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Identification of PLK1 as a proviral factor for the hepatitis B virus replication: A possible target for antiviral and anticancerous drug development

Adrien Foca

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**Identification of PLK1 as a proviral
factor for the hepatitis B virus
replication:
A possible target for antiviral and
anticancerous drug development.**

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« La science est le calcul fait au plus juste de ce qu'on croyait savoir, et qu'on ne sait pas. »

Joseph Michel Antoine Servan

אותו מפחית שלו הידע את מעלה שאינו מי

Proverbe hébreu; תלמוד

Abstract

Identification of PLK1 as a proviral factor for the hepatitis B virus replication: A promising target for antiviral and anticancerous drugs development.

Keywords: Hepatitis B virus; Polo-like-kinase 1; small-interfering RNA, lipid nanoparticle; antiviral; host-targeting agent; hepatocellular carcinoma.

Thesis research realised by Adrien Foca within the Centre de Recherche en Cancérologie de Lyon (CRCL), UMR INSERM 1052, CNRS 5286.

In highly HBV endemic regions, 70-80% of hepatocellular carcinoma cases are attributable to this virus. Despite the existence of an HBV vaccine, the World Health Organization estimates 240 million individuals are chronically infected with HBV worldwide. Current antiviral treatments to control chronic HBV infections, and consequently reduce the incidence of liver cancer, are ineffective. New and effective therapies are needed not only for fighting the virus but also to prevent HCC emergence or progression.

The polo-like-kinase 1 (PLK1), which plays pivotal roles in mitosis and is over-expressed in many human cancers, represents a promising druggable target in oncology. Beside its role during cell division, PLK1 is also thought to be involved in gene expression regulation during interphase. It was shown that the X protein (HBx) could activate PLK1 in murine cell transformation models. Yet it remained to be determined whether PLK1 could also play a role for HBV replication in non-dividing hepatocytes. Our, and collaborators, recent studies have identified a positive link between PLK1 activation and HBV replication. The goal of this thesis project was to investigate the mechanism(s) by which PLK1 exerts a positive effect on HBV replication, with the future goal of exploring PLK1 as an antiviral target.

The interplay between PLK1 and HBV replication was firstly described using the HepAD38 cellular model of HBV replication. In this context, the HBV DNA is stably integrated into the host genome, under control of a Tet-off expression system. Transcription of HBV pregenomic RNA (pgRNA), the template of viral replication, is initiated by tetracycline removal. It has been shown that in HBV-replicating HepAD38 cells, increased PLK1 expression correlates with down-regulation of two proteins that are components of chromatin modifying complexes; SUZ12 protein of the PRC2 complex, and ZNF198 of the LSD1-CoREST-HDAC1 complex. PLK1 inhibition was described to inhibit HBV replication by reducing viral transcription. How PLK1 regulates HBV transcription remains unknown. On the other hand, in HBV replication models that resemble physiologic HBV infection, comprised of Primary Human Hepatocytes (PHH) and non-transformed/differentiated HepaRG cells (dHepaRG), where HBV replicates in non-transformed and non-dividing cells, thus enabling the study of the inter-phasic role of PLK1, irrespective of its well-established cell division implication, we have demonstrated that:

1) A pharmacological inhibition of PLK1 suppressed HBV replication by a different mechanism, likely targeting the packaging of pgRNA by the HBV core antigen (HBc).

2) Knocking-down PLK1 using siRNA delivered by lipid nanoparticles (LNP siPLK1) results in a strong drop of HBV DNAs, RNAs and HBe/HBsAg secretion without affecting the cell viability.

This thesis project brought the proof of concept that PLK1 could be a drug target in HBV infection. Furthermore, the use of LNP allowed us to improve the delivery of siPLK1 to hepatocytes. Significantly, PLK1 inhibition is not toxic to quiescent cells in comparison to fast growing cancer cells, rendering PLK1 an attractive therapy target. High level of viremia in chronic HBV patients is a risk factor for progression to liver cancer. PLK1 specific inhibitors are already in clinical trials for other types of cancer (e.g., acute myeloid leukaemia) and could serve as bimodal therapy in HBV infected patients, by inhibiting virus replication as well as preventing emergence and spreading of neoplastic cells.

This project was part of a full-working group of experts and thus, has benefited of a strong support. The proximity of the oncology-specialized hospital, the Centre Léon Bérard provided us with fresh hepatic biopsy. Moreover, our project had access to liver-humanized mice, a closer model to physiological behaviors, to validate our approach.

Inhibition of cellular PLK1 is a novel antiviral approach, which in combination therapies with interferon (IFN- α) or nucleoside analogs offers great promise for curing chronic HBV infection and could even prevent emergence of cell transformation.

Résumé

Identification de PLK1 comme facteur proviral dans la réplication du virus de l'hépatite B: Une cible thérapeutique attrayante pour le développement de molécules antivirales et anticancéreuses.

Mots-clés: Hépatite B virus ; Polo-like-kinase 1 ; ARN interférents ; nanoparticules lipidiques ; antiviraux ; carcinome hépatocellulaire.

Travaux de thèse réalisés par Adrien Foca au sein du Centre de Recherche en Cancérologie de Lyon (CRCL), UMR INSERM 1052, CNRS 5286.

Dans les régions de fortes endémicités, 70-80% des carcinomes hépatocellulaires sont induits par le VHB. Bien qu'un vaccin prophylactique très efficace existe, il n'est d'aucune utilité pour les 250 millions de personnes chroniquement infectées. Les traitements actuels pour contrôler l'infection chronique par le VHB montrent des limites et le besoin de nouvelles thérapies se fait ressentir.

La Polo-like kinase-1 (PLK1), qui joue un rôle essentiel dans la mitose et est surexprimée dans de nombreux cancers, représente une cible prometteuse. Outre son rôle lors de la division cellulaire, PLK1 est impliquée dans la régulation de l'expression des gènes en interphase. Il a été montré que la protéine X du VHB (HBx) active PLK1 dans des modèles de cellules murines. Cependant, il restait à déterminer si PLK1 jouait un rôle au niveau de la réplication du VHB dans des hépatocytes quiescents. Des études récentes ont mis en évidence un lien positif entre l'activation de PLK1 et la réplication du VHB. Le but de ce projet de thèse a été d'étudier le(s) mécanisme(s) par le(s)quel(s) PLK1 jouait un rôle positif sur la réplication virale, avec pour objectif futur d'explorer l'inhibition de PLK1 comme cible antivirale.

L'interaction entre PLK1 et la réplication du VHB a d'abord été décrite à l'aide du modèle HepAD38. Dans ce contexte, l'ADN viral est intégré dans le génome hôte, sous le contrôle d'un système d'expression Tet-off. La transcription de l'ARN pré-génomique (pgRNA), à la base de la réplication virale, est initiée par la suppression de tétracycline. Dans ce contexte, l'augmentation de l'expression de PLK1 est corrélée avec la régulation négative de deux protéines; SUZ12 et ZNF198, faisant partie de complexes de remodelage de la chromatine. L'inhibition de PLK1 bloque la réplication du VHB, en agissant au niveau de la transcription virale. D'autre part, dans les modèles de réplication du VHB qui miment au mieux une infection, comprenant les hépatocytes primaires humains (PHH) et les cellules non transformées/différenciées HepaRG (dHepaRG), où le VHB se réplique dans des cellules quiescentes, nous avons mis en évidence que:

1) L'inhibition pharmacologique de PLK1 bloque la réplication virale, semblablement en perturbant l'encapsidation du pgRNA via une interaction avec la protéine core du VHB (HBc).

2) Un knocking-down de PLK1 en utilisant des ARN interférents délivrés par nanoparticules lipidiques résulte en une forte baisse de la production de pgRNA et dans la sécrétion des antigènes HBeAg/HBsAg, sans impact sur la viabilité cellulaire.

Ce projet a donc permis la preuve de concept que PLK1 pouvait être une cible thérapeutique afin de contrôler la réplication du VHB. De plus, grâce à la technologie de délivrance par nanoparticules lipidiques d'ARN interférents, nous avons pu cibler spécifiquement les hépatocytes, augmentant de ce fait la spécificité et l'efficacité de nos traitements. Un travail sur la compréhension précise des mécanismes cellulaires impliqués permettra de mieux cerner cette interaction hôte/virus afin de poursuivre le développement de stratégies antivirales innovantes portant sur l'inhibition de PLK1.

De manière significative, l'inhibition de PLK1 est non toxique pour les cellules quiescentes par rapport à des cellules cancéreuses à fort taux répliatif, ce qui fait de PLK1 une cible thérapeutique attrayante. Des inhibiteurs spécifiques sont déjà en essais cliniques pour certains cancers (e.g., Volasertib pour le traitement de la leucémie myéloïde aiguë) et pourraient servir de thérapie bimodale dans le cadre de patients infectés par le VHB, en inhibant la réplication virale, ainsi qu'en prévenant l'émergence de cellules néoplasiques.

L'inhibition de la PLK1 est une approche antivirale innovante, qui, en combinaison avec les thérapies actuelles de type IFN- α ou analogues nucléotidiques offre de grandes promesses pour endiguer l'infection chronique par le VHB mais également prévenir les événements carcinogéniques.

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List of Abbreviations

aa	amino acid
ABC	ATP-binding cassette
ADV	Adefovir dipivoxil
AFB1	Aflatoxin B1
AIP1	Actin-interacting protein 1
ALT	Alanine amino transferase
AML	Acute myeloid leukemia
APC/c	Anaphase-promoting complex
ATF	Activating transcription factor
ATM	Ataxia-telangiectasia mutated
AuAg	Australia antigen
AXL	AXL receptor tyrosine kinase
BAMBI	BMP and activin membrane bound inhibitor
BCLC	Barcelona clinic liver cancer
BCP	Basal core promoter
BDKRB2	Bradykinin B2 receptor
BRCA1/2	Breast cancer 1/2
Brd4	Bromodomain-containing protein 4
CBP	CREB-binding protein
cccDNA	covalently-closed-circular DNA
CCNE1	G1/S-specific cyclin-E1
CD	Cluster of differentiation
CDC5	PLK homolog in <i>Saccharomyces cerevisiae</i>
CDE-CHR	Cell-cycle-dependent element-cell-cycle genes homology region
CDKs	Cyclin-dependent kinases
CHB	Chronic hepatitis B
ChIP	Chromatin immuno precipitation
Chks	Checkpoint kinases
Co-REST	REST corepressor 1
CpAM	Core protein allosteric modulator
CREB	C-AMP response element-binding protein
CRISPR	Clustered regularly interspaced short palindromic repeats
CSPH	Clinically significant portal hypertension
CTD	C-Terminal domain

CTLA-4	Cytotoxic T-lymphocyte-associated antigen-4
CTLs	Cytotoxic T lymphocytes
CUL3	Cullin 3
CYLD	Cylindromatosis
DAA	Direct-acting antiviral
DDB1	DNA damage-binding protein 1
DHBV	Duck hepatitis B virus
dHepaRG	Differentiated HepaRG
DR	Direct repeat
DREAM	DP, Retinoblastoma [Rb]-like, E2F, and MuvB complex
DSB	Double strand break
dsDNA	Double stranded linear DNA
E2F1	E2F transcription factor 1
Ect2	Epithelial cell transforming 2
Enh	Enhancer
EpCAM	Epithelial cell adhesion molecule
ESCRT	Endosomal sorting complexes required for transport
ETV	Entecavir
FDA	Food and drug administration
FoxM1	Forkhead box M1 protein
FoxN1	Forkhead box N1 protein
FRG	Fah/Rag2/Il2rg triple mutants
FST	Follistatin
GAB2	GRB2-associated-binding protein 2
GBD	Global burden of disease
GLUL	Glutamate-ammonia ligase
GRIK1	Glutamate ionotropic receptor kainate type subunit 1
GSHV	Ground squirrel hepatitis virus
GTSE1	G2 and S-phase-expressed 1 protein
GWAS	Genome-wide association study
HBc	Hepatitis B core protein
HBeAg	Hepatitis B e antigen
HBsAg	Hepatitis B s antigen
HBSP	Hepatitis B splicing-regulated protein
HBV	Hepatitis B virus
HBx	Hepatitis B x protein

HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HDAC1	Histone deacetylase 1
HLA	Human leukocyte antigen
HNF4α	Hepatocyte nuclear factor 4 alpha
HOTAIR	HOX transcript antisense RNA
HPV	Human papillomavirus
HR	Homologous recombination
HTA	Host targeting agent
IARC	International agency for research on cancer
ICTV	International committee on the taxonomy of viruses
IDL	Interdomain linker
IFN	Interferon
IKK	I κ B kinase
INCENP	Inner centromere protein
IU	International unit
IV	Intravenous
KIF1B	Kinesin family member 1B
KIT	Mast/stem cell growth factor receptor or CD117
KLHL22	Kelch like family member 22
LAM	Lamivudine
L-HBsAg	Large hepatitis B s antigen
LNP	Liponano particle
LSD1	Lysine-specific histone demethylase 1
Map205	Microtubule-associated Protein 205
MAP3K8	Mitogen-activated protein kinase kinase kinase 8
M-CDK	Mitotic-cyclin-dependent kinase
MDM2	Mouse double minute 2
MET	MNNG HOS transforming protein
M-HBsAg	Medium hepatitis B s antigen
MHC	Major histocompatibility complex
MiR	Micro RNA
MLL4	Mixed-lineage leukemia 4
MRN	Mre11, Rad50 and Nbs1 complex
mRNA	messenger RNA
MTOC	Microtubule-organizing center

mTOR	mechanistic target of rapamycin
MVBs	Multivesicular bodies
MYC	avian myeloblastosis virus oncogene cellular homolog
Myd88	Myeloid differentiation primary response 88
MYT1	Myelin transcription factor 1
NAs	Nucleos/tide analogs
Nedd4	Neural precursor cell expressed developmentally down-regulated protein 4
NFkB	Nuclear factor kB
NIH	National institutes of health
NK	Natural killer
Nlp	Ninein-like protein
NTCP	Na ⁺ taurocholate cotransporting polypeptide
Orc2	Origin recognition complex subunit 2
ORF	Open reading frame
p53BP1	p53 binding protein 1
PB	Polo box
PBD	Polo box domain
PC	PreCore
PCR	Polymerase chain reaction
PDGFR	Platelet-derived growth factor receptor
pgRNA	pregenomic RNA
PHH	Primary human hepatocyte
PI3K	Phosphoinositide 3 kinase
PIV5	Parainfluenza virus 5
PLK1	Polo-like kinase 1
PLKs	Polo-like kinase(s)
Plo1	PLK homolog in <i>Schizosaccharomyces pombe</i>
Pol	Polymerase
PP2A	Phospho-protein phosphatase 2A
PRC2	Polycomb repressive complex 2
PTEN	Phosphatase and tensin homologue
RACE-PCR	Rapid amplification of cDNA-ends PCR
RAD51	RecA homolog recombination protein
RAF	Rapidly accelerated fibrosarcoma
rcDNA	relaxed-circular DNA
REST	RE1-silencing transcription factor

RET	Rearranged during transfection protein
RhoGEF	Rho guanine nucleotide exchange factor
RIP	RNA immunoprecipitation
RT	Reverse transcription
S-HBsAg	Small hepatitis B s antigen
siRNA	small interfering RNA
SLC13A2	Solute carrier family 13 member 2
Smc5/6	Structural maintenance of chromosome 5/6 complex
SNALP	Stable nucleic acid lipid particles
SP1 RNA	Spliced 1 RNA
SRPK1	SRSF protein kinase 1
STATs	Signal transducer and activator of transcription
SUMO	Small ubiquitin-like modifier
SUZ12	Suppressor of Zeste 12 protein homolog
TAF	Tenofovir alafenamide fumarate
TBV	Telbivudine
TDF	Tenofovir disoproxil fumarate
TERT	Telomerase reverse transcriptase
Tet	Tetracyclin
TFV	Tenofovir
TLR	Toll-Like receptor
TNF-a	Tumour necrosis factor alpha
TNM	Tumour, node, metastasis
TP	Terminal protein
tsg101	Tumor susceptibility gene 101
uPA	urokinase plasminogen activator
VEGFR	Vascular endothelial growth factor receptor
VPS4B	Vacuolar protein sorting 4 homolog B
vRNA	viral RNA
WEE1	WEE1 G2 checkpoint kinase
WHO	World health organization
WHV	Woodchuck hepatitis virus
WMHV	Woolly monkey hepatitis virus
ZNF198	Zinc finger protein 198

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PART I: BIBLIOGRAPHY AND STATE-OF-THE ART

Chapter 1: Hepatitis B Virus

1.1. HBV discovery: from Hippocrate to Blumberg. A story where jaundice and leukemia act as protagonists

The first descriptions of jaundice epidemics, jaundice being a common symptom of hepatitis B virus (HBV) infections, were reported in the time of ancient China and Babylonia. In Occident, Hippocrate documented an outbreak of jaundice that occurred in the Greek island of Thassos, in the fifth century B.C. Other episodes were observed in the Middle Ages, due to crowded and unsanitary conditions, mostly during times of war (Martin, 2003). The oldest evidenced case of hepatitis B was found in a 450-year-old mummy, but studies tends to indicates that HBV genotypes diversified long before the 16th century, between at least 3,000 years and possibly as long as 100,000 years ago (Kahila Bar-Gal et al., 2012; Patterson Ross et al., 2018).

As early as 1940, medical researchers had determined that there were two different forms of virus-induced jaundice; one that is transmitted as an intestinal infection (i.e., feco-oral transmission), and the other spread mainly by blood transfusion/contact. They were respectively named Hepatitis A and B viruses.

The HBV discovery came by an indirect route. In 1963, Dr Baruch Blumberg, working at the NIH, was tracking inherited polymorphic serum proteins in the blood of patients worldwide. Blumberg and colleagues used sera from multi-transfused individuals as sources of antibody to human serum proteins. The idea was that these sera could contain antibodies bound to proteins differing in sequence from those of the transfusion recipients. They screened over 20,000 sera specimens and identified a new antigen, named "Australia antigen" (AuAg) from an Australian Aborigine blood (Blumberg et al., 1965). This antigen was found to be very stable and demonstrated a relatively rare occurrence in the United States population compared to people originating from Asia and pacific islands. Moreover, sera from multi-transfused and leukemic patients exhibited high frequency of AuAg over anti-AuAg antibody. These led Blumberg and colleagues to hypothesize that AuAg predicted leukemia risk. However, in 1966, James Blair, a patient with Down syndrome, initially negative for AuAg was observed to seroconvert concomitantly with "chronic anicteric hepatitis" (Blumberg et al., 1967). In the mean time, a member of Blumberg's laboratory experienced an hepatitis due to contact with contaminated materials, again with the appearance of AuAg in the blood. These interesting observations allowed the possible association between a viral infection and AuAg, which is now named HBsAg. The ability to carry out retrospective studies with assays for AuAg confirmed a long-held suspicion that HBV was responsible for a chronic hepatitis leading to cirrhosis and liver cancer in many parts of the world (Okochi and Murakami, 1968; Prince, 1968). Eventually, in 1970, Dane observed complete HBV particle as virus-like entities in serum of patients with AuAg-related hepatitis (Dane et al., 1970)(Figure 1). Since its discovery, research works on the virus have lead to a better understanding of its life cycle and natural history, as well as to the development of viro-suppressive treatments. Work is yet to be done to cure patients from HBV infections (Figure 2).

“As ultimate reward for his work on the origins and spread of HBV, Baruch Blumberg received the 1976 Nobel Prize in Medicine.”

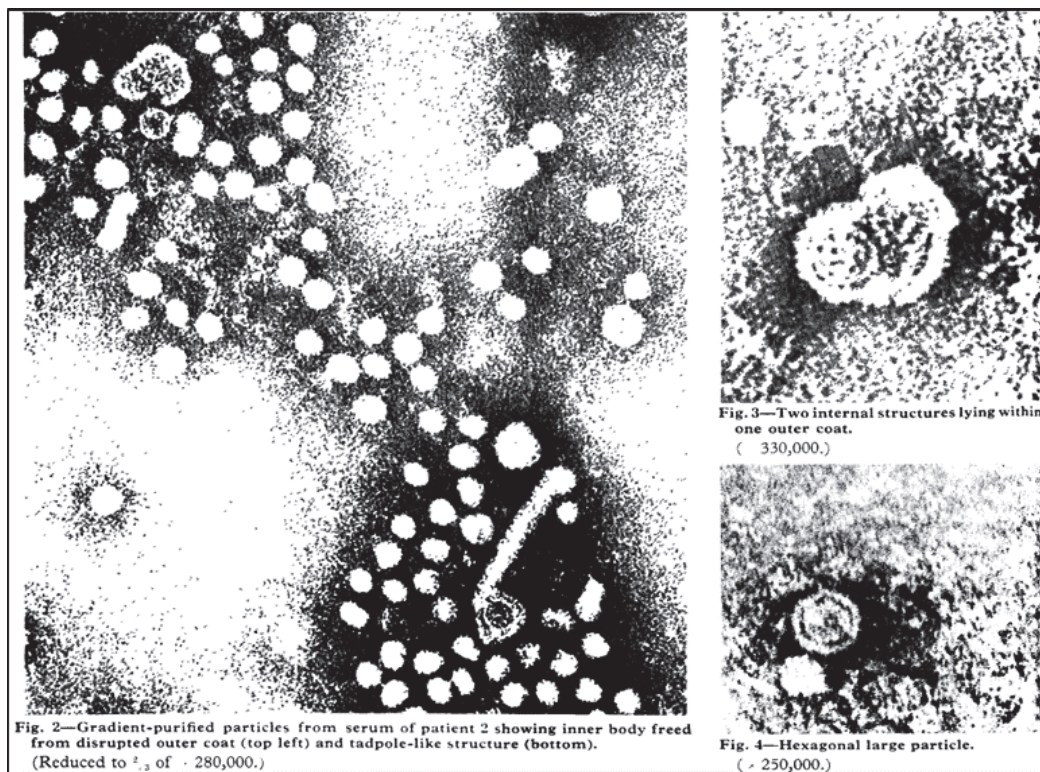


Figure 1: HBV complete virions and subviral particles. Original electron microscopy pictures taken by Dane and colleagues. Extracted from The Lancet, April 4, 1970, p695.

