.001 | <0.001 |
| G2M checkpoint | −2.01 | <0.001 | <0.001 |
| E2F targets | −2.02 | <0.001 | <0.001 |
| MYC targets V2 | −2.54 | <0.001 | <0.001 |
| MYC targets V1 | −2.64 | <0.001 | <0.001 |

[a]Normalized enrichment score.
[b]False discovery rate.

observed in the presence of preS1 to block bile acid uptake into the cells or in the absence of bile acid (Figure 5F). These results suggest that bile acid-mediated suppression of other ISGs (which may otherwise compensate the absence of an individual ISG) is necessary to observe a functional effect of *IFITM3* silencing on HCV infection. Differences in bile acid levels in cell culture medium also may explain why IFITM3 was not identified as an HCV restriction factor in previous screens (Brass et al., 2009).

### NTCP-Mediated Bile Acid Transport Affects ISG Expression and HCV Entry into Primary Human Hepatocytes

For validation in a more physiological context, we investigated the role of NTCP during HCV entry into primary human hepatocytes (PHHs). First, we silenced NTCP expression in PHHs using an NTCP-targeting siRNA. Following transfection of siNTCP, the expression of NTCP protein was reduced by ~50% (Figure 6A). The decrease in NTCP expression correlated with a significant decrease in the entry of HCVpp genotype 1b (Figure 6B).

We next evaluated whether NTCP-mediated bile acid transport affects ISG expression in PHHs. We treated PHHs with bile acid in the presence or absence of preS1 and performed genome-wide microarray analyses. GSEA showed that bile acid treatment of PHHs suppressed the expression of genes involved in the IFNα response (normalized enrichment score [NES] −2.11; p value < 0.001; false discovery rate [FDR] < 0.001) (Figure 6C) as well as other immune-related pathways. However, treatment of PHHs with preS1 under these conditions induced the expression of genes involved in the IFNα response (NES 1.5; p value = 0.014; FDR = 0.035) to restore their expression levels to normal conditions (i.e., PHHs in the absence of bile acid) (Figure 6D). These findings are consistent with our microarray analyses in Huh7.5.1-NTCP cells (Figure 4), where expression of genes involved in IFNα responses was regulated by treatment with preS1. Interestingly, *IFITM1*, *IFITM2*, and *IFITM3* were among the genes suppressed by bile acid treatment of PHHs (Figure 6C), and their expression could be rescued by the addition of preS1 (Figure 6D). Moreover, the effects we observed in PHHs were dependent on the presence of bile acid, as treatment with preS1 in the absence of bile acid did not have a major impact on the expression of these genes (data not shown).

We next evaluated the functional effect of these changes in ISG expression on HCVpp entry into PHHs. Treatment of PHHs with bile acid increased HCVpp genotype 1b entry, and the addition of preS1 under these conditions restored the level of infection to that observed in the absence of bile acid (Figure 6E). Interestingly, the concentration of bile acid required to see a robust effect for HCVpp entry into PHHs was higher than for HCVcc infection of Huh7.5.1-NTCP cells (Figure 5B), which may reflect different bile acid uptake efficiency in PHHs or that the effect is more potent when the full HCV life cycle is measured and ISGs targeting different steps of the viral infection cycle are involved. Finally, to confirm that the activity of preS1 is linked to IFN responses, we tested the effect of preS1 in the presence of an antibody blocking the type I IFN receptor. In the presence of this antibody, preS1 no longer inhibited HCVpp entry into PHHs (Figure 6F), suggesting that the inhibitory effect of preS1 against HCV entry is indeed mediated by the IFN signal transduction cascade and resulting IFN responses in PHHs.

## DISCUSSION

NTCP was recently described as a major receptor for HBV and HDV entry. Here we show that NTCP also plays a role in HCV infection. Exogenous expression of NTCP in Huh7.5.1 hepatoma cells increased HCV infection, whereas silencing of NTCP expression reduced HCV entry (Figures 1 and 2). Using microarray analyses in cell lines (Figure 4) and PHHs (Figure 6), we discovered that NTCP-mediated bile acid transport regulates innate antiviral responses, thereby inhibiting HCV infection and uncovering a role for NTCP as a regulator of antiviral immunity.

Innate antiviral responses mediated by ISGs have been shown to target multiple steps during viral infection, including entry (Liu et al., 2011; Smith et al., 2014). IFITM1, IFITM2, and IFITM3, which belong to a group of five IFN-induced transmembrane

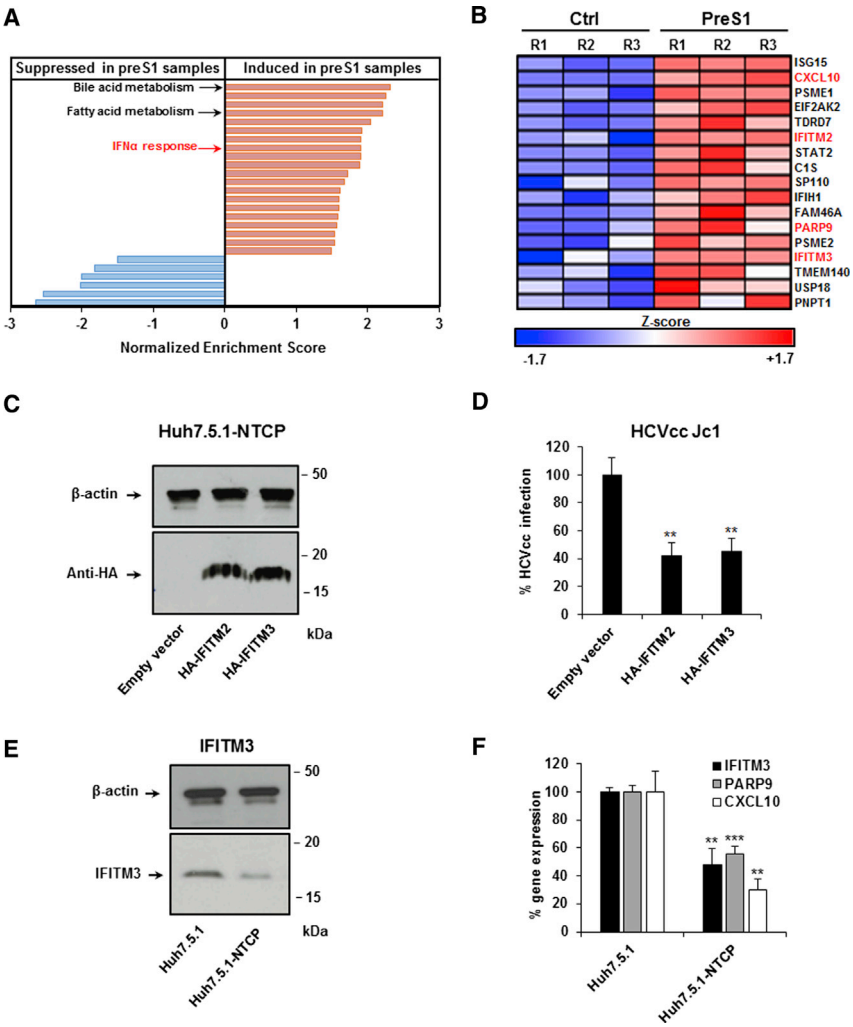


**Figure 4. Binding of HBV-Derived preS1 Peptide to NTCP Increases ISG Expression**

Huh7.5.1-NTCP cells were treated with preS1 or control peptide for 48 hr. Cells were then lysed and total RNA was extracted and purified. Total gene expression was analyzed by genome-wide microarray. Three independent biological replicates per condition from one experiment were analyzed.

(A) Schematic representation of Hallmark gene sets that are significantly induced (red, including bile acid metabolism and IFNα response) or repressed (blue) following preS1 treatment, based on Table 1. $p < 0.05$ and FDR < 0.05 were considered statistically significant.

(B) List of individual IFNα response genes that are significantly ($p < 0.05$) overexpressed following preS1 treatment. Individual *Z* scores for each sample are presented. Negative *Z* score (blue) and positive *Z* score (red) correspond to repression and induction of the indicated genes, respectively.

(C and D) IFITM2 and IFITM3 overexpression inhibits HCV infection. Huh7.5.1-NTCP cells were transfected with an empty vector, pCMV-HA-hIFITM2, or pCMV-HA-hIFITM3 for 3 days. (C) Expression of transduced proteins as assessed by anti-HA western blot is shown. (D) Transduced cells were then infected for 3 days with HCVcc (Luc-Jc1). Infection was assessed after 72 hr by measuring luciferase activity. Results are expressed as means ± SD percentage HCVcc infection compared to control cells (set at 100%) from three independent experiments performed in triplicate (n = 9).