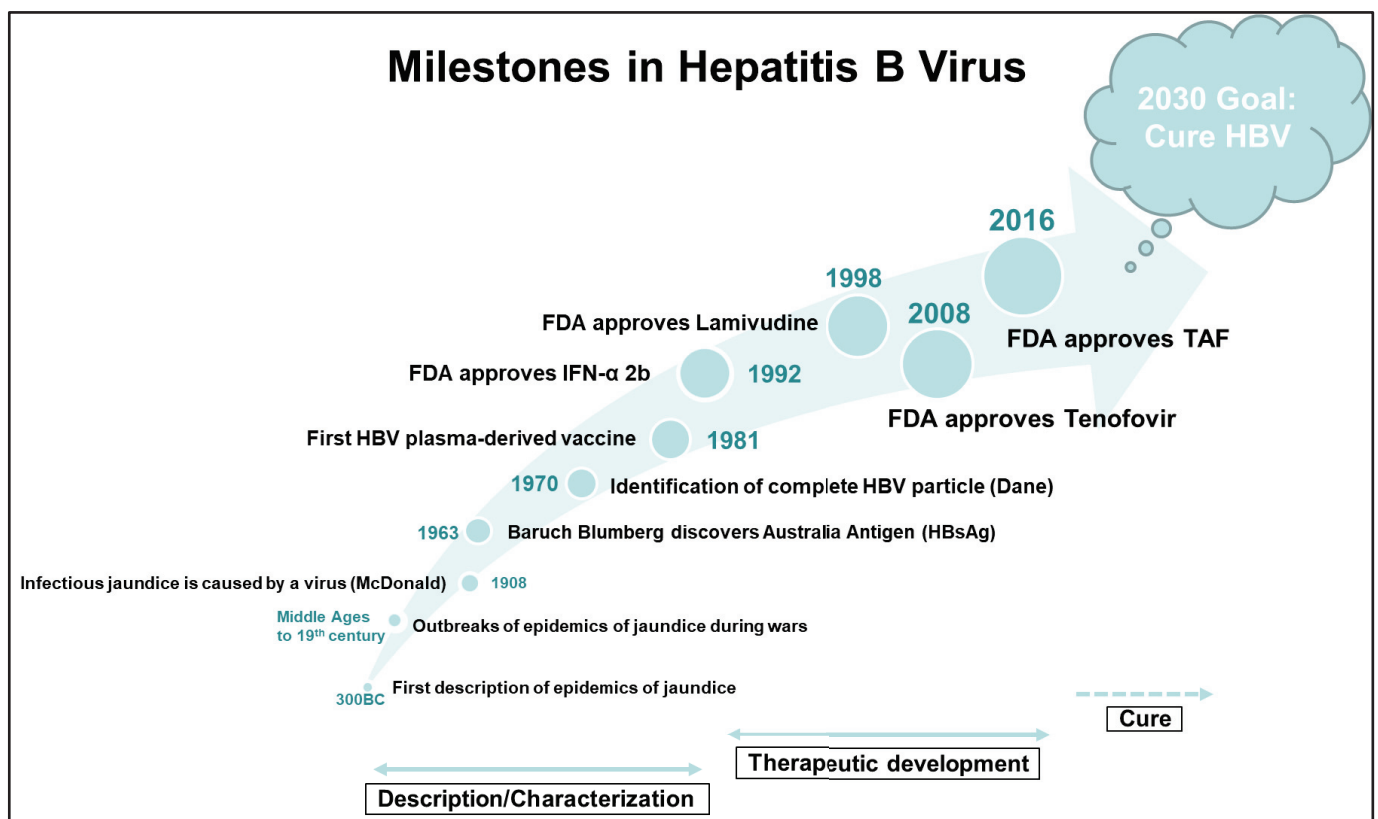


Figure 2: Milestones in HBV research. From first historical description to future goals. Principal facts depicted in three axes: “Description/Characterization”, “Therapeutic development” and “Cure”.

1.2. Classification and phylogeny of *Hepadnaviridae*

Viruses belonging to the *Hepadnaviridae* family, together with the *Spumaviridae* (foamy viruses), are the only known DNA viruses to replicate their DNA by reverse transcription of a viral pregenomic RNA template. HBV is considered as a prototype of the *Hepadnaviridae* family, which contains two genera, the orthohepadnaviruses that infect mammals, and avihepadnaviruses that infect birds (Chapter Version: ICTV Ninth Report; 2011 Taxonomy Release). This two genera assignment is based, in addition to the host specificity spectrum, on rather weak sequence homology between the two groups, despite the overall similar genome organisation. Duck hepatitis B virus (DHBV) and Heron hepatitis B virus (HHBV) are prototypes of the avihepadnavirus group. It is of importance to indicate here that DHBV served as an excellent model to study the molecular and cellular biology of *Hepadnaviridae* and develop nucleoside analogues, before the advents of transgenic/humanized animals allowing infection with human HBV. The orthohepadnavirus genus is mainly represented by four species, exhibiting a sequence divergence <35%, with HBV, WHV, GSHV, and WMHV as the prototypes (Figure 3). Another virus related to GSHV has been isolated in Arctic ground squirrel (ASHV) but has not been assigned yet due to lack of host range studies (Testut et al., 1996). Naturally, this classification is not absolute and may evolve according to future findings as well as to progress in species-to-species infection characterization. As an example of phylogenetic classification complexity, the woolly monkey hepatitis B virus is closely related in DNA sequence to HBV, with less than <20% sequence variation, but do not infect human or chimpanzee, which is highly susceptible to human HBV genotypes.

To date, eight well-characterized HBV genotypes have been identified (A to H) showing pairwise differences between 8 to 17%. Two new genotypes (i.e., I and J) have been identified in Vietnam/Laos and in the Ryukyu Islands in Japan respectively, but are not yet approved by the ICTV (International committee on the taxonomy of viruses) (Olinger et al., 2008; Tatematsu et al., 2009; Yu et al., 2010)(Figure 4). Interestingly, all these genotypes tend to exhibit different geographical distributions (Figure 5) and distinct clinical manifestations. Hence, genotype C is known to favour rapid progression to HBV-related cirrhosis, while genotype G is lacking HBeAg production due to mutation in the PreCore region (Stuyver et al., 2000). Finally, genotypes A–D, F, H and I are classified further into sub-genotypes with between 4% and 8% intergroup nucleotide divergences. Among them, the HBV F1b was recently described in Alaska native population to exhibit several core mutations, which are strongly associated with HCC development (Hayashi et al., 2018).

This diversity leads to different natural history of infection and modes of transmission, thus delineating the need of a large arsenal of therapeutic options (Liu et al., 2005).

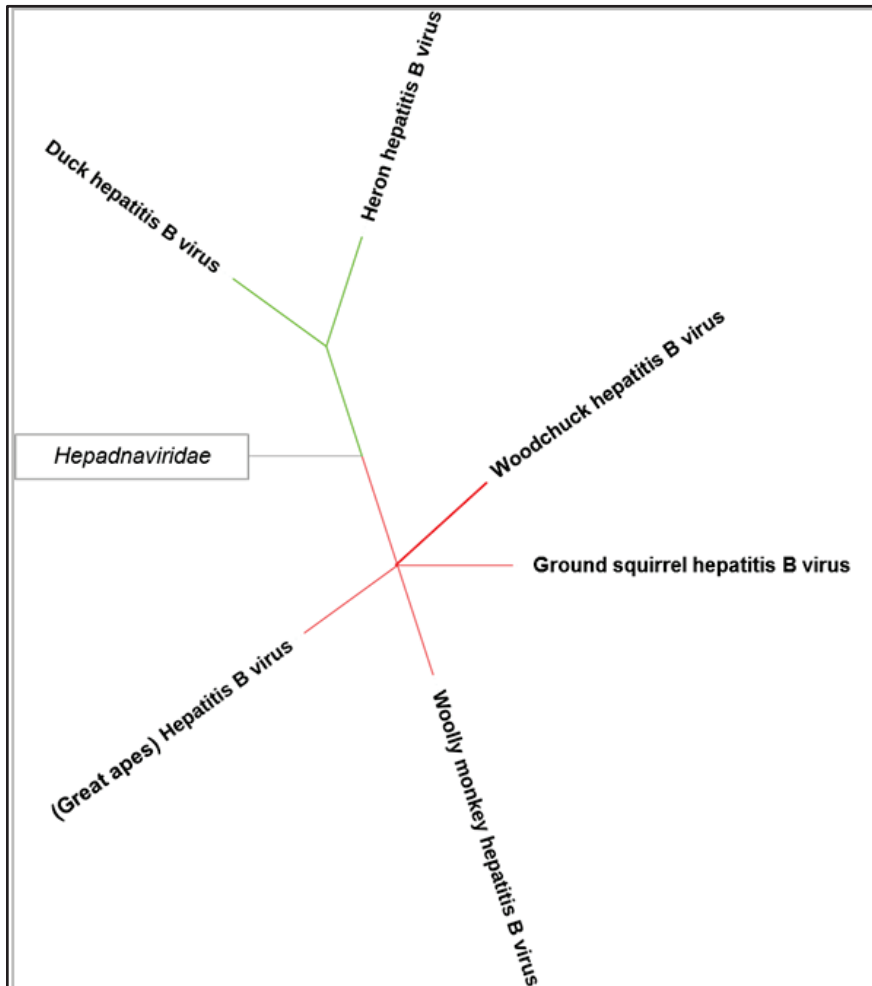


Figure 3: Phylogenetic tree of *Hepadnaviridae*. Avihepadnaviruses (*top*) and Orthohepadnaviruses (*bottom*) with prototype members. Created with itol.embl tool.

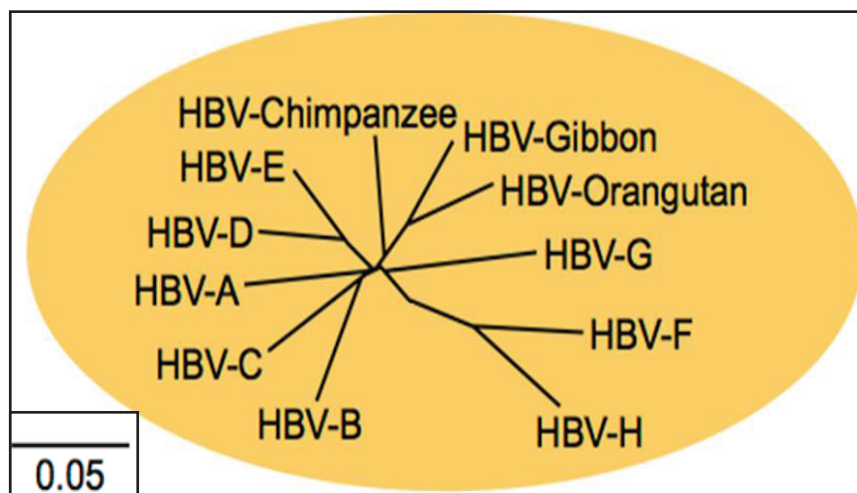


Figure 4: Phylogenetic tree of the genus *Orthohepadnavirus*, focusing on “great apes”. Complete genomes of HBV genotypes A (X02763), B (D00330), C (M12906), D (J02203), E (X75657), F (X69798), G (AF160501) and H (AY090454), and isolates found in chimpanzee (D00220), orangutan (AF193864) and gibbon (U46935), were aligned using Clustal W. Scale bar represents substitutions per site. Adapted from ICTV Ninth Report, 2011.

1.3. Epidemiology

1.3.1. HBV: a worldwide healthcare issue

The World Health Organization (WHO) reports there are around 250 million people chronically infected by HBV (CHB) worldwide, associated with a high co-morbidity due to hepatic complications. Of these 250 million, 25% could eventually die of chronic liver disease or hepatocellular carcinoma if left untreated (WHO Global hepatitis report, 2017). HBV is unevenly distributed worldwide with two highly-endemic areas, i.e., Africa and South-East Asia (Figure 6). The highest HBsAg seroprevalence is found in South Sudan with 22% of individuals being chronic carriers, while lowest-prevalence areas are observed in Northern and Western Europe. Globally, the prevalence of HBV decreased over the two past decades and now is estimated to be 3.6% of the world population (MacLachlan and Cowie, 2015; Schweitzer et al., 2015).

In the Global Burden of Disease Study 2010 (GBD 2010), 786,000 deaths were attributable to HBV, of which 341,400 were estimated to be caused by liver cancer, 312,400 by cirrhosis and 132,200 by acute hepatitis B (Lozano et al., 2012).

1.3.2. Entry into the host and transmission pathways

HBV is found in many body fluids and viral load may reach $>10^{10}$ copies per mL in blood. Thus, the rate of direct transmission is very high compared to other viruses such as HCV or HIV. Basically, HBV can be transmitted following two schemas:

Vertical transmission: defined by perinatal infection from infected mothers to the newborn or very young child. This route represents the greatest source of new infections worldwide but exhibits a clear dichotomy, depending on geographic areas. In Asia, the rate of perinatal transmission can reach 90% due to high rate of HBeAg positivity and high HBV viral load in mothers' sera. In Western Europe, North America and Africa, the risk of vertical transmission is approximately 10%, explained by lower HBeAg positivity in sera and better healthcare systems (from prevention to treatment accessibility). The high rate of chronicity in many parts of Africa is attributable to horizontal spread to young children from playmates and adults involved in their care. Hyper-immune globulin to HBsAg injection and vaccination at birth can reduce the risk of chronic infection by $>90\%$ (Beasley et al., 1983; Lo et al., 1985; Stevens et al., 1992). Some data suggest that HBeAg may cross the placenta, although, no evidences that this causes immune tolerance and facilitates chronic infection has yet been obtained.

Horizontal transmission: it regroups blood transfusion, IV (intravenous) drug use, sexual transmission and nosocomial infections. Since the 1970s, HBV infection through blood

transfusion has drastically decreased due to the abolition of paid donors and systematic screening of volunteer blood donors for serum HBsAg and anti-HBc immunoglobulins. Ongoing implementation of specific HBV-qPCR screening should decrease the risk of undetected rare variants escaping the serologic assay for HBsAg and solve the problem of occult HBV infections, in which neither HBsAg nor anti-HBc are detected. Sexual transmission still represents 30-40% of HBV infection new cases in developed countries while the role of intravenous drug use seems to be decreasing with time, currently representing only 6% of reported new cases. Nosocomial transmission is estimated to be 30% from highly viremic patients, but appears to be very rare (WHO, [guidelines for the prevention, care and treatment of persons with chronic hepatitis B infection, 2015](#)). Some cases have been reported in dialysis centers or with HBV infection from infected surgeons (Harpaz et al., 1996). Finally, percutaneous infection is still a major mode of HBV transmission, due to lifestyle in developing/developed countries (e.g., intravenous drug use, tattoos, acupuncture, and piercings) (Goldstein et al., 2002; Alter, 2003; Yang et al., 2015; Rempel et al., 2016).

1.4. Unraveling the complexity of Hepatitis B virus

1.4.1. Viral structure and subviral particles

Infectious HBV virions (Dane particles) are spherical entities of $\approx 42\text{nm}$ in diameter (depending on genotype) with no evidence of outer projections after negative staining (Transmission Electron Microscopy) observations. However, in cryo-EM, projections are distinguishable outside the envelope and are evidences of spike-like features composed of L, M, and S proteins (respectively Large, Medium and Small forms of HBsAg) (Figure 7). This envelope, formed by HBs in a 1:1:4 ratio of L, M and S, and host-cell lipids acquired from budding into multivesicular bodies (MVBs), contains a nucleocapsid of 36nm . A hundred and twenty core protein (HBc) dimers, organized in a triangulation $T=4$, compose this icosahedral capsid (Marion and Robinson, 1983; Heermann et al., 1984). A small fraction of capsids ($<5\%$) consists of only 90 dimers with a triangulation number $T=3$ but may represent dead-end products caused by an aberrant assembly process (Newman et al., 2003). Capsids contain a single copy of the partially double-stranded relaxed-circular DNA (rcDNA) genome of $\approx 3.2\text{kb}$, which is covalently linked to the viral polymerase (P) at the 5' end of the complete strand (negative strand). The viral titre of Dane particles can vary significantly among patients and can be as high as $10^{10}/\text{mL}$ of serum.

As mentioned, infected hepatocytes also produce/secrete subviral non-infectious particles, composed only by HBsAg. These latter are either spherical ($16\text{--}25\text{nm}$ of diameter) or filamentous (20nm of diameter and variable in length) and represent the most abundant particle released in the blood from infected hepatocytes (up to $10^{14}/\text{mL}$ for spheres). Filamentous structures contain mainly

L and M forms of HBsAg while M and S proteins, at a ratio of about 1:2, compose spheres. They may have various role in the HBV life cycle, including neutralization of anti-HBV antibodies or immune escape by ligand/receptor antagonization processes, thus facilitating virus spread and maintenance (Chai et al., 2008).

Eventually, in patient sera, various particles and intermediates of complete virions can be found. There are either secreted by infected hepatocytes or released upon apoptosis, but their roles are not totally deciphered (Bardens et al., 2011; Hu and Liu, 2017; Caballero et al., 2018)(Figure 8).

1.4.2. HBV genome structure and organization within Dane particles

In virions, the viral genome, as rcDNA, is about 3.2kb in length. The 5' of the negative strand is covalently linked to the viral polymerase while the 5' of the positive strand has a covalently attached 19nt oligoribonucleotide primer, which served for the minus-strand DNA synthesis. The 3' end of the positive strand terminates at a variable position, thus creating a single stranded gap that may account for up to 60% of the viral genome (Summers et al., 1975; Lien et al., 1986; Seeger and Mason, 2000). Minus strand exhibits a small 8 to 9nt-long terminal redundancy, which is required for the formation of rcDNA upon plus-strand DNA synthesis (Seeger et al., 1986)(Figure 9). A \approx 250nt overlapping between the two strands ensures circularization of rcDNA. In this overlapping, we found several regulatory elements, such as the two direct repeat regions (DR1 and DR2), which are mandatory for viral replication (Seeger et al., 1986; Wang and Tao, 1990).

A significant fraction of Dane particles (\approx 10%) contains double-stranded linear DNA (dsDNA) instead of rcDNA. These virions can enter cells but frequently lose important sequences from their ends during initiation of infection, thus playing a minor role in HBV replication (Caballero et al., 2018). This incoming dsDNA is likely involved in the early integration of HBV DNA into host genome (Yang and Summers, 1999).

The HBV genome is small and condensed, thus exhibiting a certain complexity towards its organization. The genome contains four overlapping open reading frames (ORFs), four promoters, two enhancer elements, and a single polyadenylation signal to regulate transcription of viral RNAs (Figure 9). All viral transcripts are encoded by the minus strand, are capped and polyadenylated. Transcription regulatory regions are present within ORFs and are active following the transport of the genome into the cell nucleus (Seeger et al., 1986).

HBV genotypic distribution worldwide (2017)

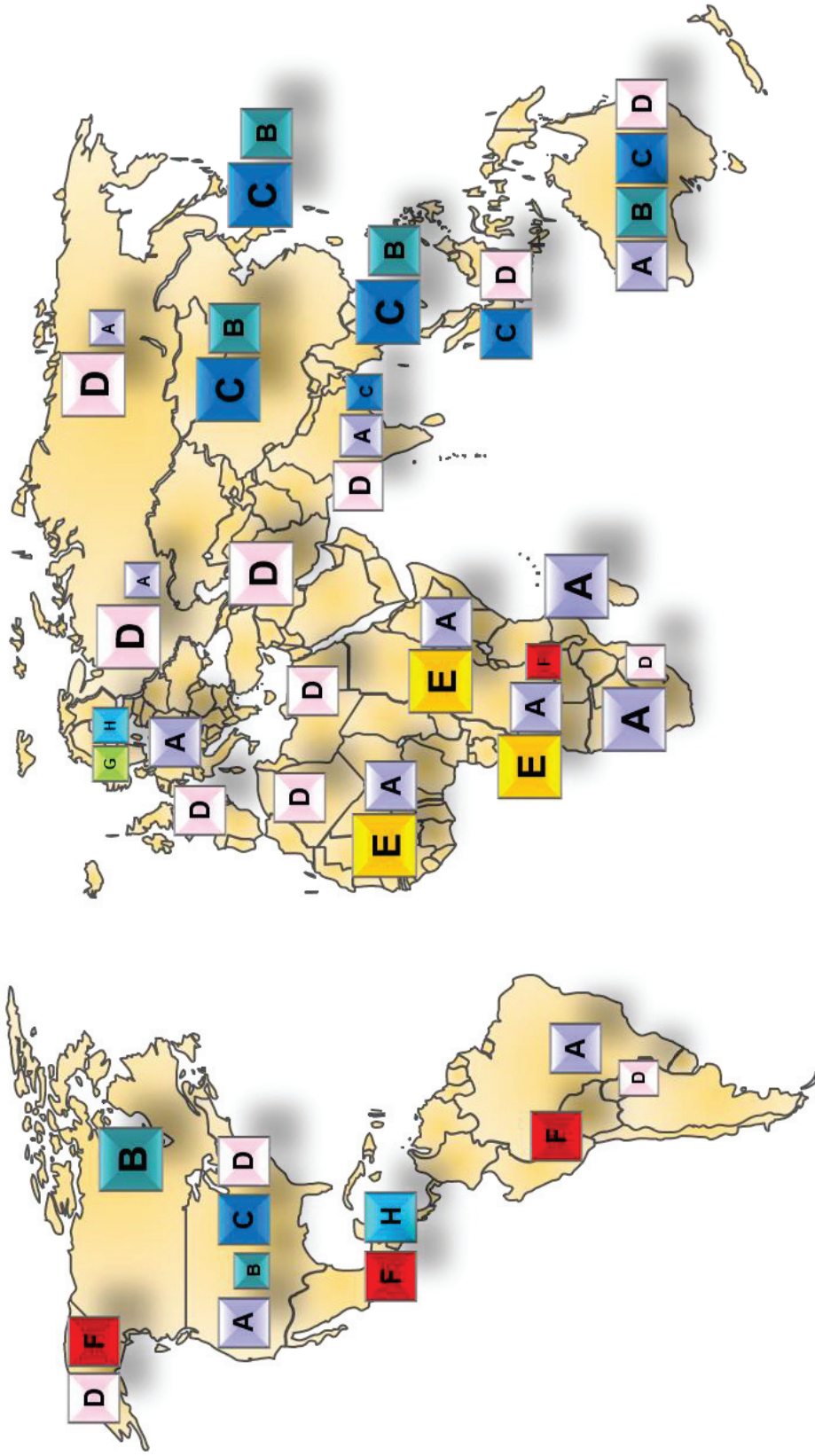
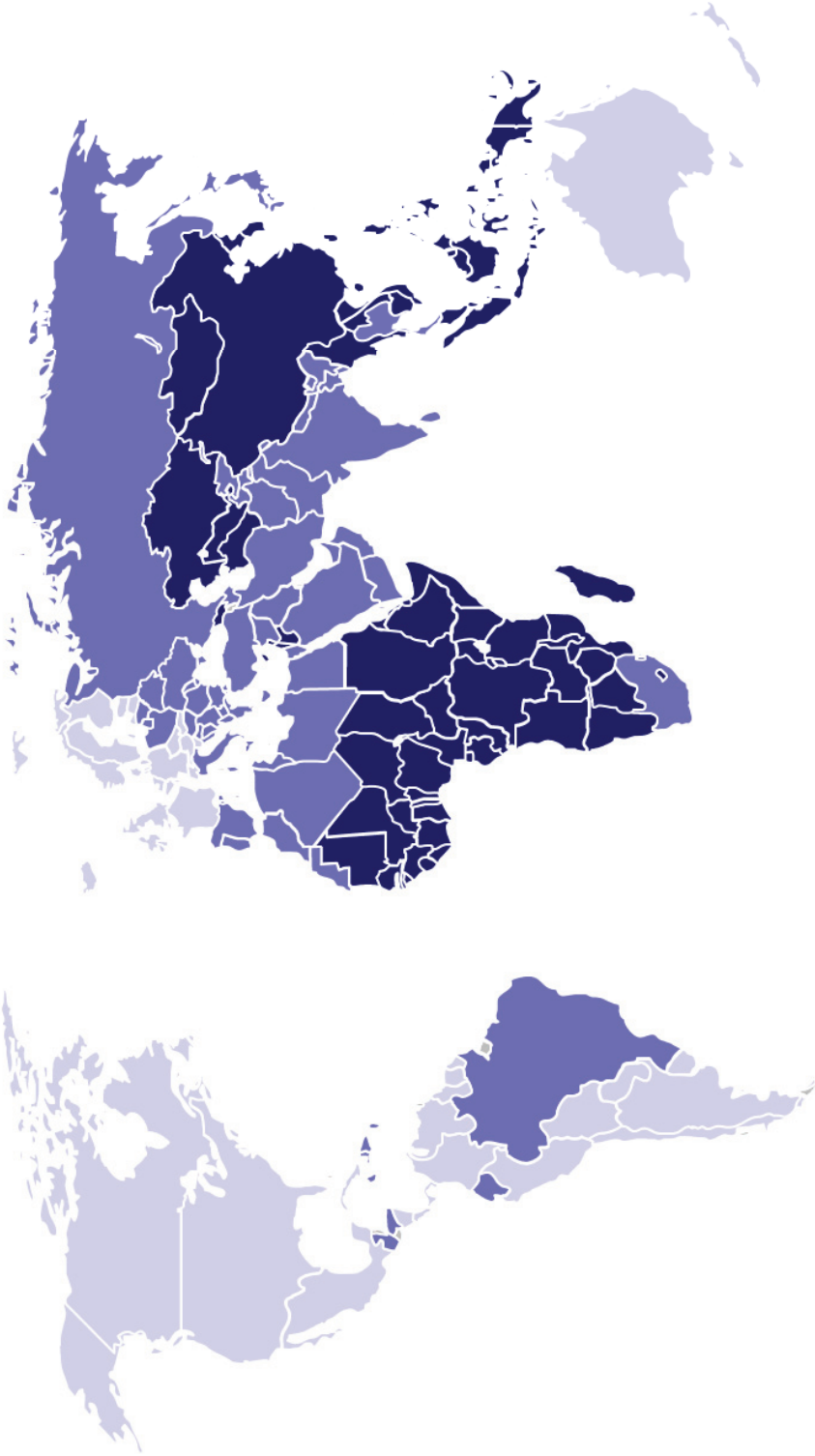


Figure 5: Global distribution of HBV genotypes. Box sizes are proportional to genotypic population found in areas. Created using WHO 2017 HBV report data.