(E) IFITM3 protein expression in hepatoma cells. IFITM3 protein expression was assessed by western blot in Huh7.5.1 and Huh7.5.1-NTCP cells. One experiment is shown.

(F) *IFITM3*, *PARP9*, and *CXCL10* expression in hepatoma cells. Basal expression of *IFITM3*, *PARP9*, and *CXCL10* mRNA was quantified by qRT-PCR in Huh7.5.1 and Huh7.5.1-NTCP cells. Results are expressed as means ± SD percentage gene expression compared to expression levels in Huh7.5.1 cells (set at 100%) from three independent experiments performed in triplicate (n = 9).

proteins (Smith et al., 2014), have been shown to exert broad antiviral activity against a range of viruses, including VSV, HIV, dengue virus, influenza virus, and Zika virus (Savidis et al., 2016; Smith et al., 2014). Interestingly, IFITM2 and IFITM3 were recently reported to restrict HCV infection at a late entry step by targeting HCV for lysosomal degradation following endocytosis (Narayana et al., 2015). IFITM1 was shown to interact with HCV co-receptors at tight junctions to disrupt the HCV entry process by alternative mechanisms (Wilkins et al., 2013).

Given that bile acids have been shown to modulate cellular antiviral responses (Graf et al., 2010; Podevin et al., 1999), we hypothesized that NTCP affects the induction of ISG expression via its bile acid transport activity. Indeed, the expression of ISGs (including *IFITM1*, *IFITM2*, and *IFITM3*) in PHHs was suppressed by the addition of bile acid (Figure 6C). However, expression of these ISGs in PHHs was restored by addition of the preS1 peptide, which blocks NTCP-mediated bile acid uptake (Figure 6D). Furthermore, modulation of the expression of IFITM3 by bile acid or preS1 treatment had a clear functional effect on HCV infection (Figures 5 and 6). Our findings are consistent with a previous report showing that bile acids affect HCV replication (Chhatwal et al., 2012), probably by similar mechanisms as those we describe here. In this study, we selected *IFITM3* as a representative ISG for functional characterization, but the expression of other ISGs is also affected by bile acids (Figure 6), likely contributing further to the overall effect on HCV infection. Since ISGs broadly restrict viral infection, this is consistent with the time-dependent effect of preS1 on VSVpp entry that we observed (Figure 3).

Our data suggest that NTCP facilitates HCV infection by modulating bile acid transport and ISG expression. ISGs also restrict HCV cell-to-cell transmission (Meredith et al., 2014), consistent with our finding that NTCP contributes to HCV cell-to-cell spread (Figures 1E–1G). It should be noted that NTCP expression did not appear to affect HCV entry in a recent Huh7 cell line model (Meredith et al., 2016). Huh7.5.1 cells are derived from Huh7.5 cells, which differ from Huh7 cells by a single point mutation in the retinoic acid-inducible gene-I (Bartenschlager