World Hepatitis B Prevalence (2017)



HBsAg prevalence:

Low exposure <2%

Intermediate exposure 2 – 7,9%

High exposure >8%

Figure 6: Hepatitis B virus infection prevalence worldwide (seroprevalence of HBsAg). Based on WHO 2017 HBV report data.

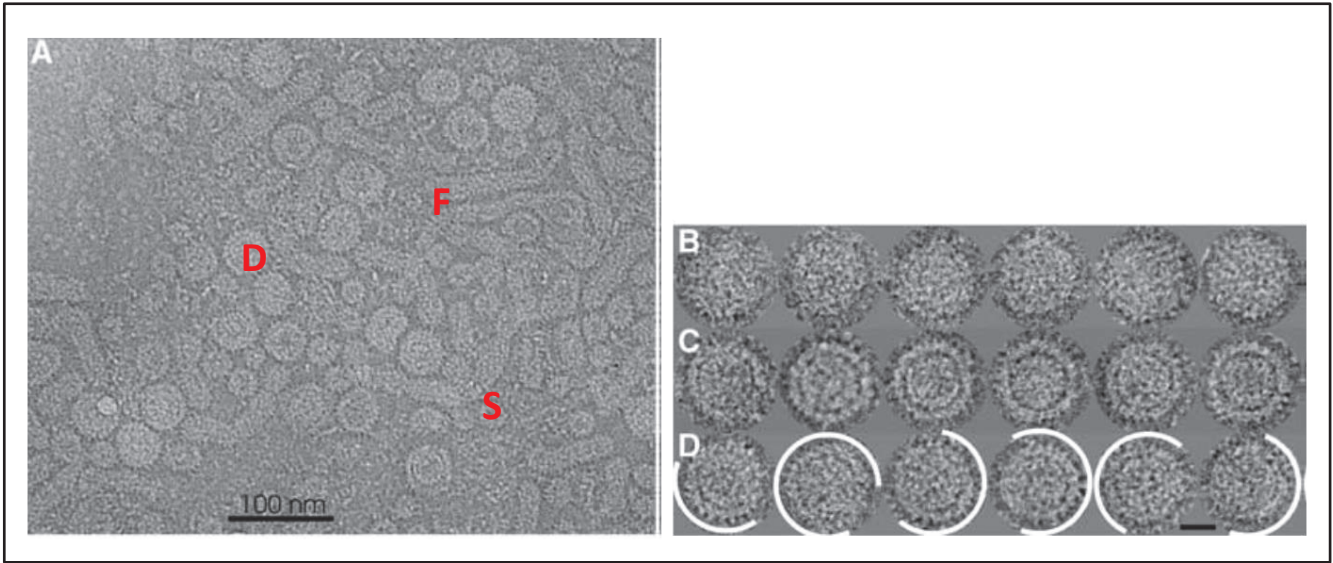


Figure 7: Cryo-EM of HBV particles. **A:** Dane particles (**D**), 22nm filamentous (**F**) and spherical (**S**) subviral particles. **B and C:** Dane particles harbouring compact and gapped morphology. **D:** particles with mixed morphology. Areas consistent with the gapped morphology are delineated in white. Scale bar for panels B, C and D equals 20nm. Adapted from Seitz S *et al.*, EMBO J, 2007.

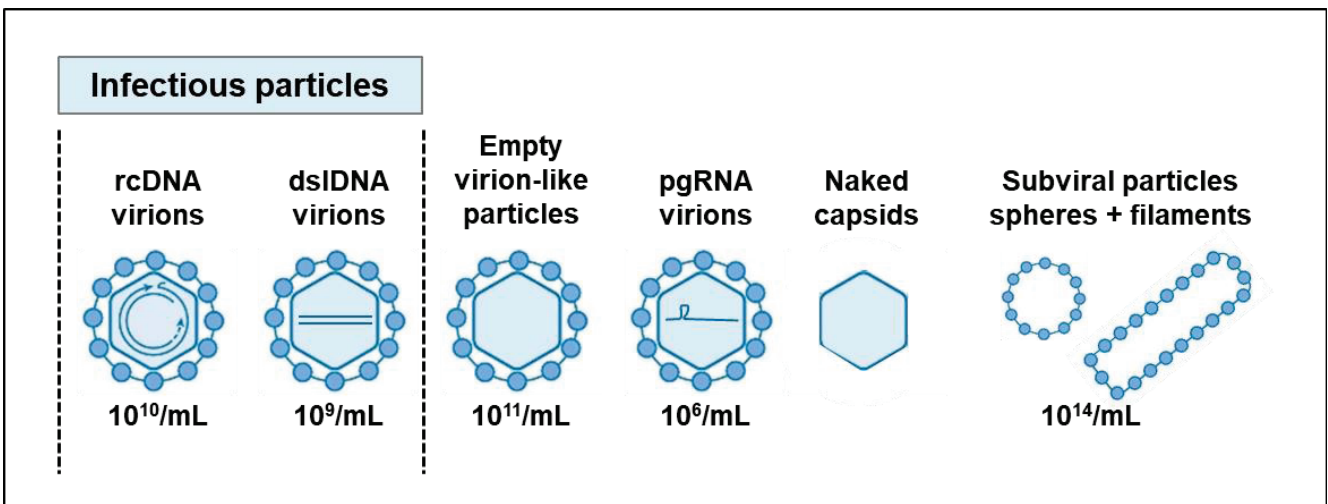


Figure 8: Various HBV particles found in patient serum.

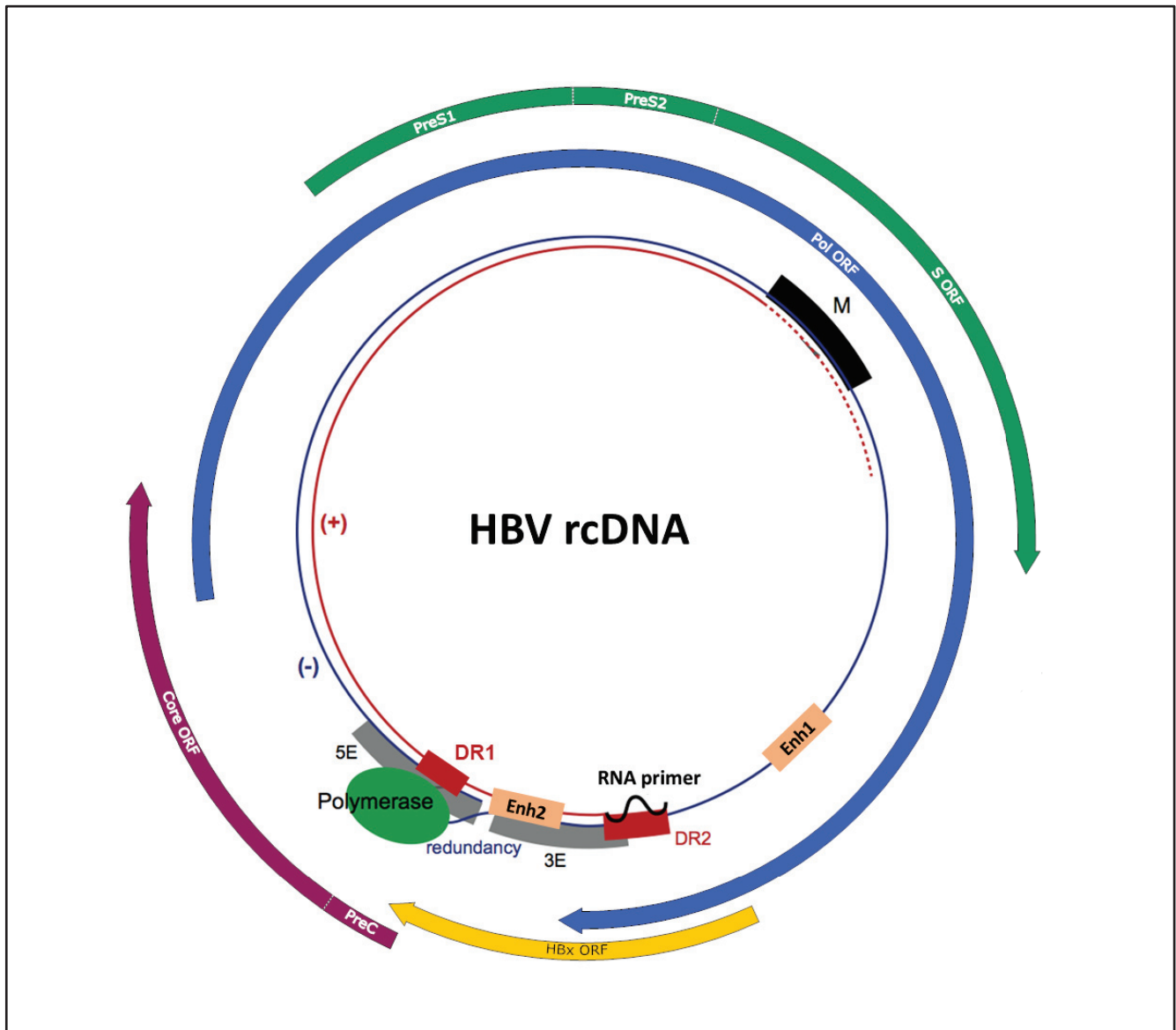


Figure 9: HBV genome organization. Key regulatory elements are positioned on the rcDNA with the four ORFs surrounding. DR1/DR2: Direct Repeat 1/2. Enh1/Enh2: Enhancer 1/2. 3E, 5E and M: *cis*-acting sequences required for rcDNA circularization. Adapted from Kann, Structure and molecular virology, 2002.

1.4.3. The cccDNA: a key genomic form for viral persistence and the main template for transcription

HBV cccDNA is formed into the nucleus of infected hepatocytes from rcDNA. This latter is converted into cccDNA by host machinery following a multiple-steps process, which is not fully characterized yet, but likely involves tyrosyl-DNA-phosphodiesterase, endonuclease, DNA polymerase, polynucleotide kinase, phosphatase and DNA ligase activities (Qi et al., 2016; Kitamura et al., 2018). Under physiologic conditions, cccDNA is associated with histones and other proteins to form a mini-chromosome and serves as template for viral RNAs transcription (Newbold et al., 1995). Although the exact location of the cccDNA in the nucleus is still elusive, studies suggest that it

localizes preferentially near to active chromatin enriched in H3K4me3, H3K4me2, as well as H3K27ac marks (ENCODE Project Consortium, 2012). In a paper published recently in *Nature Communications* by Moreau, Cournac and colleagues, it was reported that HBV cccDNA contacts preferentially CpG islands enriched in Cfp1, which is a factor required for its transcription (Moreau et al., 2018). Theoretically, the cccDNA is persistently sustained into quiescent cell without affecting viability as the cellular Pol II considers it as a normal and functional chromosome. Furthermore, the cccDNA pool can be maintained through a rcDNA recycling loop from neosynthesized capsids (Ko et al., 2018). However, the cccDNA can be eliminated, during apoptosis of hepatocytes and/or successive mitosis, which leads to cccDNA dilution and loss as it has no centromere. Up to now, no therapeutic is available to directly target the cccDNA in an efficient manner, highlighting the need of fundamental research efforts towards understanding its biology.

The cellular Pol II carries out the transcription of the cccDNA, leading to the main production of 2 genomic and 3 sub-genomic viral RNAs. The major transcripts that are detected in HBV-infected livers are 3.5kb, 3.5kb, 2.4kb and 2.1kb in length and termed pregenomic (pg), pre-C/C, pre-S and S mRNAs respectively. A minor transcript, the 0.7kb X mRNA, has occasionally been detected in infected tissues and evidenced more consistently in cells transfected with subgenomic HBV DNA (Moolla et al., 2002; Shiromoto et al., 2018)(Table I). New tools, such as deep RNA sequencing or 5' RACE-PCR should help deciphering further these viral RNAs *in vivo*.

Name	Size	Encodes
Pregenomic mRNA	3.5 kb	Viral Pol and HBc and serves as template for rcDNA synthesis
Pre-C/C mRNA	3.5 kb	HBeAg
Pre-S mRNA	2.4 kb	L-HBsAg
S mRNA	2.1 kb	M and S-HBsAg
X mRNA	0.7 kb	HBx

Table I: List of HBV viral mRNAs transcribed from cccDNA.

Name	ORF	Size (kDa)	Principal functions
Polymerase (Pol)	Pol	90	Reverse transcription of pgRNA into rcDNA. RNase H activity
Pre core (HBeAg)	Pre-C/C	15 – 17	Secreted as dimers. Tolerogenicity. Bait
Core (HBc)	Pre-C/C	21	Capsid formation. Decorates cccDNA
L-HBsAg	S	42	Envelope protein. Contain the PreS1 domain that allow interaction with entry receptor. Tolerogenicity
M-HBsAg	S	31	Envelope protein. Tolerogenicity
S-HBsAg	S	27	Envelope protein. Contain main humoral epitopes. Tolerogenicity
HBx	X	17	Trans activator. Linker between host bait and cullin-ligase complexes. Still elusive
HBSP	“Pol”	12.4	Viral persistence. Decrease liver inflammation

Table II: List of the eight HBV proteins.

1.4.4. HBV viral proteins

The HBV genome harbours four ORFs, encoding 8 viral proteins. Seven are described as principal and the eighth, the hepatitis B spliced protein (HSBP), which is produced by alternative splicing of pgRNA was solely recently described and found in liver biopsies (Soussan et al., 2000).

1.4.4.1. Large, Medium and Small envelope proteins; How to build HBV envelope the right way

The three viral envelope proteins L, M and S are synthesized from two mRNAs, both originating from the S ORF (Table I and II). The 2.4kb mRNA encodes L-HBsAg while the 2.1kb encodes M- or S-HBsAg, depending on the AUG used. The initiation codon for S-HBsAg is preferentially recognized by the ribosomal machinery, explaining the large excess of S- compared to M-HBsAg (4:1 ratio) (Sheu and Lo, 1992). The three forms of HBsAg share the same STOP codon, thus differing only in their N-ter domains. S-HBsAg is a 226aa polypeptide; M has a 55aa additional domain called PreS2 and L possesses S, PreS2 and a 108-119aa PreS1 domain in N-ter (Figure 10). These three proteins are synthesized in the ER and matured in the Golgi apparatus as their fate is to be eventually secreted. Regarding principal post-translational marks, L-, M- and S-HBsAg are either N or O-glycosylated. Furthermore, the L form is myristoylated on its second aa, which favours a topology of L-HBsAg that makes the preS1 attachment site less accessible to neutralizing antibodies (Bremer et al., 2011). The three forms of HBsAg harbour four transmembrane domains organized as described in Figure 10B.

The S domain is cysteine-rich, resulting in disulfure bridges formation, mandatory for homo-heterodimerization and subsequent envelope assembly. The 124-147 region is known as the “a” determinant, which is the main target of neutralizing antibodies (Wounderlich and Bruss, 1996). The M form of HBsAg seems to be unnecessary for neither formation nor infectivity of HBV particles (Bruss and Ganem, 1991). The L protein is found under two topologies at the ER membrane:

- i-preS: the first transmembrane domain is not translocated the right way and stays cytosolic, thus allowing interaction with newly assembled capsids.
- e-preS: the first transmembrane domain is translocated through the ER, facilitating the downstream interaction with the NCTP HBV receptor (Prange, 2012).

Beside the involvement of L, M and S proteins in envelope formation, they are also found in subviral particles. These latter are known as bait and hold a key role in immunity control. Spheres are mostly composed by M and S forms whereas filaments harbour also the L protein (Gilbert et al., 2005; Chai et al., 2008).

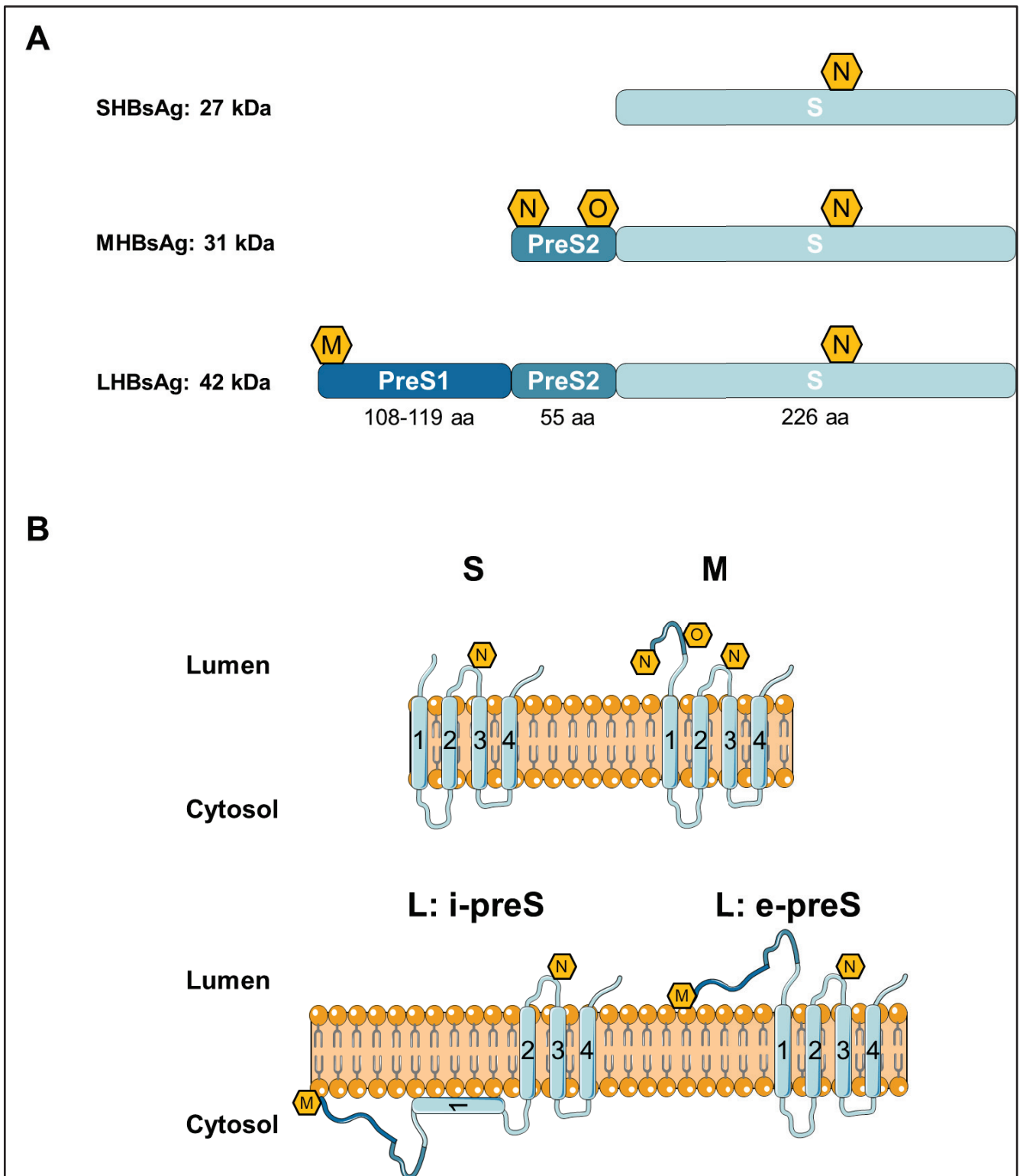


Figure 10: Schematic representations of HBV envelope proteins. **A:** Principal features of S, M and L HBsAg. **B:** Topology of HBV envelope proteins as processed in the ER. Light blue: S domain. Middle blue: PreS2 domain. Dark blue: PreS1 domain. N-ter are positioned at left. Principal post-translational modifications are shown in orange hexagons. N: N-glycans. O: O-glycans. M: myristic acid.

1.4.4.2. HBc: the Swiss army knife of HBV

“A review I have written and published in Antiviral Research details the diverse functions of the core protein in the viral life cycle. Please confer to Appendix”

The capsid protein or HBc, is a 183aa long polypeptide of 21kDa, which has been classically viewed as a structural protein that self-assembles to form the viral nucleocapsid. However, accumulated evidences support that HBc functions extend well beyond this structural role. HBc seems to be involved in nearly every stage of the HBV life cycle, including subcellular trafficking and release of HBV genome, RNA metabolism and trafficking, capsid assembly and transport, and reverse transcription (Nassal, 1992; Rabe et al., 2003; Perlman et al., 2005; Basagoudanavar et al., 2007). Moreover, recent findings implicate an active role for HBc in epigenetic regulation of the viral and host genomes. Collectively, this multiplicity of functions elevates HBc as an excellent target for mechanism-based antiviral therapeutics (Durantel and Zoulim, 2016).

1.4.4.3. HBeAg: the HBV precore protein

HBeAg is a non-structural protein, which is related to the HBV core protein and circulates in HBV infected patients' bloodstream. HBeAg is encoded by the 3.5kb Pre-C/C mRNA, giving rise to a 25kDa precursor, similar to HBc but exhibiting an additional 29aa at its N-ter (Messageot et al., 2003). The initiation AUG codon involved in HBeAg translation is located 87nt prior to the AUG used for HBc synthesis. This 25kDa precursor is addressed to the ER, where a furin cleaves the peptide signal in N-ter, leading to a 22kDa intermediate. This latter is then cleaved in the C-ter arginine rich domain and HBe secreted as a 17kDa protein that assembles in dimers (Ito et al., 2009).

HBeAg is not directly involved into HBV replication as HBe defective HBV genotype G is infectious and replicates *in vitro* alone (Chudy et al., 2006) and in human in the presence of genotype A (Stuyver et al., 2000). However, HBeAg is found among all *Hepadnaviridae*, highlighting its importance. Indeed, as HBeAg mimics HBc, it serves as a tolerogenic bait, extinguishing anti-HBV immune response through a saturation of lymphocytes T MHC-I receptors. HBeAg may cross the placenta, leading to tolerogenicity in child while HBeAg negative status in the mother can lead to fulminant hepatitis for the child (Liang et al., 1991; Chen et al., 2004).

Clinically, the level of circulating HBeAg witness the HBV replication rate and the seroconversion of HBeAg to anti-HBeAg evokes the end of active replication.

1.4.4.4. The viral polymerase (Pol)

The viral polymerase is the largest HBV protein, with a length of 845aa for 90kDa, and is the sole enzyme coded by HBV. Currently, in clinic, direct acting antivirals are focused on targeting this protein (e.g., nucleoside/tide analogues NUCs). The Pol contains four distinct domains and exhibits several enzymatic activities:

- Terminal protein domain: aa 1-180. Mandatory for the covalent attachment to the ϵ packaging signal of pgRNA. The Y63 residue of the TP domain and the minus strand 5' end of the pgRNA are engaged in a phosphodiester bond. This domain has a primase activity, allowing the reverse-transcription initiation through the synthesis of a 3-4nt primer (Zoulim and Seeger, 1994; Lanford et al., 1997)(**Figure 11 & 14 and section "1.4.5. HBV replication strategy and spreading"**).
- Spacer domain: aa 181-349. Functions not well elucidated yet. Could be of importance for flexibility of HBs proteins by interacting with the PreS domain and thus responding to immune pressure.
- Pol/RT domain: aa 350-693. The domain polymerase/reverse-transcriptase holds two functions. In the one hand, the RNA-dependent polymerase which ensure the pgRNA reverse-transcription into the minus strand DNA and in the other hand, the DNA-dependent polymerase require for the subsequent plus strand DNA synthesis. This domain is divided in several subdomains, where the so-called "C" contains the YMDD catalytic site, which is involved in resistance to NAs (nucleoside analogues, *i.e.*, LAM, TDF, TAF) (Liaw, 2002; Cai et al., 2016; Calica Utku and Karabay, 2016).
- RNaseH domain: aa 694-845. Is responsible for pgRNA degradation after completion of the minus strand DNA synthesis (Wei and Peterson, 1996).

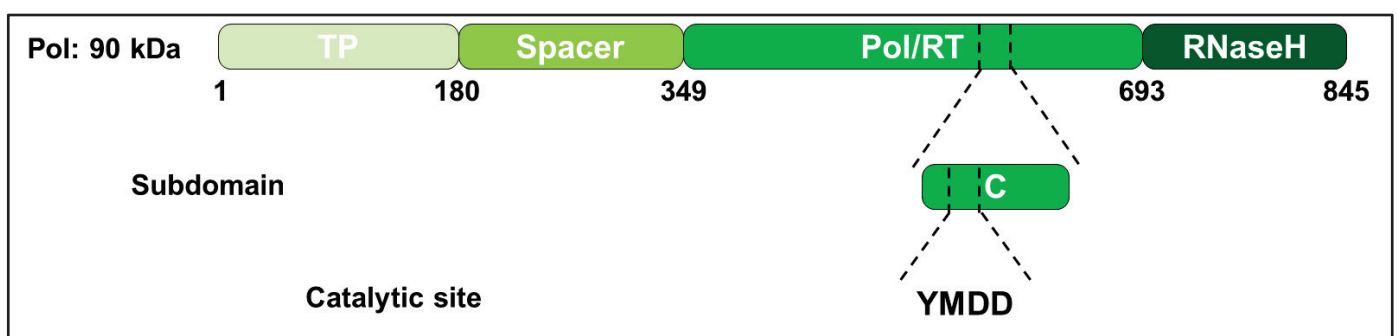


Figure 11: Schematic representation of the different domains of the viral polymerase. The catalytic site YMDD within de C subdomain is a mutational hot point and is critical for resistance to NAs.

1.4.4.5. HBx: an enigmatic multifunctional protein endowed with weak oncogenic properties

HBx is a non-structural protein of 17kDa, involved in establishment and maintenance of infection. Indeed, a HBV mutant lacking HBx exhibits a lower amount of transcripts synthesized from the cccDNA and is unable to maintain the infection in a stable fashion (Lucifora et al., 2011). HBx is an enigmatic protein found in high copy numbers in the cytoplasm of infected cell (up to 80,000 copies) but can also be found in the nucleus, especially in close vicinity to the cccDNA. HBx indirectly increases the cccDNA transcription by recruiting histones acetylases, leading *in fine* to better HBV replication. Recently, a study has shown that X protein is involved in the degradation of the SMC5/6 complex through a DDB1/E3 ubiquitin ligase-dependent mechanism. This way, the cccDNA is no more “recognized” by the SMC5/6 complex and can be transcriptionally active (Decorsière et al., 2016).

Eventually, HBx seems to be involved in the gene expression modulation of several host cell genes, involved especially in cell-cycle, apoptosis and DNA repair pathways (Figure 12). Thus, HBx seems to be a good candidate as pro-oncogenic protein even if the limits of its perimeter of action are still blurry (Andrisani and Barnabas, 1999; Ma et al., 2014). Please refer to section “HBV putative onco-proteins: the *trans*-effect” for details.

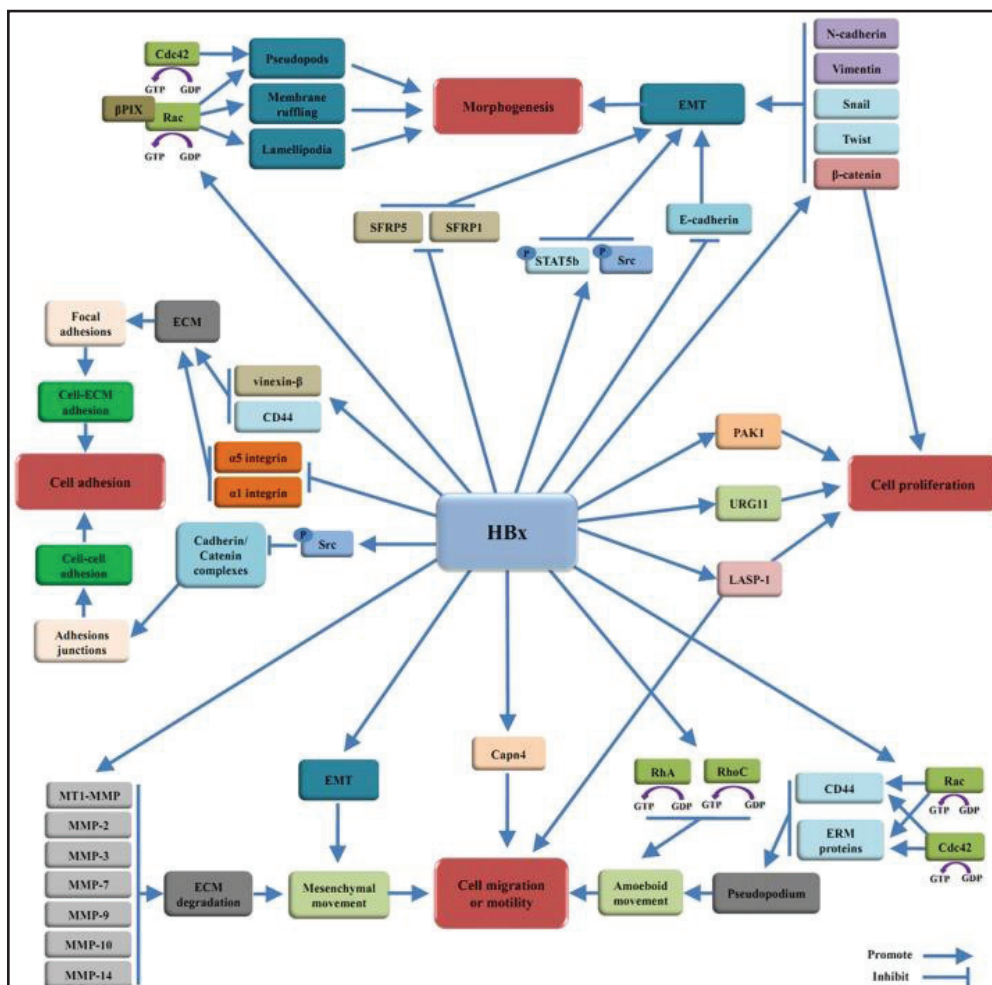


Figure 12: HBx as the center of a hub in cellular pathways involved in oncogenic process. Extracted from Kong F. et al., Onco. letters, 2017.

1.4.4.6. The Hepatitis B splicing-regulated protein (HBSP)

As human transcripts, HBV RNAs are subjected to splicing events, which increase the genetic diversity. Among all the HBV spliced RNAs, the variant SP1 of the pgRNA is the best characterized. The SP1 RNA, of 2.2kb, results from 1.3kb intron removal from the pgRNA and accounts for up to 30% of total pgRNAs amount. SP1 is translated into a 93aa long protein of 12.4kDa, the hepatitis B splicing-regulated protein (HBSP) (Soussan et al., 2000). HBSP and the viral polymerase share the same AUG codon and thus exhibiting a 46aa homology at their N-ter. The following sequence of HBSP is encoded by the original nucleotidic arrangement found in the spliced variant SP1 (Figure 13).

As SP1 shares homology with pgRNA, it can be encapsidated the same way and reverse transcribed, yet leading to defective HBV particles formation. These latters are found to be enhanced prior to development of HCC (Bayliss et al., 2013). HBSP seems to be involved in reducing HBV-induced liver inflammation, lowering the efficacy of immune responses and *in fine*, promoting HBV infection and maintenance (Soussan et al., 2003; Pol et al., 2015). However, the roles of spliced HBV RNAs and HBSP need still to be further defined (Chen et al., 2015).

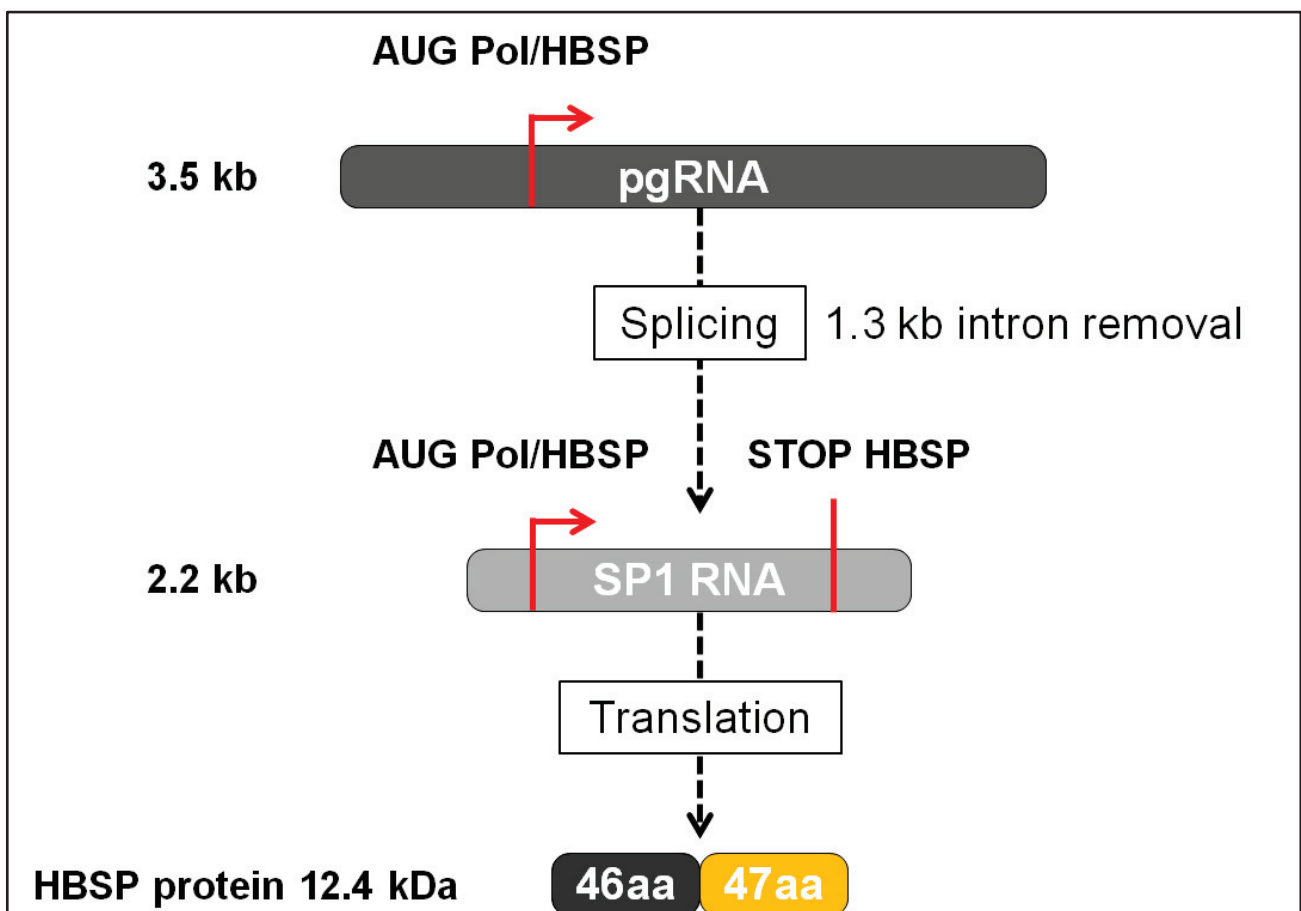


Figure 13: Schematic representation of HBSP synthesis from pgRNA splicing. 1.3kb intron is removed from pgRNA by splicing, giving rise to SP1 RNA. This latter is translated in the 12.4kDa HBSP protein. The first 46aa (dark grey) are common with the polymerase as HBSP shares the same AUG. The following 47aa (orange) comes from the unique rearrangement of the SP1 RNA sequence.

1.4.5. HBV replication strategy and spreading

“Involvement of HBc in HBV replication cycle is not extensively detailed hereafter as it is the focus of a review I have written in Antiviral Research. Please confer to the Appendix section.”

Hepatitis B virus mainly infects liver cells and replicates exclusively in quiescent hepatocytes. The entry is mediated through a low affinity interaction between HBV virion and heparan sulfate proteoglycans present at the hepatocytes cell surface (Schulze et al., 2007). The following step is characterized by a higher affinity interaction involving the PreS1 domain of the HBV large envelop protein and its receptor, the sodium taurocholate cotransporting polypeptide (NTCP) on the basolateral side of hepatocytes (Yan et al., 2012). The downstream internalisation of virions is poorly understood although glypican 5 has been recently identified as co-entry factor (Verrier et al., 2016a). After viral entry, nucleocapsids are transported to the nuclear pore, where the viral rcDNA is released and then converted into cccDNA, which is responsible for viral persistence. This latter serves as template for viral mRNAs transcription and proteins are synthesized in the cytosol. HBV can also be integrated into host genome, preferentially from dsDNA-containing particles, which facilitate homologous recombination (Tu et al., 2017). Some genes, as hTERT have been highlighted as non-random integration site of HBV genome, which are of importance in viral oncogenic events occurrence (confer section “2.3.4.1.1. HBV dsDNA integration: the cis-effect”) (Horikawa and Barrett, 2003). Once exported to the cytosol, the pgRNA is both used for the translation of the core (HBc) and viral polymerase (Pol) and as the template for reverse transcription within nucleocapsid. The polymerase would preferentially bind to the pgRNA used for its synthesis, on the packaging signal epsilon (ϵ) at the 5' end of the mRNA, while the translation is still ongoing. This process warrants a good stoichiometry between the genetic template and HBV pol before encapsidation of this ribonucleoprotein complex. The covalent attachment of the polymerase to the ϵ domain of the pgRNA leads to HBc dimers recruitment and capsid formation (Figure 14). Cellular chaperones, such as HSP90, and the arginine-rich CTD domain of HBc are required for correct interaction with the pol- ϵ complex (Nassal, 1992; Hu et al., 2004). Capsid formation and subsequent reverse transcription of the pgRNA into rcDNA are two intimately linked processes, dependent on a dynamic phosphorylation of HBc CTD. Several kinases as, SRPK1, CDK2 and PLK1 are known to phosphorylate HBc CTD in a kinetic-dependent manner, thus regulating subcellular localization of Core protein and reverse-transcription of pgRNA in the capsid (Daub et al., 2002; Ludgate et al., 2012; Diab et al., 2017). For more details, see the dedicated review I have written focusing on the diverse functions of HBc in the viral life cycle (confer Appendix review (Diab et al., 2018)).

The first step in pgRNA reverse transcription is the minus-strand DNA synthesis, beginning from the bulge of the ϵ loop. The polymerase remains covalently linked to the 5' end of minus-strand DNA upon all subsequent steps of DNA synthesis, virus assembly and virions release. The RNA template is digested by the RNaseH activity present near the C-terminus of the polymerase, except of the 5' region, which serves as priming for the positive-strand synthesis (Summers and Mason, 1982).

Due to the location of DR1 within the terminal redundancy on the pregenome, the minus-strand DNA exhibits a short terminal redundancy region, which is crucial for rcDNA circularization (**Figure 9**). The plus-strand DNA synthesis is incomplete and reaches approximately 60% the genome in length, probably due to steric factors imposed by the nucleocapsid and the polymerase itself or a lack of incoming dNTP within the capsid (Summers and Mason, 1982; Clark and Hu, 2015). The fate of freshly formed rcDNA containing capsids is dual. On the one hand, they can enter back to the nucleus, thereby amplifying the cccDNA pool, in what is called the “recycling loop”. On the other hand, rcDNA containing capsids bud from intracellular membrane containing envelope proteins to form virions that are then secreted.

The budding process of mature rcDNA capsids through the interaction with envelope proteins (L, M and S) is still a poorly understood mechanism. The N-terminal domain of L-HBsAg seems to play an important role in this interaction as M and S proteins alone cannot support translocation of capsids across membranes (Halverscheid et al., 2008). Reports provided evidence for a mechanism in which rcDNA containing capsids interact with envelope proteins and bud at MVB membranes (Watanabe et al., 2007). Indeed, the envelopment and secretion of Dane particles appear to be dependent on vacuolar protein sorting proteins AIP1 and VPS4B. The L-HBsAg binds to α -taxilin mediating thus a connection with tsg101 component of the ESCRT complex. Surprisingly, secretion of subviral particles occurs through the ER-Golgi compartment and do not depend on MVBs (Patient et al., 2007). However, as filaments contain a significant amount of L-HBs, some data suggest a possible release of these latter by MVBs (Jiang et al., 2015).

*A schematic HBV replication cycle highlighting the principal steps is available in **Figure 15**.*

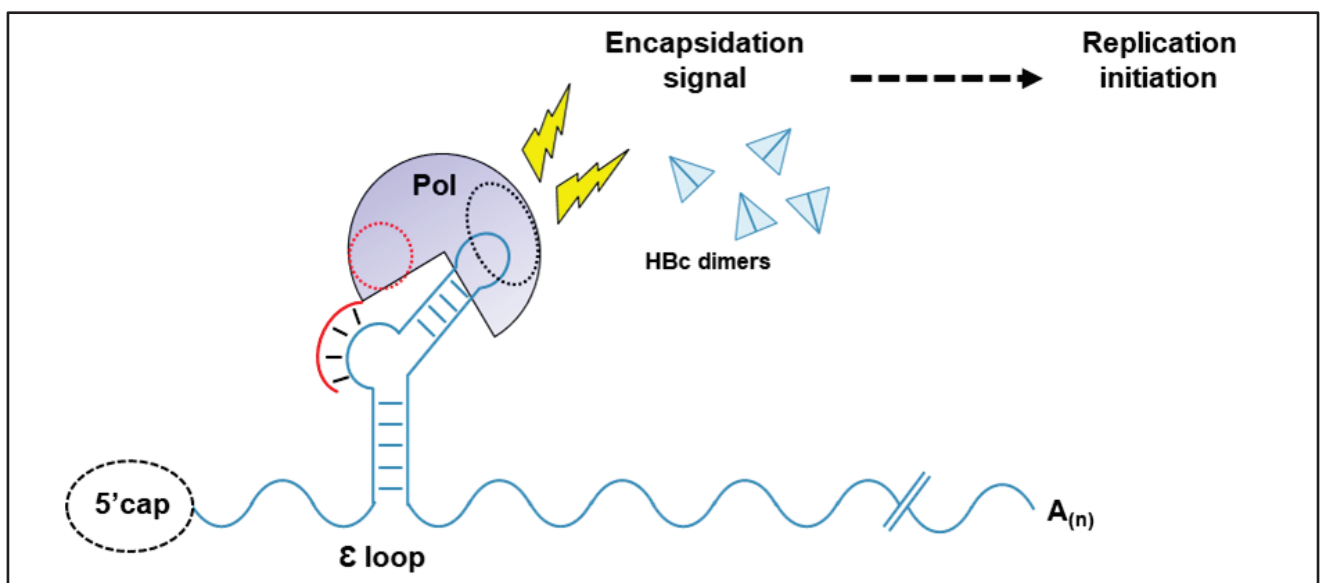


Figure 14: Representation of the viral polymerase and pgRNA interaction, leading to its encapsidation. Dashed red circle: terminal protein (TP) of the polymerase. Black dashed circle: reverse transcriptase (RT) domain of the polymerase. A_(n): poly-A tail. Red line: 4nt primer synthesized by the TP, initiating the reverse transcription of the pgRNA.

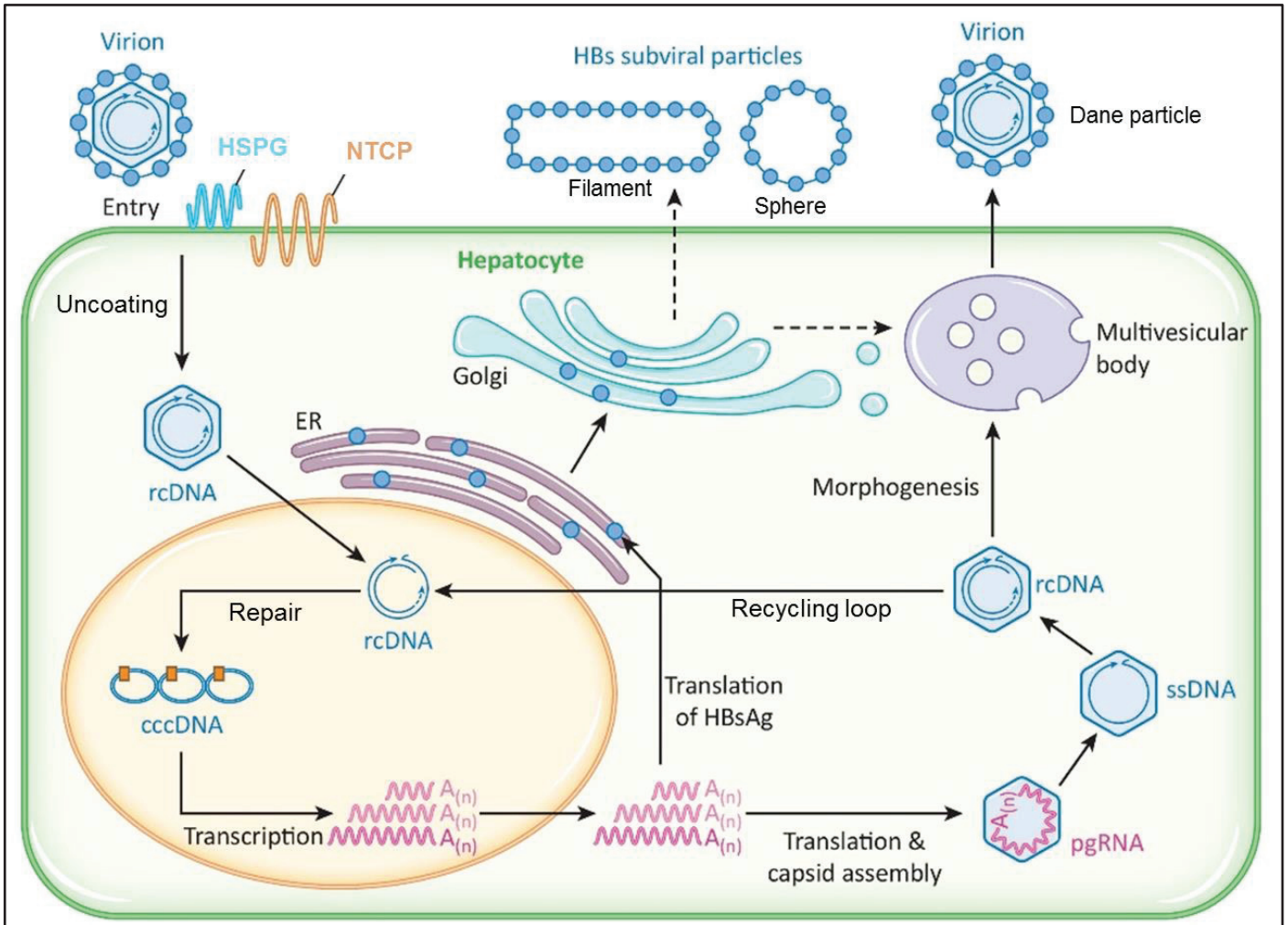


Figure 15: Simplified HBV replication cycle. Please refer to the section: “1.4.5. HBV replication strategy and spreading” for details. Extracted from Shih C *et al.*, Trends in Microbiology, 2018.