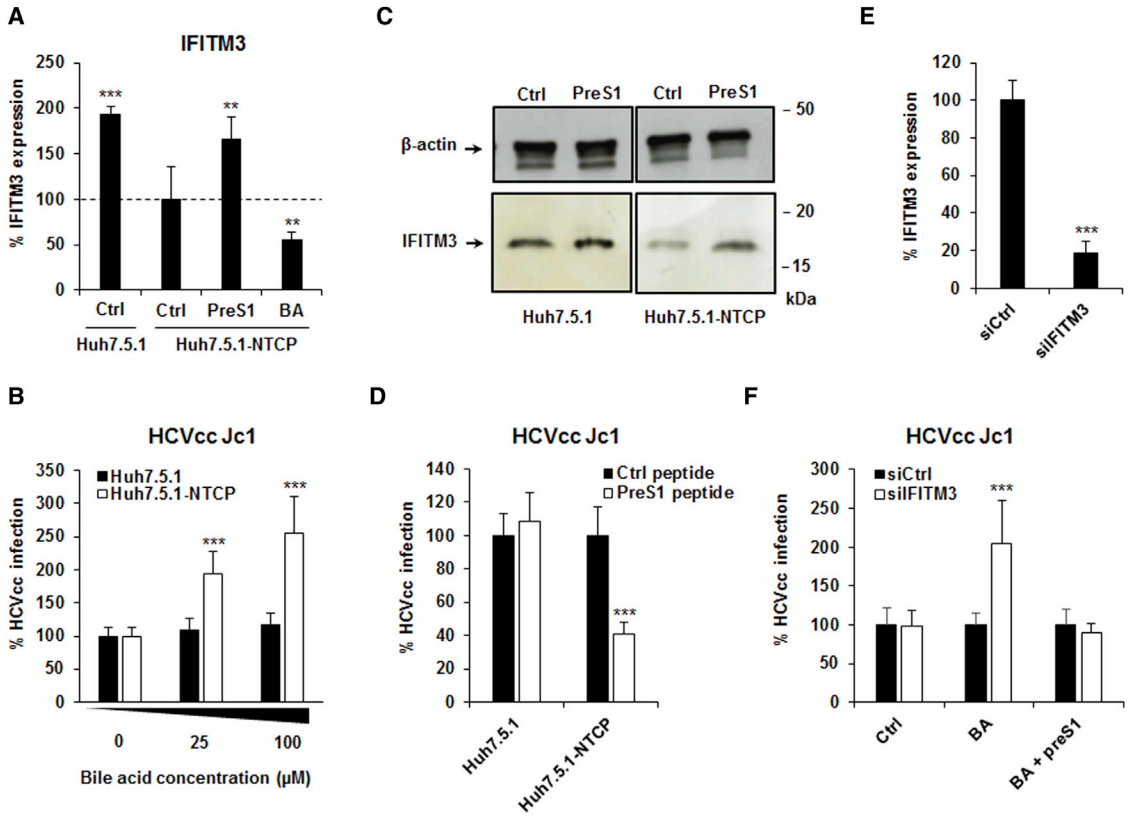


**Figure 5. Bile Acid Uptake Enhances HCV Infection by Decreasing ISG Expression**

(A) The impact of bile acid and preS1 treatment on *IFITM3* expression. Huh7.5.1-NTCP cells were treated with the bile acid (BA) sodium taurocholate (100 μM) or with 200 nM preS1 for 72 hr. IFITM3 expression was then quantified by qRT-PCR. Results are expressed as means ± SD percentage IFITM3 expression compared to untreated (Ctrl) Huh7.5.1-NTCP cells (set at 100%) from three independent experiments performed in duplicate (n = 6).

(B) The impact of bile acid on HCVcc infection. Huh7.5.1 and Huh7.5.1-NTCP cells were treated 0, 25, or 100 μM sodium taurocholate for 72 hr and then infected with HCVcc (Luc-Jc1). Results are expressed as means ± SD percentage HCVcc infection compared to untreated (Ctrl) Huh7.5.1 and Huh7.5.1-NTCP cells (both set at 100%) from three independent experiments performed in triplicate (n = 9).

(C and D) Effect of preS1-mediated inhibition of bile acid uptake on IFITM3 protein expression and HCVcc infection. Huh7.5.1 and Huh7.5.1-NTCP cells were treated with sodium taurocholate (100 μM) in the presence or absence of preS1 peptide for 72 hr. (C) IFITM3 protein expression was assessed by western blot. One experiment is shown. (D) Cells were then infected by HCVcc (Jc1) for 3 days. Results are expressed as means ± SD percentage HCVcc infection compared to Huh7.5.1 and Huh7.5.1-NTCP cells treated with control peptide (both set at 100%) from three independent experiments performed in triplicate (n = 9).

(E and F) Silencing of *IFITM3* expression increases HCV infection in a bile acid-dependent manner. Huh7.5.1-NTCP cells were transfected with siRNA control (siCtrl) or siRNA targeting IFITM3 (siIFITM3) and then treated for 72 hr in the absence or presence of BA (100 μM) in the presence of either preS1 or Ctrl peptide (200 nM). (E) Then 3 days after transfection, silencing efficacy was assessed by measuring expression of *IFITM3* mRNA by qRT-PCR. Results are expressed as means ± SD percentage *IFITM* expression from four independent experiments performed in triplicate (n = 12). (F) Cells were then infected with HCVcc Luc-Jc1. Infection was assessed after 72 hr by measuring luciferase activity. Results are expressed as means ± SD percentage HCVcc infection compared to cells treated with siCtrl (set at 100% for each condition) from four independent experiments performed in triplicate (n = 12).

and Pietschmann, 2005; Zhong et al., 2005). This may explain the differences observed between the two cell lines. Moreover, differences in bile acid concentrations in cell culture medium may be responsible for differences in the effect of NTCP on cell entry of HCV (Figure 5C). However, our loss-of-function studies and microarray analyses in PHHs (Figure 6) clearly demonstrate the impact of NTCP on HCV infection in primary cells with physiological innate immune responses.

For HBV/HDV infection, viral entry requires direct interaction with NTCP (Ni et al., 2014; Yan et al., 2012). Furthermore, HBV binding to NTCP may interfere with the physiological function of NTCP (i.e., bile acid uptake), and NTCP ligands can abrogate HBV/HDV infection (Oehler et al., 2014; Yan et al., 2014). In contrast, NTCP modulates HCV entry independently of direct binding mechanisms. We confirmed the inhibitory effect of the HBV preS1-derived peptide on HDV infection following 1-hr treatment (Figure 3D), but we did not see a corresponding effect on HCV infection under these conditions. Furthermore, the effect of NTCP on HCV infection does not appear to involve HCV E2 or binding factors CD81 and SR-BI (Pileri et al., 1998; Scarselli et al., 2002) (Figures 3A and 3B). NTCP expression did not modulate the expression of canonical entry factors CLDN1 and OCLN or EGFR either (Figure 3C).

Other mechanisms may contribute to the effects of NTCP on viral entry and infection that we observed. Indeed, modulation of NTCP bile acid transport activity by preS1 affected IFN