1.5. *In vivo* and *in vitro* models for HBV replication study

In this part, we will discuss in a non-exhaustive way, the different models used for the study of HBV. I decided to focus on historically important breakthroughs as well as on *in vivo* and *in vitro* systems that have been used during my PhD.

1.5.1. Natural infection models

As explained in the part “1.2. Classification and phylogeny of *Hepadnaviridae*”, solely differentiated hepatocytes of few species are susceptible to a HBV infection followed by a complete replication cycle. Among them, higher primates, and in particular the chimpanzee has been employed historically to study virus transmission, host response and vaccination. Studies showed that these apes were protected from HBV infection by the vaccination comprised of HBsAg purified from virus carriers as well as from recombinant HBsAg produced in yeast. More recently, chimpanzees were used to show that vaccination was not protecting against some NA-escape HBV variants. Particularly, they helped confirming observations made in vaccinated infants who experienced breakthrough infections due to LAM resistance, which induce mutation in the overlapping S gene (Kamili et al., 2009). Finally, in this model, studies have demonstrated the key roles of CD4 and CD8 T cells in the clearance of HBV infections and further documented lack of induction (or rapid suppression) of innate immune responses by the primary infection (Thimme et al., 2003; Wieland et al., 2004a, 2004b). However, improvement in ethical domain as well as the need of highly specialized personal and expensive infrastructures led to decay in the use of those animals (Dandri and Petersen, 2017).

Other surrogate models, based on host-adapted viruses have been of importance for the study of HBV. Among them, Woodchuck infected by WHB was the first model suggesting causality between HBV infection and progression to HCC. Indeed, 100% of neonatally infected woodchucks developed liver cancer within 2 to 4 years. Strikingly, 20% of those that resolved the infection after neonatal inoculation were subjects to HCC, highlighting that HBV leaves a suitable landscape for cancer development, even after the resolution of the infection (Korba et al., 1989).

1.5.2. From transgenic mice to humanized infectable mice

As mice are useful models in biology (inexpensive, easy-handling, rapid growth, tools at disposition ...), several transgenic mice have been developed to study the HBV liver-specific pathogenesis, either by expression of the entire virus or by individual gene products (Kim et al., 1991). However, this unusual route of partial infection led to the introduction of bias and resulted in rapid resolution of HBV infection (Iannacone and Guidotti, 2015).

About 25 years ago, Brinster and colleagues created transgenic mice that express the serine protease urokinase-type plasminogen activator (uPA) in hepatocytes. This allowed the repopulation of the murine hepatic liver-shape with human hepatocytes, rendering mice susceptible to HBV infection. Based also on repopulation of mice livers by human hepatocytes, the FRG model was developed. These mice are immune-deficient Fah knockout lacking the genes for Rag-2 and the common gamma chain of the IL receptor. These animals breed normally while on nitisinone (NTBC). These FRG mice can be readily repopulated with human hepatocytes after pre-treatment with a vector expressing urokinase 1–3 days prior to transplantation (Azuma et al., 2007). The selective pressure to regenerate the liver is controllable in the FRG model but is lacking in the uPA/SCID animals. Indeed, as the mice breed normally under NTBC, no liver damages are observable, thus their handling is easier compared to uPA models. Utilization of these systems has been limited because of the technical challenge of acquiring and transplanting human hepatocytes, which is done by injection of hepatocytes into the spleen. However, improvement in techniques and handling led now to >95% of primary human hepatocytes (PHH) replenishment with low mortality rate and incoming double humanized-mice model (liver and immune system) is a trend on which research efforts are made (Dusséaux et al., 2017; Kremsdorf and Strick-Marchand, 2017).

1.5.3. *In vitro* models for the study of HBV

Historically, most of the studies on the HBV life cycle were performed in hepatoblastoma cell lines where HBV DNA was transfected, due to a lack of susceptibility towards HBV infection. In 2002, Gripon and colleagues developed and characterized a human hepatoma cell line infectable by HBV, called HepaRG (Gripon et al., 2002). Up to recently, this latter, with PHH cultures, were the only two *in vitro* models allowing studies of the HBV complete life cycle, from virions entry to release. However, the identification, in 2012, of the sodium-taurocholate cotransporting polypeptide (NTCP) as the main HBV receptor led to engineered cell line development, allowing proper infection by HBV (*i.e.*, HepG2-NTCP, Huh7-NTCP) (Yan et al., 2012; Verrier et al., 2016b).

Chapter 2: From HBV-infected liver to hepatocellular carcinoma

2.1. Clinical manifestations of chronic HBV infections and pathogenesis

2.1.1. Natural history of the disease

Following acute infection, approximately 95% of adults and 5% to 10% of newborns recover, while the remainder evolve towards chronic viral infection. Not all patients with chronic HBV infection, which is characterized by the persistence of HBsAg for more than 6 months, have chronic hepatitis (CHB), which is evidenced by elevated ALT/ALAT, inflammation and fibrosis progression. The natural history of chronic HBV infection has been schematically divided into five phases, taking into account the presence of HBeAg, HBV DNA levels, alanine aminotransferase (ALT) values and eventually the grade of liver inflammation (**Figure 16**). Since 2017, guidelines from EASL recommend a new nomenclature based on the description of infection vs. hepatitis. However, the global HBV community is not united as one and the ancient nomenclature is presented here (**EASL 2017 Clinical Practice Guidelines**):

- **Phase 1 “Immune tolerant”**: characterized by the presence of HBeAg in the serum, high viral load, no to low liver necroinflammation and normal ALT (<40 IU/L). The rate of spontaneous HBeAg loss is very low in this phase. These patients are highly contagious due to the high levels of HBV DNA. The high level of HBV DNA integration events and clonal hepatocyte expansion suggest that hepatocarcinogenesis could be already underway in this phase.
- **Phase 2 “Immune (re)active”**: presence of serum HBeAg, high levels of HBV DNA and elevated ALT. The liver is moderately inflamed and subject to a rapid fibrosis progression. The outcome of this phase is variable. Most patients achieved HBeAg seroconversion and HBV DNA suppression and enter the HBeAg negative infection phase. Those patients who fail to control the infection progress to the HBeAg-negative CHB phase for many years.
- **Phase 3 “HBeAg-negative chronic HBV infection”**: previously known as “inactive carrier” phase, is characterized by serum anti-HBe antibodies, low viral replication, HBV DNA <2,000 IU/mL and normal ALT. HBsAg loss and/or seroconversion may occur spontaneously in 1–3% of cases per year, typically in patients with HBsAg <1,000 IU/mL. Nevertheless, viral infection is not resolved, as cccDNA remains active in hepatocytes.

- **Phase 4 “HBeAg-negative chronic hepatitis B”:** characterized by detectable anti-HBe in the serum and fluctuating moderate to high levels of serum HBV DNA and ALT levels. The liver shows fibrosis and necroinflammation. Most patients harbour HBV variants with precore and/or the basal core promoter (BCP) mutants, abolishing HBeAg production.
- **Phase 5 “HBsAg-negative”:** also known as “occult infection”. Serum negative HBsAg with or without anti-HBsAg and presence of anti-HBcAg. Normal ALT levels and undetectable HBV DNA. However, the cccDNA is frequently detected in the liver but could be silenced somehow (Levrero et al., 2009). If cirrhosis has developed before HBsAg loss, patients remain at risk of HCC, therefore the surveillance should continue (Figure 17).

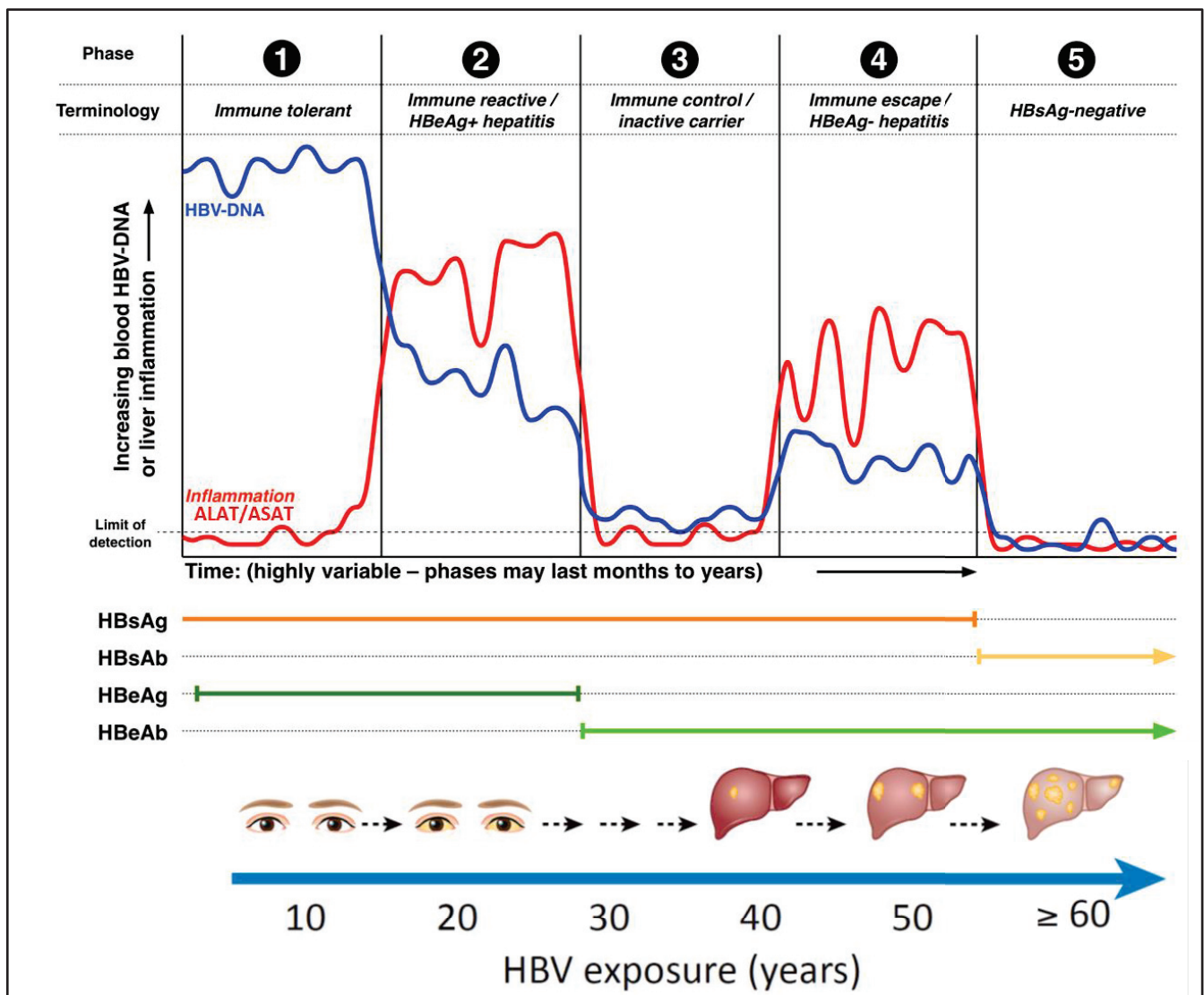


Figure 16: Natural history of hepatitis B. Evolution of seric markers over the course of the disease. Witness of HBV replication rate, inflammation and immunologic status.

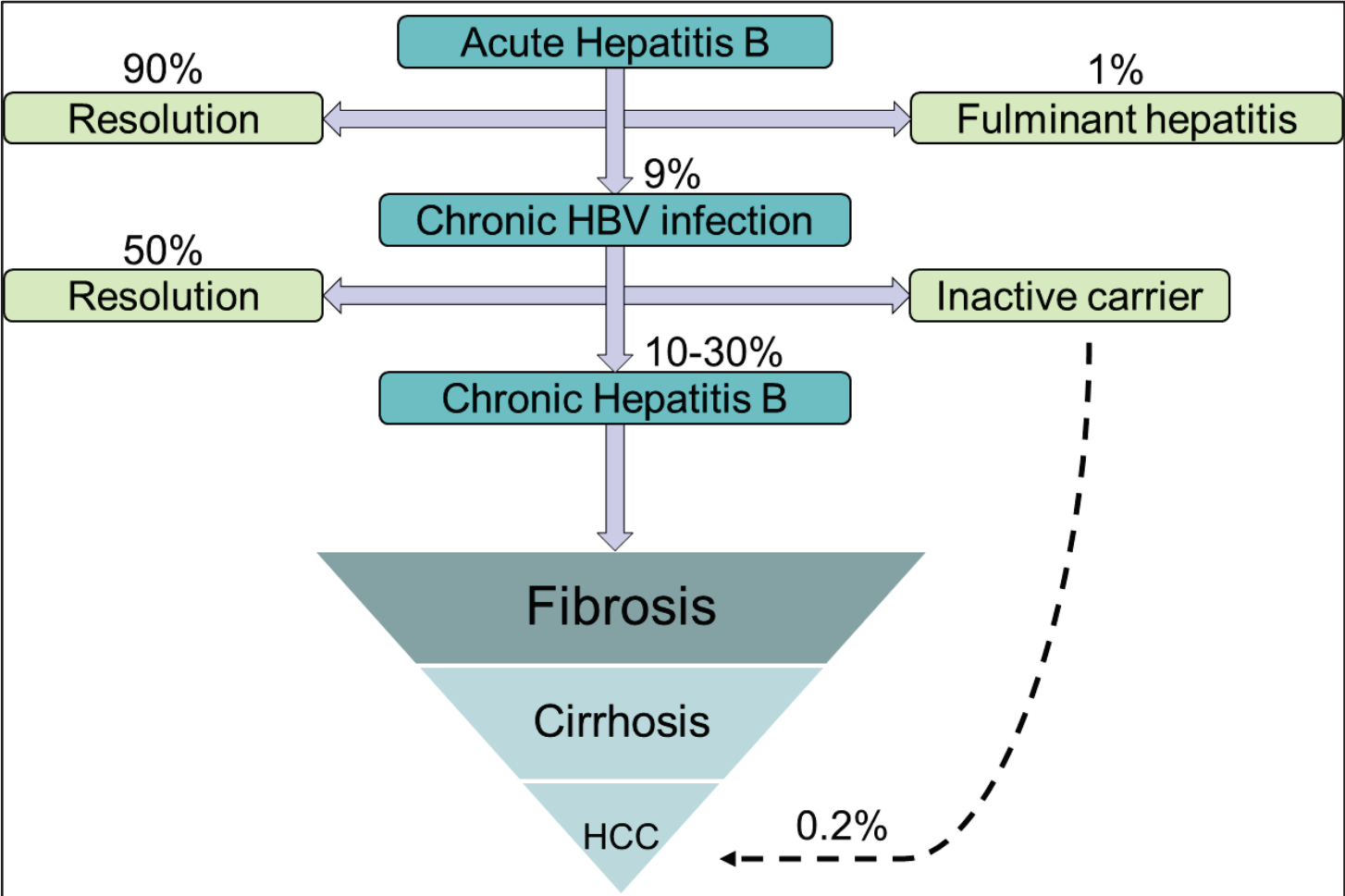


Figure 17: Pathology of HBV infection. Progression of HBV infection through different clinical states. % represents the progression from one stage to another per year.

2.2. Prevention and Control

2.2.1. Vaccines and other prevention strategies

The first generation of vaccines containing S alone were produced by processing of HBsAg purified from plasma of HBV carriers. Nowadays, vaccines are obtained from the yeast *Saccharomyces Cerevisiae* expressing S from recombinant DNA. In Europe a vaccine, containing both the S and M envelope proteins expressed in a Chinese hamster ovary cell line, is also available. This latter seems to result in lowering the frequency of nonresponders to the classical vaccine (Holzer et al., 2003). These vaccines are administered by intramuscular injection in the deltoid region and are highly protective with an efficacy > 95% for children or young adults. About 5% of those vaccinated fails to respond with development of antibodies to the vaccine (Hollinger et al., 2007; Zanetti et al., 2008). The protection last for more than 10 years and although anti-HBs antibodies titer may decline to undetectable levels, the immunity persists, indicating the existence of an immunologic memory (Osioy, 2018). An universal vaccination program has been recommended by the WHO in order to eradicate HBV by 2030. As example, the implementation of such a program in Taiwan resulted in a loss of HBsAg prevalence: from 9.8% in 1988 to 1.3% in 1994 in children (Chen et al., 1996; Ni and Chen, 2010). In Italy, the same mass vaccination program was implemented with similar results (Tosti et al., 2016). In France, since January 1st of 2018, the vaccination is mandatory again for <18 months child, due to resurgence of HBV, imputable to population movements and migration (LOI n°2017-1836 Art.L.3111-2.-I.)(Coppola et al., 2015).

Furthermore, vaccination campaigns have not only decreased the incidence of HBV infection and the prevalence of chronic carriers, but also the incidence of HCC, thereby making HBV vaccine the first to have proven efficacy in cancer prevention (Stroffolini et al., 2000).

Eventually, other prevention strategies, such as screening the blood products, proper sterilization of medical instruments, antenatal screening of pregnant women and avoidance of risky behaviours may preclude HBV transmission. General education should be advocated in addition to vaccination as in highly endemic regions, the knowledge awareness among the healthy population is not satisfying yet (Yasobant et al., 2017).

2.2.2. Antiviral therapies

The main goal of therapy is to improve survival and quality of life by preventing disease progression and subsequent HCC development. The suppression of HBV DNA to undetectable levels and normalisation of ALT levels as well as HBeAg seroconversion are considered as clinical endpoints of therapy. The loss of HBsAg is perceived as the optimal treatment endpoint, termed “functional cure”, but is only rarely achieved with the current antiviral arsenal and should be an overall objective for HBV eradication.

Currently, there are two main therapeutical options for CHB patients: treatment with a NA or with IFN- α . This latter has been used since the 1970s (Greenberg et al., 1976). Although IFN- α has a direct antiviral effect, suppressing HBV replication, its major effect is considered immunologic. Nowadays, IFN- α is used under a pegylated form, thus increasing its stability (Craxi and Cooksley, 2003). However, this antiviral strategy tends to lose fields toward more specific and well-tolerated NAs. In Europe, six NAs have been approved for the treatment of HBV infection, classified into:

- Low barrier against HBV resistance: Lamivudine (LAM), Adefovir dipivoxil (ADV) and Telbivudine (TBV).
- High barrier to HBV resistance: Entecavir (ETV), Tenofovir disoproxil fumarate (TDF) and Tenofovir alafenamide fumarate (TAF).

The main advantage of treatment with a NA exhibiting a high barrier to resistance is its predictable long-term antiviral efficacy as well as its favourable safety profile (Lok et al., 2016; Terrault et al., 2016). Currently, management of HBV infection with antiviral therapy is recommended only for patients with active liver disease and not for patients that do not have elevated ALTs and/or evidence of inflammatory liver disease. This has been mediated by the fact that NAs at disposition, until recently, exhibited a low barrier resistance (*i.e.*, LAM). The higher barrier to entecavir and tenofovir resistance may lead to a change in treatment guidelines (Table III).

However, a long life treatment is mandatory to prevent HBV reactivation upon treatment arrest, as NAs are ineffective on suppressing the cccDNA. CccDNA loss under NAs therapies is believed to reflect the death of infected hepatocytes rather than intracellular instability, at least in the context of chronic infections. NAs, which act by inhibiting HBV DNA synthesis, thus indirectly lower the amount of cccDNA, as some infected hepatocytes die and others proliferate to maintain the overall liver cell population (Summers et al., 1990; Fourel et al., 1994; Zhu et al., 2001).

Drug	Year 1	Year 2	Year 3	Year 4	Year 5
Lamivudine	24	38	49	67	70
Adefovir	0	3	11	18	29
Entecavir	0.2	0.5	1.2	1.2	1.2
Entecavir (LAM resistant)	6	15	36	46	51
Telbivudine	4	17	-	-	-
TDF	0	0	0	0	0
TAF	0	0	-	-	-

Table III: Incidence of drug resistance during antiviral therapy. Data are presented in %. No evidence of resistance has been shown after 8 years of TDF treatment (long term treatment with TDF for chronic hepatitis B infection is safe and well tolerated and associated with durable virologic response with no detectable resistance: 8 year results from two phase 3 trials).

2.2.3. Future insights in anti-HBV drug development

The currently approved therapies exhibit an overall satisfactory antiviral effect by decreasing the HBV DNA to undetectable levels, good HBeAg seroconversion and limited side-effects. The issue concerning resistance to NAs (up to 80% of patients treated for 5 years with lamiduvine) is partially solved with the availability of innovative, high barrier resistance molecules such as ETV, TDF and TAF. Nowadays, the life-long treatment need and low HBsAg seroconversion are the two remaining problems and need to be addressed by the scientific/medical HBV community.

In this respect, the step forward in anti-HBV drug development is focused on targeting the cccDNA, by preventing its formation or promoting its degradation/silencing. Another trendy way to moving forward antiviral strategy is to focus on the HBc protein, which is involved in several HBV life cycle steps, as detailed in the review I have written (*confer* **Appendix** review (Diab et al., 2018)). Regarding pre-clinical efforts, you can refer to the study I get involved in, that deciphers the anti-HBV activities of two CpAMs being trialed by the Janssen pharmaceutical group (*confer* **Appendix** paper Lahlali et al., 2018)).

An innovative approach resides in the use of siRNA, targeting either HBV directly or proviral host factors. In this regard, the compound ARB-1467 from Arbutus Biopharma, comprises three siRNA triggers that target all four HBV transcripts and has been shown in preclinical studies to reduce all viral antigen levels as well as HBV DNA. Furthermore, these siRNA utilizes Arbutus' proprietary lipid nanoparticle (LNP) platform to improve proper delivery. The ARB-1467 is currently under phase 2a clinical trial in subjects with CHB infection receiving nucleos(t)ide analogue therapy (NCT02631096). In my second study, we took advantage of the LNP technology to deliver siRNA targeting PLK1, a host kinase considered as a HBV proviral factor (*confer* **Second study: Antiviral activity of PLK1-targeting siRNA delivered by lipid nanoparticles in HBV-infected hepatocytes**).

Finally, other anti-HBV strategies such as restoring immunity (i.e., TLR-7/8 agonists, reverse T-cell exhaustion), targeting proviral host factors (i.e., HTA), genomic approaches (siRNA or CRISPR/Cas9) and RNA destabilizers are under early-drug development and could reinforce the anti-HBV arsenal, allowing a more personalized medicine (Gane, 2017; Gilani et al., 2018). The management of HBV infection should come from either a breakthrough innovation or more probably from combining already approved NAs with new molecules.