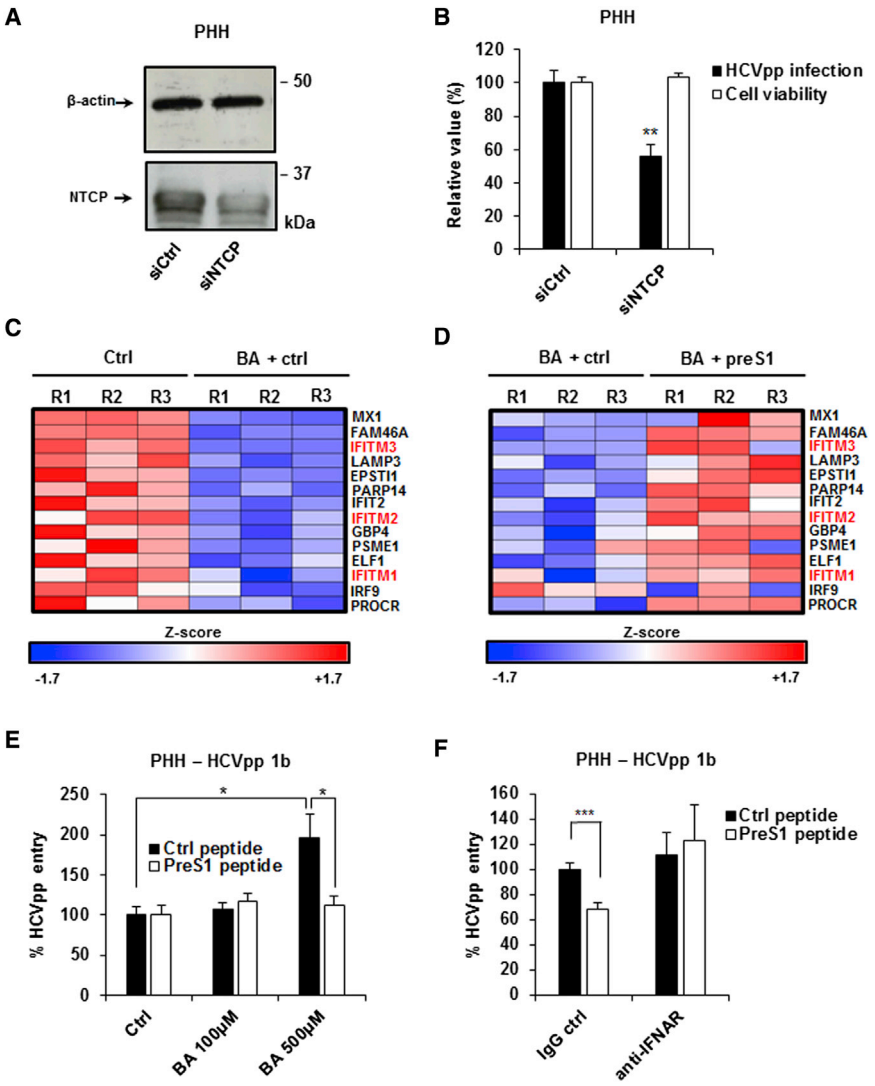


**Figure 6. NTCP-Mediated Bile Acid Uptake Modulates ISG Expression and HCV Entry in PHHs**

(A and B) Silencing NTCP expression in PHHs. PHHs were transfected with siRNA control (siCtrl) or siRNA targeting NTCP (siNTCP). 3 days after transfection, siRNA efficacy was assessed by western blot (A), and cell viability was assessed using PrestoBlue reagent (B). Results are expressed as means ± SEM percentage cell viability compared to cells treated with siCtrl from two independent experiments performed in quintuplicate (n = 10) (B). 3 days after NTCP silencing, PHHs were incubated with HCVpp (genotype 1b). Infection was assessed after 72 hr by measuring luciferase activity. Results are expressed as means ± SEM percentage HCVpp entry from three independent experiments performed in triplicate (n = 9) (B).

(C and D) Bile acids modulate the expression of ISGs in PHHs. PHHs from a single donor were treated with the bile acid (BA) sodium taurocholate (500 μM) in the presence or absence of the preS1 peptide (400 nM) for 48 hr. Cells were then lysed and total RNA was extracted and purified. Total gene expression was analyzed by genome-wide microarray. Three independent biological replicates per condition were analyzed. (C) Individual IFNα response genes that are significantly ($p < 0.05$) repressed following BA treatment are shown. Individual *Z* scores for each sample are presented. Negative *Z* score (blue) and positive *Z* score (red) correspond to repression and induction of the indicated genes, respectively. (D) Effect of preS1 treatment on the expression of IFNα response genes presented in (C). Individual *Z* scores for each sample are presented.

(E) Effect of bile acid and preS1 treatment on HCVpp infection in PHHs. PHHs were treated with increasing concentrations (0, 100, and 500 μM) of BA in the presence of either preS1 or Ctrl peptide (400 nM) for 72 hr and then infected with HCVpp (genotype 1b). Infection was assessed after 72 hr by measuring luciferase activity. Results are expressed as means ± SEM percentage HCVpp entry compared to untreated PHHs in the presence of the control peptide (set at 100%) from four independent experiments performed in triplicate (n = 12).

(F) PreS1 inhibition of HCVpp entry is dependent on the IFN-signaling pathway in PHHs. PHHs were treated with 500 μM BA in the presence of preS1 or a scrambled control peptide (400 nM), with or without treatment with an antibody targeting the type I IFN receptor (IFNAR) for 72 hr. Cells were then infected with HCVpp (genotype 1b) and infection was assessed after 72 hr by measuring luciferase activity. Results are expressed as means ± SEM percentage HCVpp entry compared to PHHs treated with the control peptide and an IgG control (set at 100%) from three independent experiments (one performed in triplicate and two in quintuplicate; n = 13).

responses, but also bile acid metabolism and cholesterol homeostasis in Huh7.5.1-NTCP cells as well as other pathways (Table 1). In particular, modulation of bile acid metabolism and cholesterol homeostasis by preS1 would alter cellular cholesterol pools, which has been shown to affect the activation of type I IFN responses (York et al., 2015) and may potentially contribute to the effect on HCV infection that we observed. Furthermore, modulation of signaling and transcription factor targets (Table 1) may have additional effects on viral replication and translation.