According to the WHO and following model previsions, the incidence of chronic infections should reduce by 90% and mortality by 65% (compared to 2015), which would eliminate hepatitis B as public health threat by 2030.

However, as mentioned earlier, available treatments are limited and the cccDNA elimination seems far to be reachable. Although potent inhibition of viral replication could be completed, the risk of developing HCC is still a major threat. Moreover, we still do not know how to manage HBV integration events, which account also for HCC development.

2.3. HBV-induced HCC: a threat to global health

2.3.1. Generalities

HCC management is a global challenge for the WHO, with 800,000 deaths/year worldwide (**Figure 18**). Among them, >340,000 are related to HBV etiology with an increasing incidence in developing countries. HCC is currently the main concern for CHB patients with up to a 100 fold increased risk of developing liver cancer compared to general population. Of the 257 million people chronically infected, <5% are estimated to be efficiently treated ([Rapti and Hadziyannis, 2015](#)). The first line of defense towards HBV-induced HCC is thereby improvements in prevention/diagnosis and proper access to antiviral therapies; especially in HBV highly-endemic regions.

However, patients who have been treated with appropriate NAs have still a risk of developing HCC of 5 fold the normal ([El-Serag, 2012](#)). This latter point highlights the need to put efforts in fundamental research in order to better understand HBV biology (i.e., role of cccDNA and integrated DNA, host/virus interactions, long-lasting immunity modulation...). Thus, the ultimate goals in the treatment of chronic HBV infection are to suppress viral replication, eliminate the cccDNA and *in fine* prevent the life-limiting outcomes of liver failure and liver cancer.

2.3.2. Epidemiology

Soon after HBV discovery, the scientific community aimed for a potential link between HBV and HCC. In a cohort of 17 chronically infected patients, Sherlock and colleagues found five HCC positives, which lead the HBV community to focus further on a possible link between the virus and liver carcinogenesis. HCC develops rarely during the first 4 decades of life, except in HBV hyper endemic regions, bringing a new clue in the HBV-HCC correlation ([El-Serag, 2012](#)). Definitive support in favor to the hypothesis that HBV plays a role in HCC development came from epidemiologic studies. Particularly, a prospective study on >22,000 Taiwanese men has revealed that HBsAg positive individuals have a relative lifetime risk of developing HCC multiplied by 20 compared to uninfected ([Beasley et al., 1981](#)). Finally, the strongest evidence was found in WHV-infected woodchucks, where, as explained before, 100% of individuals develop HCC within 3 to 4 years post-infection ([Korba et al., 1989](#)).

Most of HBV-infected patients who develop HCC have cirrhosis (70-90% of HBV-induced HCC) secondary to the necroinflammation. The seroprevalence of HBsAg among patients with HCC varies globally with 3% in Sweden, 55% in Greece and can reach up to 70% in South Korea. In accordance to several studies, the HCC incidence rate is 0.02 per 100 person-years in inactive carriers, 0.3 in subjects with chronic HBV without cirrhosis, and 2.2 in subjects with cirrhosis ([El-Serag, 2012](#)). Several factors have been reported to increase the HCC risk among HBV-infected patients such as

demographic ones, viral (HBV genotypes, replication level and co-infection) and environmental (alcohol, tobacco and exposure to aflatoxin).

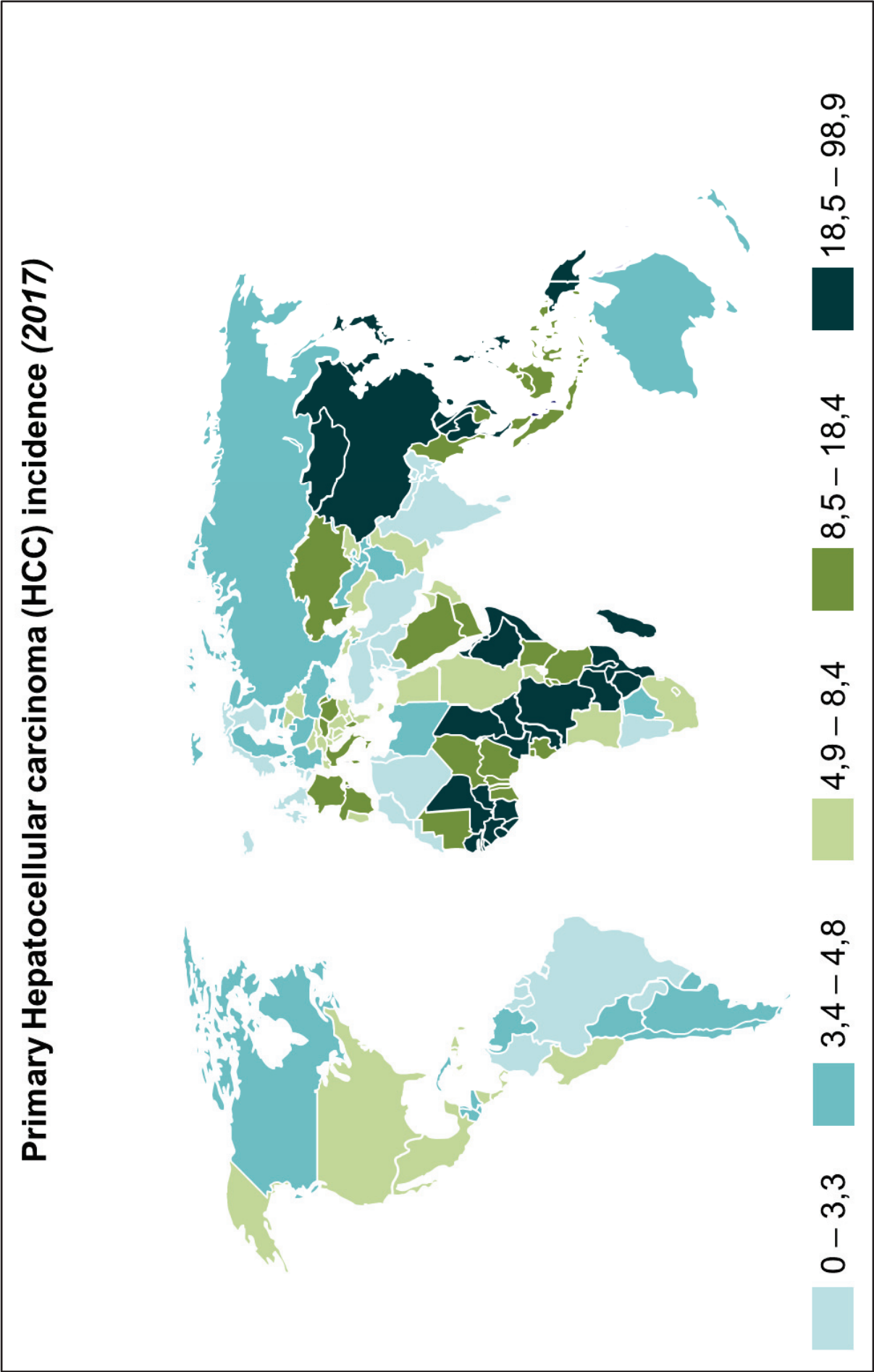


Figure 18: Global incidence of hepatocellular carcinoma. Incidence means number of cases for 100,000 individuals.

2.3.3. Factors increasing the HCC risk among HBV-infected patients

2.3.3.1. Sex: Men at risk

It is well known that men have an overall increased risk for HCC development (sex ratio male-to-female up to 5), which is partially explained by the higher exposure to alcohol and viral hepatitis (Liu et al., 2017)(Figure 19). However, the risk remains increased when these two confounders are adjusted and men have an increased risk for cirrhosis and subsequent HCC, especially in HBV-infected group.

Moreover, the viral load is usually higher in men. A study focusing on >18,000 Taiwanese and Shanghainese HBV-carriers has shown that high serum level of testosterone is associated with HCC, suggesting an involvement of androgens in both viral replication intensity and carcinogenesis (Yuan et al., 1995). In transgenic mice model, studies have shown that HBV can increase the transcription of androgen receptors through the HBx protein and conversely, androgens bind directly the viral genome, leading to higher HBV transcription (Chiu et al., 2007; Yeh and Chen, 2010).

Finally, some papers reported that oestrogen can be favourable and protects against HBV infection progression. On the one hand, estradiol has been described as a potent antioxidant, which suppresses hepatic fibrosis and decreases induction of redox sensitive transcription factors, hepatocyte apoptosis and hepatic stem cells activation by inhibiting reactive oxygen species production in primary cultures (Shimizu et al., 2007). On the other hand, the frequency of NK cells in the liver of chronically HBV-infected females was higher than in males and correlated with plasma level of estradiol (Macek Jilkova et al., 2017). These two latter points could partially explain the ability of females to better deal with HBV disease.

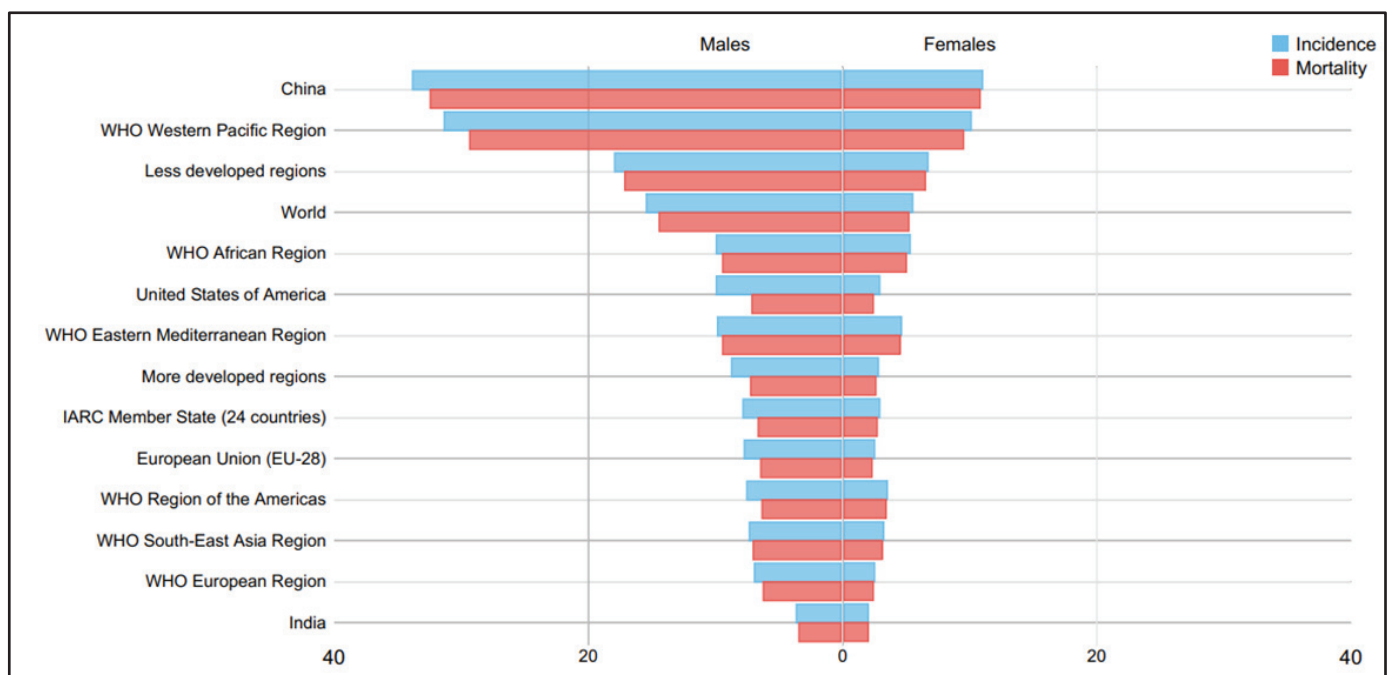


Figure 19: Estimated age-standardised incidence and mortality of liver cancer worldwide Male/Female. Cases per 100,000. Globocan 2012.

2.3.3.2. The impact of HBV genotypes on HCC development

As largely developed in the section “1.2. Classification and phylogeny of *Hepadnaviridae*”, due to genetic variability, the human HBV is further classified in eight (A to H) distinct, well-established genotypes. These latter seem to affect both natural course of HBV infection and clinical outcomes.

Genotypes B and C are more common in Asian population and studies have shown that HBV genotype C was strongly associated with progression of chronic infection to severe liver diseases, such as cirrhosis and HCC. In a meta-analysis investigating the correlation between HBV genotypes and HCC, HCC detection was 12% for genotype B and 25% for genotype C, with an odd ratio of 2.05 (Wong et al., 2013).

However, a study from Taiwan demonstrated that genotype B led more rapidly to HCC development, with most of patients under 50 years old and no evidence of cirrhosis (Kao et al., 2003). Another Taiwanese study, focusing on children HBV carriers highlighted that genotype B was involved in 74% of HBV-related HCC (Ni et al., 2004).

These different clinical outcomes depending on HBV genotypes are mostly due to sub types variations or well-identified mutations in the HBV genome. Focusing on the BCP, T1762 and A1764 are known to increase HCC incidence while mutations found in the precore region such as G1896A are associated with a decreased risk of HCC (Liu et al., 2006; Yang et al., 2008). A study from China, conducted on HBeAg-positive patients infected with the C1 sub-genotype, reported that, in addition to T1762/A1764 BCP mutations, V1753 and A1768 mutations were closely correlated with HCC. Moreover, the increased risk of HCC mediated by these BCP mutations could be partially based on modifications of HBx biological functions (Li et al., 2013).

Furthermore, antiviral drug-selected mutants such as the hepatitis B virus rtA181T/sW172* which has a dominant negative secretion defect of HBsAg and alters the profile of viral rebound, may lead to missed diagnosis if viral load is used as the only criterion for drug failure. Thus, implementing HBV polymerase chain reaction sequencing is mandatory to detect these drug-selected mutant, in order to prevent undetected HBV infection and subsequent risk for HCC progression (Warner and Locarnini, 2008).

Finally, a recent study published in *Hepatology* identified a specific mutation in the core region of HBV genotype F1b within an Alaskan native population. The A2051C mutant led to upregulation of proliferation and/or carcinogenesis related-genes such as MYC, GAB2, BDKRB2, FST and MAP3K8 in human hepatocytes infected with genotype F1b. Moreover, this mutation was associated with better viral replication and an enhanced stability of HBc dimerization. This specific HBV genotype is then associated with quicker progression to fibrosis and HCC than other genotypes (Hayashi et al., 2018)(Figure 20).

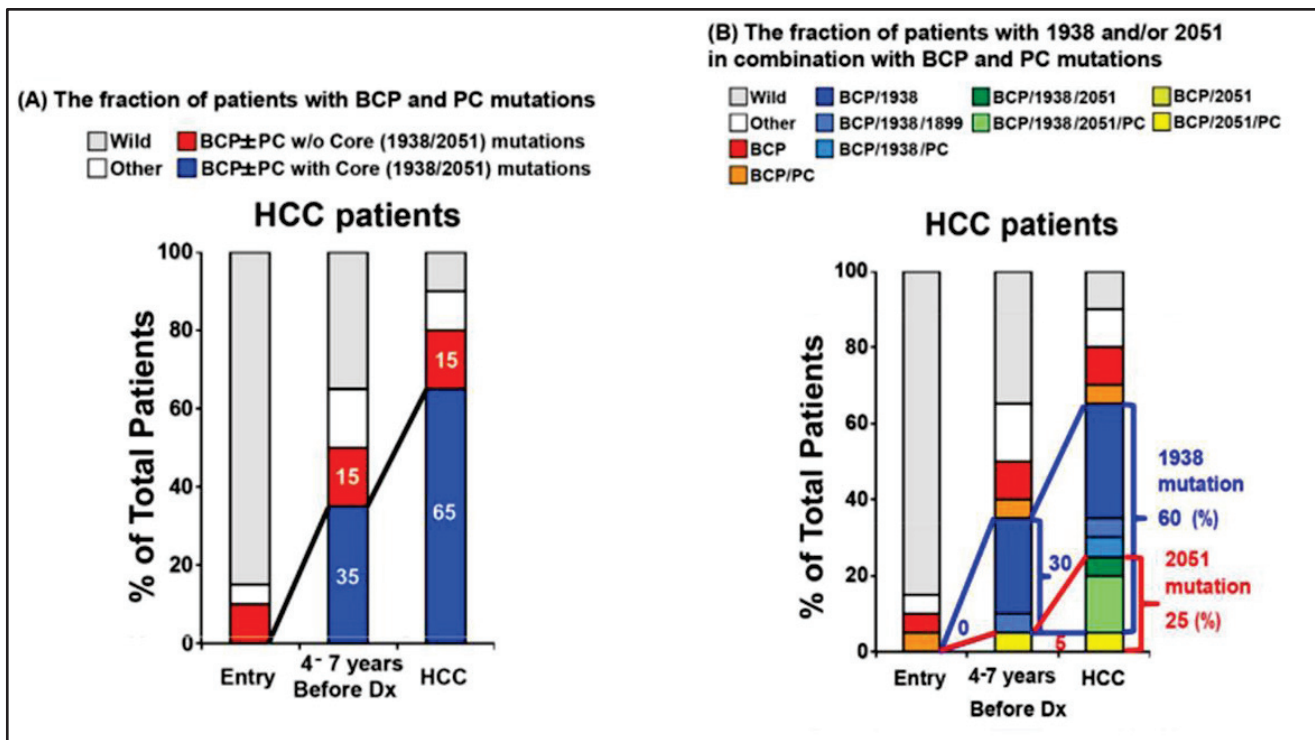


Figure 20: Viral mutation pattern in HBV infected patients over a longitudinal period. Among all the patients with HCC, 65% exhibits BCP/PC and 1938/2051 mutations (panel A, blue). 25% of HCC presents the A2051C mutant with or without other mutations in BCP or precore regions (panel B, yellow + light and dark green). Dx means diagnosis. Extracted from Hayashi S *et al.*, Hepatology, 2018.

2.3.3.3. Genetic factors

Several genetic factors have been associated with an increased risk of HBV-related HCC. Among them, the p53 mutant R249S is the most widely described and believed to directly cooperate with HBx for HCC development, in a pathway that bypasses cirrhosis (Ortiz-Cuaran *et al.*, 2013). This p53 specific mutant is found to be induced mainly by aflatoxin exposure and its involvement in liver carcinogenesis is discussed in a further extent in the following section "2.3.3.4. Aflatoxin B₁ and HBV: the two-headed monster".

Regarding TNF- α , IL-23R, CD3G, MiR-106b-25 cluster, non-classical HLA, IL-18, and CTLA-4, specific SNP alleles have been described to increase the HBV-related HCC risk albeit the mechanisms have still to be determined (Xu *et al.*, 2014).

Recently, new tools such as genome-wide association studies (GWAS) helped to identify five independent loci that are significantly associated with HBV-related HCC: rs17401966 at KIF1B, rs9272105 at HLA-DQA1/DRB1, rs455804 at GRIK1, rs7574865 at STAT4, and rs9275319 at HLA-DQ (Zhang *et al.*, 2010; Li *et al.*, 2012; Jiang *et al.*, 2013). However, these studies were Asia-centric and descriptive thus, the biological process underlying is yet to be deciphered. Moreover, the role of these loci in carcinogenesis may be controversial, as the rs17401966 at KIF1B was not found to be associated with HBV-related HCC development in Taiwanese patients (Sopipong *et al.*, 2013).

Interestingly, familial predisposition as a risk factor for HBV-related HCC has been suggested. In a study published in 2000 by Yu and colleagues, the HCC risk associated with having parents and/or siblings with HCC was evaluated in 4808 male HBV-positive patients. The main conclusion was that first-degree relatives of patients with HBV-related HCC appear to be at increased risk for liver cancer development (Yu et al., 2000). Shared environment, habits but also genetic background are regrouped under this "familial predisposition" term. Regarding genetics, a GWAS has identified GLUL and SLC13A2/FoxN1 haplotypes for familial HBV-related HCC. To date, this is the sole genome-wide association study to identify genetic factors for familial HBV-related HCC, which differ from sporadic HCC (Lin et al., 2017).

2.3.3.4. Aflatoxin B₁ and HBV: the two-headed monster

Aflatoxin B₁ (AFB₁) is a Mycotoxin produced by *A. flavus* and *A. parasiticus* fungi and is mostly found in contaminated food, due to warm and damp storage conditions of corn and peanuts. AFB₁ is a powerful hepatocarcinogen, leading the International Agency for Research on Cancer to classify it as a class-1 carcinogen (Overall evaluations of carcinogenicity IARC). Once ingested, AFB₁ is metabolized within the hepatocytes in AFB₁-*exo*-8,9-epoxide, which can bind to and damage DNA, leading especially in a mutation at the serine codon 249 of the tumour suppressor p53. This specific event is detected in ≈50% of HCC from aflatoxin-endemic areas patients, most of whom had HBV infections (Bressac et al., 1991; Turner et al., 2002). Areas where aflatoxin is a recurrent environmental problem correlate with highly endemic HBV regions (Figure 21).

There is evidence from both epidemiological studies and animal models that the two factors can act synergistically to increase the HCC risk, but the underlying mechanisms of interaction are yet undefined. As example, studies in China have shown that AFB₁ increased the risk of HCC by 4 fold and HBV alone by 7 fold. However, when the two factors are presents, the carriers had as much as a 60-fold increase in risk of HCC (Qian et al., 1994). Thereby, the prevention of HBV-induced HCC by establishment of vaccination programs in highly endemic areas would reduce the effects of AFB₁ on liver carcinogenesis risk (El-Serag, 2012).

Regarding the mechanism involved, albeit being not well deciphered yet, it was assumed that most of the pathogenesis was imputable to the R249S p53 mutant. Indeed, R249S has lost ability to bind p53 response elements and to transactivate downstream genes, *in fine* increasing proliferation and clonogenic properties in HCC. The mutant p53 R249S was found to form a complex with HBx in PLC/PRF/5 cells, but the interplay between these two factors is still unprecised (Gouas et al., 2010). In 2010, Jiang and colleagues has demonstrated that HBx variants may cooperate with p53 R249S mutant, inducing aneuploidy, anchorage-independent growth and proliferation in a telomerase-immortalized normal human hepatocyte-derived cell line (Jiang et al., 2010).

However, the increased risk of HCC found in HBV-carriers and patients exposed to AFB₁ concomitantly, is not only driven by the p53 axis. Recently, the autophagy-related protein p62 has been pointed out as a possible prognosis factor for HCC in AFB₁-HBV population. Indeed, p62 expression was found to be correlated with high NRF2 and Nqo1, promoting tumour recurrence. Finally, this pathway downregulates AKR7A3, which is mandatory for liver detoxification of aflatoxin B1 (Xiang et al., 2017).

From a fundamentalist point of view, meta-analysis of correlation between AFB₁ and HBV genotypes in HCC risk would be useful to further decipher the underlying mechanism involved.