Our results demonstrate that NTCP, acting by distinct mechanisms, is relevant for the three major viruses causing chronic hepatitis and liver disease. This finding could contribute to the development of antiviral strategies targeting NTCP. Targeting viral cell entry with receptor antagonists, antibodies, peptides, and receptor kinase inhibitors has provided perspectives to prevent and treat chronic hepatitis B and C infections (Colpitts et al., 2015). Myrcludex B, a preS1-derived peptide targeting NTCP (Ni et al., 2014; Volz et al., 2013), has been shown to protect against HBV infection (Petersen et al., 2008), to modulate viral spread in animal models (Volz et al., 2013), and to decrease HDV viral load in patients in a phase II clinical trial (Bogomolov et al., 2016). Here we show that preS1-NTCP binding enhances the expression of ISGs. These data suggest that myrcludex B may inhibit HBV

infection by a dual mode of action, by interfering with viral binding and potentially increasing ISG expression. NTCP-targeting agents in clinical development also may inhibit HCV infection, which is of interest particularly for patients with HBV/HDV/HCV co-infections.

NTCP is a member of the solute carrier (SLC) family of proteins, a group of membrane proteins having crucial roles in many physiological functions (César-Razquin et al., 2015). However, these proteins are relatively uncharacterized. Here we uncover NTCP as a mediator of innate antiviral responses in hepatocytes and establish a role for NTCP in the entry process of multiple viruses. Our data uncover an important role of SLCs in virus-host interactions by linking their function to the regulation of innate immune responses. Moreover, bile acid transport through SLCs profoundly affects liver gene expression (Table 1).

Overall, we have identified NTCP as a regulator of innate immune responses in the human liver. These findings improve the understanding of virus-host interactions in the human liver, and they may open perspectives for the development of broad antiviral therapies targeting hepatotropic viruses that cause chronic liver disease and cancer.

## EXPERIMENTAL PROCEDURES

### Cell Lines

The sources for 293T and Huh7.5.1 cells have been described (Lupberger et al., 2011). Huh7.5.1 cells were seeded in six-well plates at 50% confluency 1 day prior to transduction with human NTCP-expressing VSVpp (GeneCopoeia). Cells were incubated in DMEM (Gibco) containing 10% fetal bovine serum (Dutscher). After 3 days, the cells were expanded and selected for NTCP expression with 1.8 μg/mL puromycin.

### PHHs

Liver tissue from patients undergoing surgical resection was obtained with informed consent from all patients. The respective protocols were approved by the Ethics Committee of the University of Strasbourg Hospitals (CPP 10-17). PHHs were isolated from liver resection tissue and cultured in William's E medium (Sigma) (Krieger et al., 2010; Lupberger et al., 2011).

### Viral Infection

Lentiviral pseudoparticles expressing HCV envelope glycoproteins (HCVpp strains HCV-J [1b], JFH1 [2a], NIH S52 [3a], and UKN4.21.16 [4]), VSVpp, MLVpp, as well as cell culture-derived HCVcc (Luc-Jc1 and Luc-JcR2A) were generated as described (Fofana et al., 2010; Lupberger et al., 2011). Huh7.5.1 cells and PHHs were infected as described (Krieger et al., 2010; Lupberger et al., 2011). Pseudoparticle entry and HCVcc infection were assessed by measuring luciferase activity 72 hr post-infection (Krieger et al., 2010; Lupberger et al., 2011). HDV production and infection have been described (Verrier et al., 2016) (see the Supplemental Experimental Procedures).

### Gene Expression Analyses in Huh7.5.1-NTCP Cells and PHHs Treated with Bile Acids and preS1 Peptide

Huh7.5.1-NTCP cells were treated with preS1 or a control peptide (200 nM) for 48 hr. Alternatively, PHHs were treated with bile acids (500 μM) in presence of preS1 or a control peptide (400 nM) for 48 hr. Total RNA from three biological replicates per condition from one experiment was then extracted using the ReliaPrep Kit (Promega), and 200 ng was subjected to genome-wide transcriptome profiling using HumanHT-12 beadarray (Illumina), following the manufacturer's protocol. Raw scanned data were normalized by cubic spline algorithm using Illumina normalizer module of GenePattern genomic analysis toolkit (http://software.broadinstitute.org/cancer/software/genepattern), as previously described (Hoshida et al., 2008). Modulated molecular pathways were determined using GSEA (Subramanian et al., 2005).