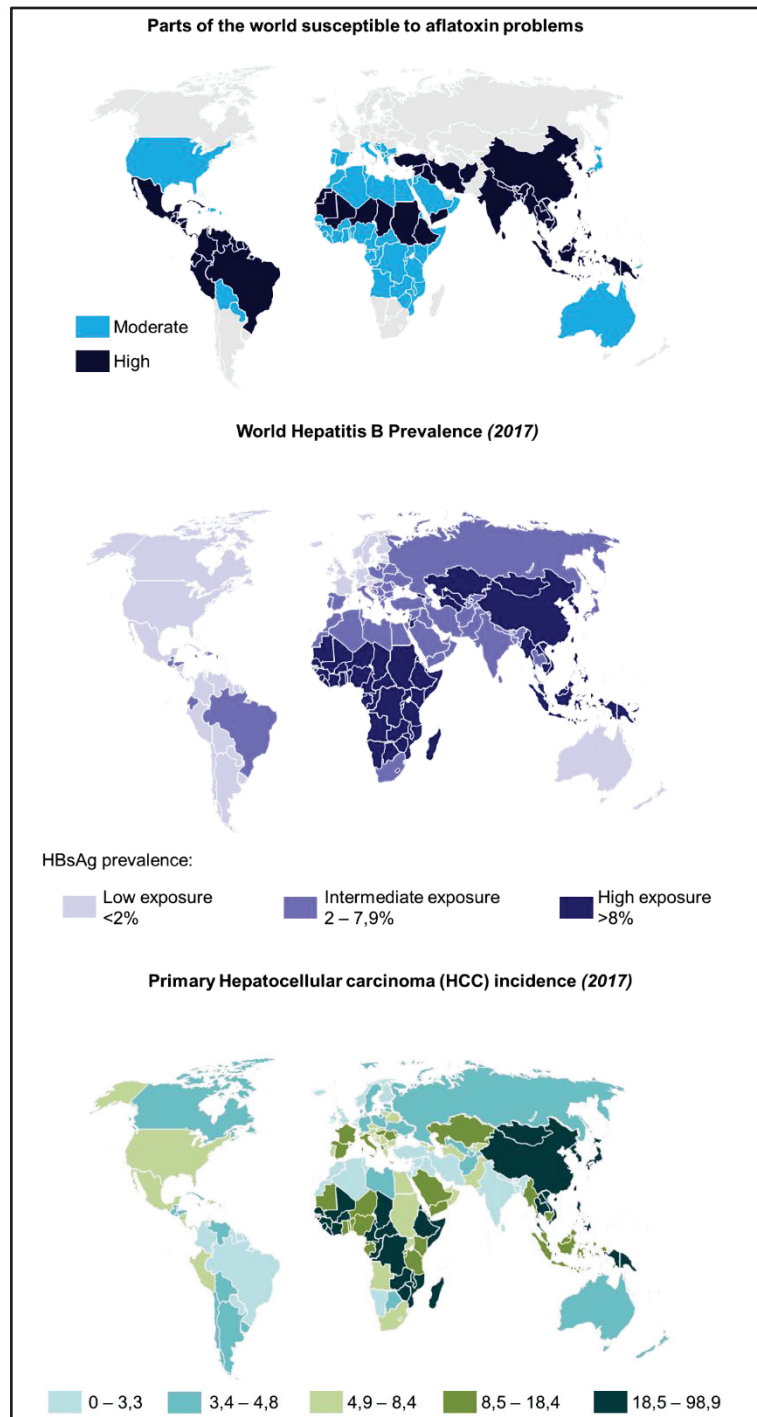


Figure 21: World maps presenting repartition of aflatoxin, HBV and HCC globally. Incidence means number of cases for 100,000 individuals.

2.3.3.5. Herbal traditional Chinese medicine and HBV-related HCC

Aristolochic acids are known to be carcinogenic, mutagenic and nephrotoxic phytochemicals since the early 2010s (Shaw, 2010). They are commonly found in birthwort plants which are used in an extensive manner in Chinese traditional medicine. Recently, it was suspected that aristolochic acid-induced mutations may be associated with HBV to fasten the development of HCC. A retrospective, population-based (>800,000 individuals) study conducted in Taiwan by Chen and colleagues highlighted a significant dose-response relationship between the consumption of aristolochic acid and HCC in patients with HBV infection (Chen et al., 2018). Although the use of herbal medicine containing aristolochic acid has been largely banned worldwide, the community highlights the need of a close follow-up of HBV positive patients who have taken those herbs.

2.3.4. Mechanisms of HBV-induced HCC

The pathogenesis of HBV-induced HCC is a multifactorial process with still elusive mechanisms. However, the field has been driven by two hypotheses; on one hand, the virus is thought to act directly on carcinogenesis while on the other hand, HCC formation might be a consequence of chronic necroinflammation due to the immune response, unveiling an indirect role of HBV. Actually, both contribute to HCC development.

2.3.4.1. HBV direct-associated hepatocarcinogenesis

2.3.4.1.1. HBV dsDNA integration: the *cis*-effect

A significant fraction of virions (≈10%) contains dsDNA as incoming genomic material, instead of rcDNA. These entities can both give rise to cccDNA from being converted by cellular enzymes, yet, dsDNA can also integrate into the host cell genome at double-strand DNA breaks by non-homologous end joining (NHEJ) (Bill and Summers, 2004; Tu et al., 2018)(Figure 22). Although the reimport of dsDNA-containing nucleocapsids occurs under HBV replication, it appears to play minor role in HBV integration events. Interestingly, complete HBV genome integration virtually never occurs, thus, in most of the cases, these inserts cannot serve as template for viral replication.

Up to 85% of HCC from HBV etiology contains HBV integrated DNA and through clinical progression of chronic hepatitis B, dsDNA virions fraction increases and can reach up to 35%, suggesting a correlation between these two factors (Bréchet et al., 2000). Integration events are observed in all known hepadnaviruses, including woodchuck, ducks, as well as in chimpanzees and humans (Yang and Summers, 1999; Summers and Mason, 2004; Mason et al., 2009, 2016). Since hepatocytes are the only liver cells clearly susceptible to HBV infection/replication, it was hypothesised that HCC arise from dedifferentiation of hepatocytes. The first evidence for a direct

role of HBV in the liver carcinogenesis was fuelled by the observation of clonal HCC tumours with respect to the viral integration site (Esumi et al., 1986).

Nowadays, integration of HBV DNA, also named the *cis*-effect, is one of the reported drivers of HCC development by inducing pro-carcinogenic pathways. Among them, chromosomal instability, insertional mutagenesis in key cancer-associated genes, transcription of downstream cancer-associated cellular genes, and formation of a persistent source of viral protein expression were reported (Budzinska et al., 2018). However, the contribution of each of these mechanisms is still unclear, as no sequence consensus integration sites have been found. HBV seems to be able to integrate widely into the host genome, but occurrence in chromosomes 13, X and Y appeared to be less frequent (Murakami et al., 2005; Jiang et al., 2012a). Finally, the number of HBV integrations is associated with lower patient survival as it maximizes the risk of insertion within critical genes.

Nevertheless, several hot point sites of HBV DNA integration into the host genome have been reported. Indeed, large-scale analysis using the Alu-PCR approach revealed that integration events occur frequently in actively transcribed regions. Recurrent HBV insertions nearby the putative cancer-related TERT, the epigenetic regulator MLL4 and the cell-cycle CCNE1 genes have been highlighted (Paterlini-Bréchet et al., 2003; Murakami et al., 2005; Sung et al., 2012)(Figure 23).

Regarding the rate of integration in different stages of infection, a recent study has shown that HBV insertion in human genome is an event frequently found in mild phases of chronic hepatitis. Using Alu-PCR followed by a downstream Sanger sequencing of the amplicons, they found integration events in 39/74 biopsies (52%), which is far below the 85-90% expected. However, the use of a single primer spanning a human-HBV region could have missed several insertional events (Larsson et al., 2018).

Moreover, HBV integration has been associated with important genomic alterations, such as chromosomal translocations, inverted duplication events and large deletions (Yaginuma et al., 1985; Hino et al., 1986; Tokino et al., 1987). Some of these latter could explain the relatively rare occurrence of HCC on non-cirrhotic liver.

At last, HBV integration can act in *trans* on carcinogenesis. Although integrated viral sequences are defective for replication, it can result on HBx and/or HBs mutants' production, which is described to disrupt and redirect cellular functions, leading to HCC development. Indeed, several studies have shown that >40% of the integration breakpoints on the HBV genome occurred within a 1,800bp range, where the regulatory DR1 region is located and near the 3' end of HBx gene, leading to virus-human fusion protein and/or mutated HBx production (Jiang et al., 2012b; Sung et al., 2012)(Figure 24). In this regard, a study published in 2015 by Quetier and colleagues reported that C-terminal-truncated HBx proteins (Ct-HBx) production that is due to HBV integration is a potent cell transforming factor. In mice treated with DEN in order to initiate the carcinogenesis, Ct-HBx expression was correlated with higher incidence and number of liver lesions and associated with

inflammation. Thus, they speculated that truncated HBx may therefore facilitate HCC development in chronically infected patients (Quetier et al., 2015).

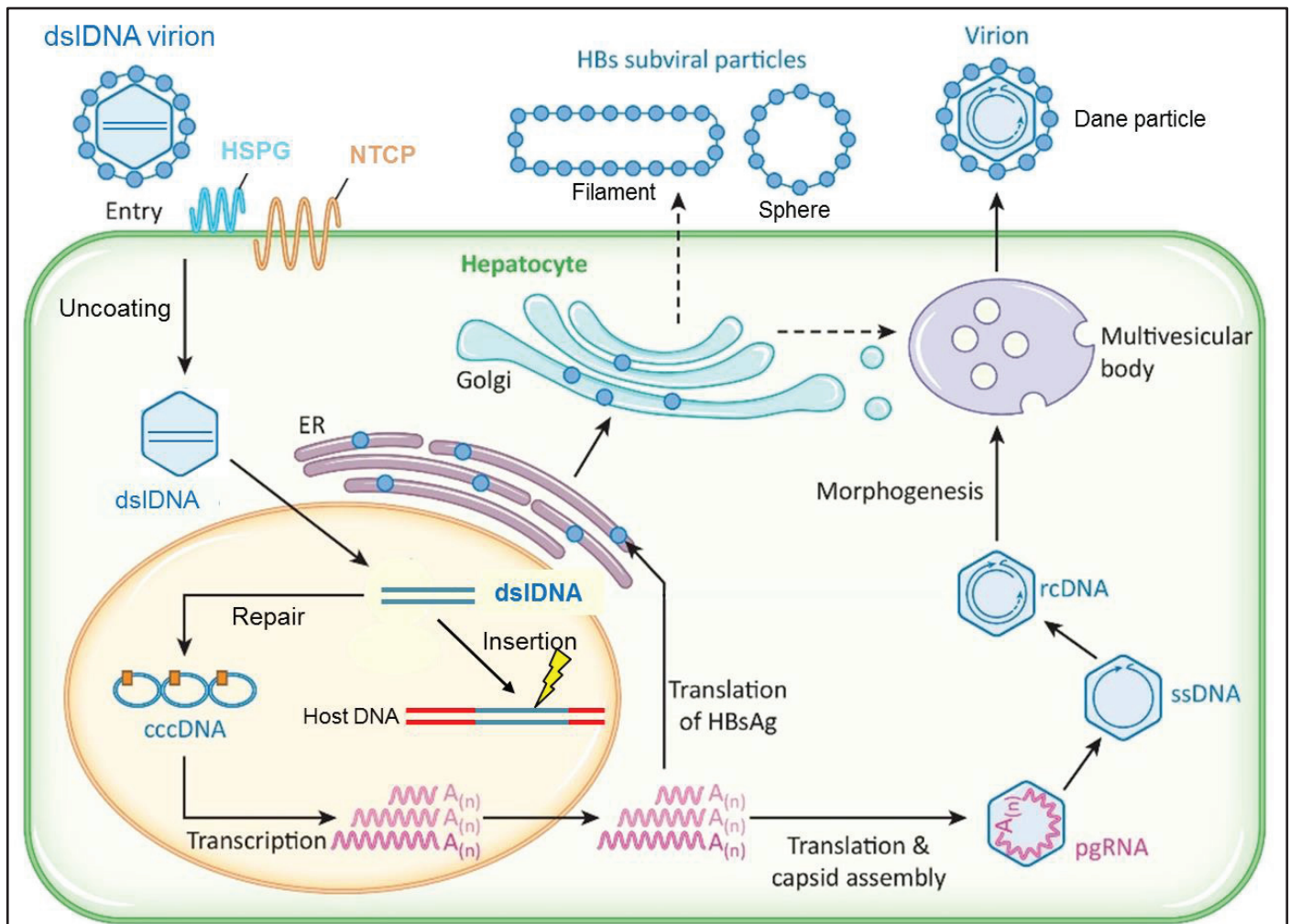


Figure 22: Infection of a hepatocyte by dsDNA virion. Once in the nucleus, the HBV dsDNA can be repaired into cccDNA or integrate the host genome at double-strand DNA breaks through NHEJ. cccDNA originating from HBV dsDNA has the same fate as cccDNA from rcDNA and permits establishment of long-lasting infection and fully functional viral replication cycle. Adapted from Shih C *et al.*, Trends in Microbiology, 2018.

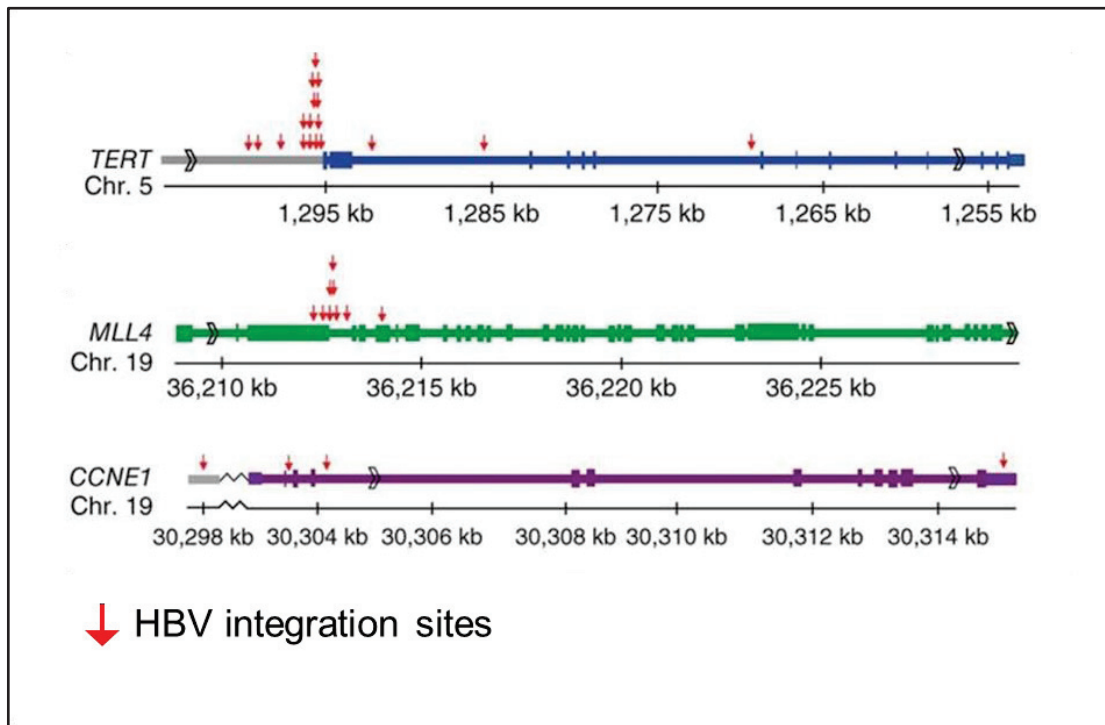


Figure 23: Mapping of HBV integration sites. Each arrow represents a HBV breakpoint from one clinical sample. Boxes represent exons. Extracted from Sung WK *et al.*, Nature Genetics, 2012.

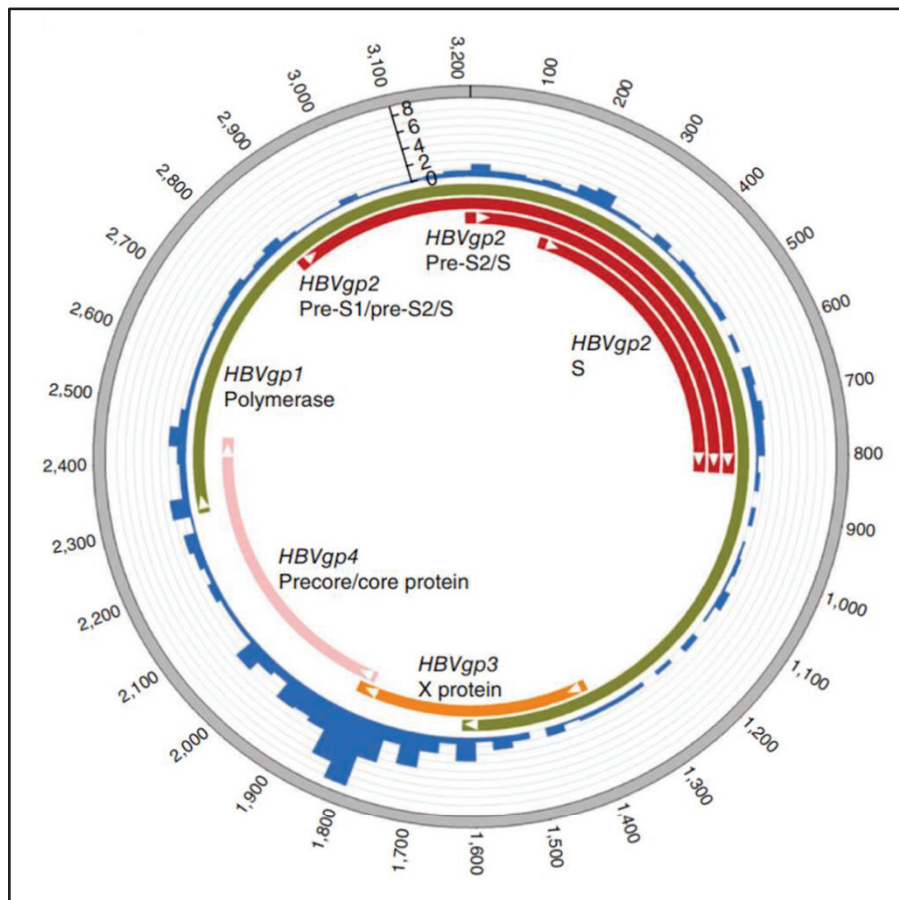


Figure 24: Visualization of breakpoints in HBV genome. The frequency of integration breakpoints at different loci in the HBV genome is shown as a blue histogram, highlighting the 1,800bp region, where the HBx gene is located. Extracted from Sung WK *et al.*, Nature Genetics, 2012.

2.3.4.1.2. HBV putative onco-proteins: the *trans*-effect

This second HBV direct-associated hepatocarcinogenesis, called the *trans*-effect, highlights the involvement of viral gene products as potential onco-proteins. Although HCC development occurs decades after chronic infection, indicating that HBV cannot transform hepatocytes in a single hit event, the hypothesis gained momentum with the discovery that transgenic mice expressing the entire coding sequence of HBx developed HCC (Kim et al., 1991). From this study, it appears that HCC development under HBx overexpression was mostly due to the selection of the mouse strain, CD1, known to develop spontaneous hepatomas at an increased rate. Thus, HBx was described to promote but not necessarily initiate carcinogenesis. Supporting this hypothesis, in another background, HBx transgenic mice treated with diethylnitrosamine (highly mutagenic/oncogenic compound) were more susceptible to HCC than nontransgenic individuals were (Slagle et al., 1996). Furthermore, in 2003, a study has shown a collaborative influence of HBx and c-Myc that result in HCC development after a prolonged latent period (Lakhtakia et al., 2003). Finally, several cell-culture based studies have demonstrated effects of HBx on proliferation and cell viability, strengthening observations made *in vivo* (Gearhart and Bouchard, 2011; Zhang et al., 2015; Fan et al., 2016; Wang et al., 2017b).

However, conflicting results emerged from these studies, describing opposite effects and suggesting that HBx could be cytostatic, or even induces apoptosis (Schuster et al., 2000). The main caveat regarding these studies is the use of irrelevant systems, where HBx is highly expressed while in chronically infected liver, its level is barely detectable (Su et al., 1998; Neuveut et al., 2010). Thus, the expression of HBx protein was described to potentiate the induction of liver disease in a long run manner, thereby classifying HBx as a weak oncogene.

Focusing on the underlying mechanism of HBx-induced HCC, albeit being not precisely well-defined, it is believed to involve multifunctional aspects of HBx, acting in a timely fashion in order to support HCC initiation and/or development. HBx is able to directly sequester p53, leading to p53-mediated apoptosis inhibition, which is thought to be one of the main mechanisms responsible for HBx-induced hepatocarcinogenesis (Chan et al., 2016). Another major axis is the CREB/ATF pathway activation, which is involved in liver metabolism, cell proliferation and hepatocarcinogenesis (Abramovitch et al., 2004). In HBx-transfected cells, a study from Cougot and colleagues has shown that HBx interacts with CBP/p300, playing a decisive role in the downstream CREB-dependent transcription. In this model, HBx seems to increase CREB/ATF DNA-binding affinity as well as to enhance the recruitment of CBP/p300 to CREB/ATF bound to cellular DNA. Furthermore, a microarray-based screen for HBx target genes allowed the authors to unveiled activation of two CREB-responsive genes; IL-8 and PCNA in primary human hepatocytes and confirmed their results in HepaD38, where the level of HBx is significantly increased after tetracycline removal (Cougot et al., 2007). As CBP/p300 recruitment represent the limiting event for activating CREB target genes, HBx could overwhelm this issue and therefore predispose cells to transformation.

HBx was also found to interact with DNA repair protein such as DDB1, however studies tend to conclude that HBx regulates the function of DDB1 in both positive and negative manners, thus playing different roles in viral replication and carcinogenesis. In 2016, Decorsière and colleagues uncover a novel role for the Smc5/6 complex, which can act as a restriction factor blocking specifically extrachromosomal DNA transcription. HBx, by hijacking the DDB1-containing E3 ubiquitin ligase complex to target Smc5/6, promotes the cccDNA transcription, and therefore contributes to HCC development (Decorsière et al., 2016).

The group of Dr Andrisani has been largely involved in the understanding of HBx-induced transformation. Notably, they have shown that HBx and PLK1 where inherently linked and playing crucial functions in oncogenic transformation, mostly by enhancing the degradation of repressing factors involved in the inhibition of cellular proliferation. This axis is extensively developed in the section **“3.3.9. PLK1 as a keystone in virus-induced oncogenesis? The example of HBV-related HCC”**.

At last, HBx has been shown to inactivate BubR1, an effector of several kinases, including PLK1, involved in centrosome dynamics and mitotic spindle formation. Several studies have linked HBx expression to DNA re-replication, leading to partial polyploidy, which is highly associated with carcinogenesis (Andrisani and Barnabas, 1999; Rakotomalala et al., 2008; Studach et al., 2009a).

In summary, despite a wealth of information about biological activities of HBx, it is still hard to claim that HBx is involved as a strong oncogene in HCC development.

Interestingly, HBs has also been suggested as a potential oncogene, yet, to a lesser extent than HBx. Notably, overexpression of M truncated protein in transgenic mice induced the activation of the c-Raf/Erk2 pathway, leading *in fine* to an increased hepatocyte proliferation. After 1-2 years, these mice developed small liver tumours (Hildt et al., 2002). However, these observations are still on debate regarding their relevance and transposition to human carcinogenesis.

In addition, the intact L protein also has been shown to transactivate viral and host genes. Recently, a study published in *The Journal of Pathology* by Li et al., has shown that L-HBsAg is involved in HCC development through induction of cytokinesis failure, leading to aneuploidy in hepatocytes. This phenotype appears to be mediated *via* DNA damage and G2/M checkpoint override. Interestingly the authors have shown that this mechanism was supported by a PLK1 upregulation and that inhibiting PLK1 resulted in G2/M checkpoint restoration and in a decrease of tumour formation incidence, tumour volume and tumour weight (Li et al., 2018). This study strengthens evidences that HBs could act as an oncogene but also identifies a potential target to interfere with the L-HBsAg-induced HCC, which may be full of interest for patient failing to seroconvert after antiviral therapy.