### Statistical Analysis

Each experiment was performed at least two times in an independent manner. The number of independent experiments as well as the total number of biological replicates (n) are indicated in the figure legends. Statistical analyses were performed using Mann-Whitney U test by comparing values from every biological replicate per study (indicated by "n" in the figure legends); $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***) were considered statistically significant. Significant p values are indicated by asterisks in the individual figures. For microarray analyses, two-tailed unpaired Student's t test was performed by comparing the values from three biological replicates ($p < 0.05$ was considered statistically significant).

## ACCESSION NUMBERS

The accession number for the datasets reported in this paper is GEO: GSE85092.

## SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and two figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2016.09.084.

## AUTHOR CONTRIBUTIONS

M.B.Z. and T.F.B. designed and supervised research. T.F.B. initiated the study. E.R.V., C.C.C., C.B., L.H., L.Z., F.X., C.T., E.C., R.G., and C.S. performed experiments. E.R.V., C.C.C., C.B., L.H., L.Z., F.X., C.T., E.C., R.G., C.S., Y.H., M.B.Z., and T.F.B. analyzed data. P.P. and F.-L.C. contributed key reagents. E.R.V., C.C.C., J.A.M., M.B.Z., and T.F.B. wrote the paper. E.R.V. and C.C.C. contributed equally to this work as first authors. M.B.Z. and T.F.B. contributed equally to this work as senior authors.

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# Approches de génomique fonctionnelle pour la caractérisation de facteurs hépatiques impliqués dans l'infection par le virus de l'hépatite B

**Résumé :**

L'infection chronique par le virus de l'hépatite B (HBV) est un problème majeur de santé publique et est la principale cause de développement de maladies hépatiques chroniques progressives et de carcinome hépatocellulaire. Alors que le génome et la structure du virus ont été étudiés en détail, le manque de modèles d'étude *in vitro* efficaces a été un frein pour la découverte et la compréhension des interactions moléculaires entre le virus et son hôte. Or, une meilleure compréhension des interactions virus-hôtes est primordiale pour le développement de nouveaux antiviraux efficaces. Les récents progrès sur la compréhension de l'entrée virale ouvrent la voie au développement de nouveaux modèles d'infection adaptés aux études de génomiques fonctionnelles à haut débit, opportunité unique pour découvrir de nouvelles cibles thérapeutiques à grande échelle. La mise au point de modèles d'infection non disponibles au laboratoire au début de ma thèse et leur validation dans des études sur les interactions virus-hôte, a permis l'étude d'un mécanisme viral encore mal compris qu'est la détection et l'échappement du HBV à la réponse immunitaire innée. Ces modèles ont par la suite permis de mettre au point et de réaliser un criblage fonctionnel à haut débit qui a identifié CDKN2C comme facteur d'hôte impliqué la réplication virale du HBV. Ainsi, les travaux réalisés dans le cadre de cette thèse ont permis la mise en évidence et la compréhension de différents mécanismes d'interactions du virus avec la cellule hôte lors de l'infection. Ils ouvrent la voie au développement de nouveaux systèmes d'infection plus physiologiques et la caractérisation de nouvelles cibles thérapeutiques permettant d'éradiquer le VHB.

**Mots Clés** : virus hépatite B, interactions virus-hôte, génomique fonctionnelle.

**Abstract :**

Chronic infection by hepatitis B virus (HBV) is a major public health issue and remains the principal cause of progressive chronic liver disease and hepatocellular carcinoma. While the genome and structure of the virus have been studied in great details, the lack of efficient cell culture models has impaired the understanding of molecular interactions between virus and its host cells. However, a better understanding of virus-host interactions is crucial for the development of new efficient antiviral strategy. Recent advances in the characterization of viral entry paved the way to the development of novel infection models available for innovative high-throughput functional genomic studies a unique opportunity to discover new therapeutic targets. The development of these models which were not available in the laboratory and their validation in virus-host interactions studies allowed me the investigation of poorly known viral mechanism as viral detection and escape of HBV from the innate immune response. These models were subsequently used in a functional high-throughput screen that identified CDKN2C as a cellular host factor involved in HBV viral replication. Thus, these works highlight new mechanisms of virus host interactions. They are the starting points of novel research programs contributing to the development of more physiological infection system and characterization of new therapeutic target to eradicate HBV.

**Key words:** hepatitis B virus, virus-host interaction, functional genomic