2.3.4.2. HBV indirect-associated hepatocarcinogenesis

HCC development in patients is explained mostly by regeneration of hepatocytes, a compensatory effect to HBV-induced death. Hepatocytes are a self-renewing population with no impact on carcinogenesis as it occurs on a sporadic basis to maintain the liver mass. However, under persistent injuries, such as those observed in chronic hepatitis B, the high regeneration rate leads to fibrosis and cirrhosis, a favourable ground for HCC development. Of note, all factors inducing chronic liver disease are associated with an increased risk of HCC, and not necessarily due to HBV etiology only.

Regarding HBV, several studies have suggested that high levels of L-HBsAg are toxic for hepatocytes and transgenic mice developed HCC under this condition (Chisari et al., 1989). Due to high apoptosis rate, macrophages recruitment is increased and leads to free radicals generation, which are known to further expand hepatocyte DNA damages. In support to this hypothesis of HBV-induced carcinogenesis, Hagen and colleagues have detected deoxyguanosine adducts on DNA isolated from the livers of these mice (Hagen et al., 1994). In summary, L-HBsAg overexpression leads to high death and compensatory proliferation but also to hepatocyte DNA damage attributable to free radicals generated by macrophages.

The detail of HBV-induced liver carcinogenesis is still to be deciphered, as it remains elusive. The combination of viral gene expression, modification of cell physiology and changes in the microenvironment of the hepatocytes results in increased cell proliferation, which is one of the main hallmarks of cancer. However, the time course of occurrence of these events is still fuzzy. In one hand, viral mechanisms that contribute to hepatocyte transformation include modulation in gene expression caused by HBx and/or HBV insertions, HBsAg (mainly truncated M and full size L), along with inhibition of DNA repair and apoptosis pathways. On the other hand, cellular mechanisms include hepatocyte killing by CTLs, induction/propagation of DNA damage via free radicals generated by macrophages and expression of cytokines providing a proliferative stimulus to hepatocytes.

2.3.5. Prevention and treatments of HBV-related HCC

As already discussed, the prevention of HBV-related HCC depends mainly on the management of HBV infection. Considerable progress has been made in the last decades, especially thanks to large-scale programs of vaccination against hepatitis B virus. One of the most striking examples is found in Taiwan, where a universal infant vaccination program was introduced in the 80s. The decrease in the rate of HBsAg-positivity among children was associated with a significant childhood HCC reduction from 0.52/100,000 for those born between 1974 and 1984 to 0.13 for those born between 1984 and 1986 (Ni and Chen, 2010). However, childhood chronic hepatitis B infection and subsequent HCC have not been eliminated by the universal vaccination program, thus efficient treatments for hepatocellular carcinoma are still needed.

2.3.5.1. Staging and prognostic assessment

As for all oncologic pathology, the HCC staging is crucial for the choice of the adapted therapeutic protocol to follow and should help define outcome prediction. Since most of patients have associated liver disease, the prognostic assessment should not only focus on the tumour stage but also on the level of liver necroinflammation and the degree of liver function impairment. In this respect, the Barcelona Clinic Liver Classification (BCLC) system established in 1999 is the most commonly used staging system for hepatocellular carcinoma. Since its publication, it has been extensively validated and updated according to the results of investigations that have incorporated strong evidence that has modified medical practice. Other relevant classification systems are the Cancer of the Liver Italian Program; le Groupe d'Etude et de Traitement du Carcinome Hépatocellulaire; Tumour, Node, Metastasis (TNM); the Chinese University Prognostic Index; the Japanese Integrated Staging; the Taipei Integrated Scoring System; and, more recently, the Hong Kong Liver Cancer staging system (Yau et al., 2014). Basically, all these classification methods are constructed on the same basis albeit Asian systems tend to be oriented with personalized systemic therapies instead of surgical approaches. Recently, the community tried to improve staging system with implementation of tumour biomarkers, such as the level of α -fetoprotein in serum, microvascular invasion and tumour differentiation status (Llovet et al., 2012; Forner et al., 2018). These latter might have prognostic power but to date have no value in modifying treatment decisions.

2.3.5.2. Treatments

As HCC is considered chemorefractory and we still have no efficient enough molecules, the aim of treatment is to increase survival while maintaining the highest quality of life. This reason highlights the difficulties to evaluate the strength of any treatment approach as they are compared to untreated patients and effectiveness is always a reflex of a survival gain of few months. The potentially useful treatment number is large and goes from liver transplantation to selective tyrosine kinase inhibitors. The next chapters' goal is to give an overview of existing options for HCC management and to point out issues regarding HCC cure.

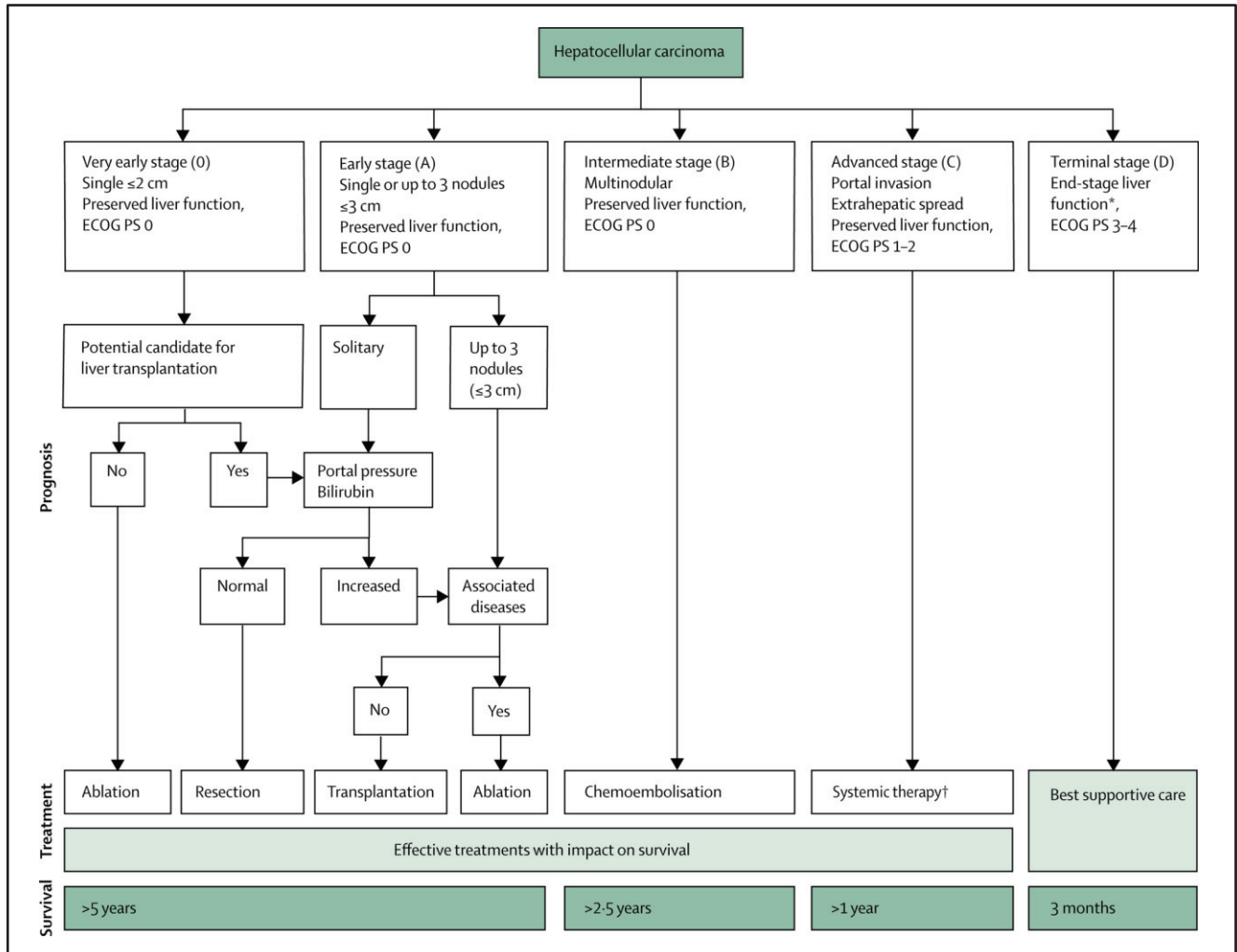


Figure 25: Barcelona Clinic Liver Cancer (BCLC) staging and treatment strategy. The BCLC system establishes a prognosis in accordance with the five stages that are linked to first-line treatment recommendation. ECOG PS=Eastern Cooperative Oncology Group Performance Status. *Patients with end-stage cirrhosis due to heavily impaired liver function. †Currently, Sorafenib followed by Regorafenib has been shown to be effective. Lenvatinib has been shown to be non-inferior to Sorafenib, but no second-line option after Lenvatinib has been explored. Extracted from Forner A *et al.*, The Lancet, 2018.

2.3.5.2.1. Liver resection

For patients without cirrhosis, liver resection is the treatment of choice for hepatocellular carcinoma. Indeed, major liver resections can be done without main issues regarding necroinflammation and regeneration in this case. Usually, this option is of interest for early tumour and is associated with good survival. Although the tumour size is not an excluding criteria, the risk of vascular invasion and dissemination increases with diameter (Fuks et al., 2012). In Europe and USA, level of bilirubin and portal pressure are the variables used to select appropriate candidates (European Association For The Study Of The Liver and European Organisation For Research And Treatment Of Cancer, 2012), while the Japanese use the indocyanine green retention rate (Kokudo et al., 2015). Patients without clinically significant portal hypertension (CSPH) and normal bilirubin reach 70% survival at 5 years, whereas survival decrease to <50% when both factors are present (Ishizawa et al., 2008). The main issues with liver resection are the invasiveness of the procedure and the increased risk of recurrence due to dissemination. Indeed, at 5 years, *de novo* tumours complicate 70% of the cases. A less invasive alternative option to liver resection is laparoscopic surgery, which exhibits similar long-term survival but is not useful regarding the recurrence issue (Han et al., 2015)(Figure 26). However, as HBV-related HCC occurs mostly on cirrhotic liver (>70%), the liver resection approach is uncommonly used and effectively manages a very small fraction of HBV-related HCC. Of course, liver transplantation is the best option but is limited by the availability of organs and only patients those exhibits high recurrence risk factors such as microvascular invasion or satellite nodules after liver resection are indicated for *ab initio* liver transplantation (Ferrer-Fàbrega et al., 2016).

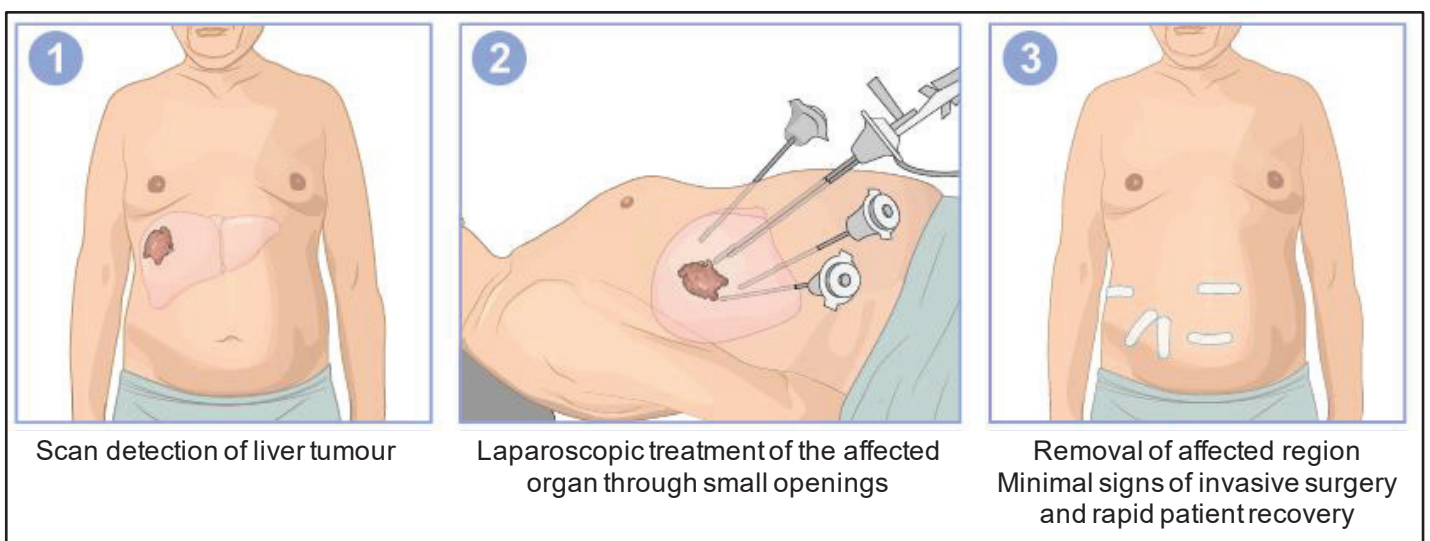


Figure 26: Laparoscopic resection for HCC located on superficial liver areas. This approach exhibits high survival for superficial early stage tumours. Extracted from Galle PR et al., J Hepatol., 2018.

2.3.5.2.2. Liver transplantation

The liver transplantation is the best therapeutic option available as it can theoretically cure both the tumour and underlying cirrhosis. The best post-liver transplantation survival, of 70% at 5 years with recurrence <15%, is found when the initial tumour is unresectable, <5cm or three nodules <3cm each, defined by the Milan criteria (Mazzaferro et al., 1996). The main limitation regarding liver transplantation is donor shortage. Even with dedicating all transplants to HBV-related HCC patients, it will be sufficient for barely 25% of them. This limitation imposes waiting list, a time under which tumours might progress and impede transplantation. Thus, it is crucial to refine liver transplantation indications according to donor availability in each allocation area, the proportion of enlisted patients without hepatocellular carcinoma versus those with hepatocellular carcinoma as well as the dynamics of the waiting list (Mazzaferro, 2016; Sapisochin and Bruix, 2017).

2.3.5.2.3. Percutaneous tumour ablation

For early-stage HCC, image-guided tumour ablation is a widely accepted and used treatment option. Ablation induces necrosis either by temperature shock (radiofrequency, microwave or cryoablation) or by chemical stress (mostly percutaneous ethanol injection) (Forner and Bruix, 2010). Among them, radiofrequency ablation is preferred and recommended as first-line technique as it allows better disease control and outcomes compared to others (Germani et al., 2010)(Figure 27). Regarding small tumours, especially for patients with Child-Pugh stage A liver disease, ablation exhibits non-inferior survival compared to surgical resection, challenging this latter, in terms of cost-efficiency and invasiveness (Hasegawa et al., 2013). However, focusing on HBV-related HCC, such therapies could result in HBV reactivation in 15-30% even in HBsAg negative patients, highlighting the need of expert-oriented choice for local ablation (Yoshida et al., 2008; Jang, 2014).

2.3.5.2.4. Transcatheter tumour therapy

Image-guided transcatheter tumour therapies take advantage of the highly-developed vascularization system of the tumour to deliver drugs, embolic particles, or radioactive devices (Forner et al., 2014). Among them, solely transarterial chemoembolization has shown survival benefit (Llovet and Bruix, 2003). Polyvinyl alcohol spheres enabled the slow release of chemotherapy, increasing the survival time up to 40 months while reducing adverse events (Burrel et al., 2012). This kind of approach is particularly recommended for patients with compensated liver, unresectable large tumour (Malagari et al., 2012). Finally, transarterial chemoembolization might be full of interest for patients on the waiting list for liver transplantation as it exhibits a nice delay in tumour progression.

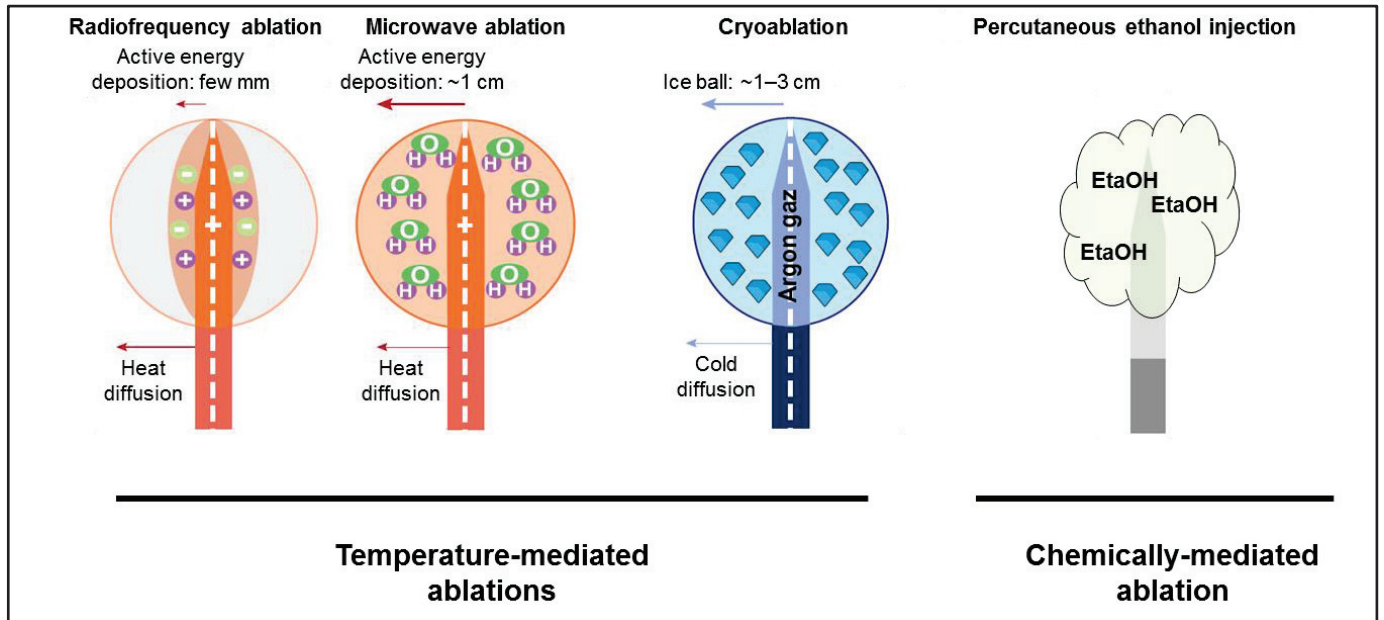


Figure 27: Presentation of commonly used percutaneous ablation techniques for HCC. Adapted from Galle PR *et al.*, J Hepatol., 2018.

2.3.5.2.5. Systemic therapies

Until 2008, no efficient systemic therapy for hepatocellular management was available. This is in part explained by the fact that somatic mutations found in HCC remain undruggable (TERT, CTNNB1, TP53 and ARID1A). The understanding of molecular mechanisms driving liver carcinogenesis has led to the development of targeted therapies. A large number of molecules have been tested or are currently under development (Nault, 2017), but to date, only Sorafenib and Regorafenib have been shown to improve survival versus placebo (Llovet *et al.*, 2008; Bruix *et al.*, 2017). Both are orally available multikinase inhibitors targeting RAF, KIT, RET, VEGFR1 and PDGFR, exhibiting antiproliferative and antiangiogenic effects, albeit underlying mechanisms are not well known yet. Sorafenib was the first evaluated one, in two positive randomised placebo-controlled trials: one multicentre trial done predominantly in Europe and the USA, and another trial done in the Asia-Pacific area (Llovet *et al.*, 2008; Cheng *et al.*, 2009). Several promising agents with suggestive findings from early-stage studies failed to demonstrate survival benefit over placebo as first or second-line treatment for HCC (Table IV). Regorafenib was the only drug that demonstrated survival

benefit as a second-line treatment and was evaluated in the RESORCE phase 3 trial for patients with hepatocellular carcinoma but were tolerant to Sorafenib (Bruix et al., 2017).

Recently, Lenvatinib was described to be non-inferior to Sorafenib in first-line treatment but it targets the same population, thus limiting its interest (Spallanzani et al., 2018).

Finally, promising results have been announced for Cabozantinib as a second-line treatment. This is another tyrosine kinases inhibitor acting on VEGF receptors 1, 2, and 3, MET, and AXL, which are implicated in the progression of HCC and the development of Sorafenib resistance. The first data from a clinical trial phase 3 were published in July 2018 in the *New England Journal of Medicine*. The aim was to evaluate Cabozantinib as compared with placebo in previously treated patients with advanced HCC. 707 patients were involved in this trial and among the treated arm, the median overall survival was 10.2 months with Cabozantinib versus 8.0 months with placebo. As mentioned, eligible patients have previously received at least Sorafenib as first-line therapy and were still under disease progression, rendering Cabozantinib a promising drug for advanced, Sorafenib-resistant HCC (Abou-Alfa et al., 2018; NCT01908426).

	Study (year)	Randomisation	Time to progression		Survival	
			Months	p value	Months	p value
First line						
Sorafenib*	Llovet et al (2008) ⁷³	Sorafenib (n=299) vs placebo (n=303)	5.5 vs 2.8	<0.001	10.7 vs 7.9	<0.001
Sorafenib*	Cheng et al (2009) ⁷⁴	Sorafenib (n=150) vs placebo (n=76)	2.8 vs 1.4	<0.001	6.5 vs 4.2	0.001
Sunitinib	Cheng et al (2013) ¹²⁹	Sunitinib (n=530) vs sorafenib (n=544)†	3.6 vs 3.6	NS	7.9 vs 10.2	NS
Brivanib	Johnson et al (2013) ¹³⁰	Brivanib (n=577) vs sorafenib (n=578)	4.2 vs 4.1	NS	9.5 vs 9.9	NS
Sorafenib plus erlotinib	Zhu et al (2015) ¹³¹	Sorafenib plus erlotinib (n=362) vs sorafenib (n=358)	3.2 vs 4.0	NS	9.5 vs 8.5	NS
Linifanib	Cainap et al (2015) ¹³²	Linifanib (n=514) vs sorafenib (n=521)	5.4 vs 4.0	0.001	9.1 vs 9.8	NS
Sorafenib plus doxorubicin	Abou-Alfa et al (2016) ⁸³	Sorafenib plus (n=173) doxorubicin vs sorafenib (n=173)	NA	NA	9.3 vs 10.5	NS
Lenvatinib*	Kudo et al (2017) ⁷⁵	Lenvatinib (n=478) vs sorafenib (n=476)†‡	8.9 vs 3.7	<0.001	13.6 vs 12.3	<0.001
Second line						
Regorafenib*	Bruix et al (2016) ⁷⁶	Regorafenib (n=379) vs placebo (n=194)	3.9 vs 1.5	<0.0001	10.6 vs 7.8	<0.0001
Brivanib	Llovet et al (2013) ¹³⁶	Brivanib (n=263) vs placebo (n=132)	4.2 vs 2.7	0.001	9.4 vs 8.2	NS
Everolimus	Zhu et al (2014) ¹³⁷	Everolimus (n=362) vs placebo (n=184)	2.9 vs 2.6	NS	7.6 vs 7.3	NS
Ramucirumab	Zhu et al (2015) ¹³⁸	Ramucirumab (n=283) vs placebo (n=282)	3.5 vs 2.6	<0.0001	9.2 vs 7.6	NS
Tivantinib	Rimassa et al (2017) ¹³⁹	Tivantinib (n=226) vs placebo (n=114)	NA	NA	8.4 vs 9.1	0.81

NS=non-significant. NA=not available. *Agents with survival benefit. †Open-label trial. ‡Non-inferiority design.

Table IV: List of targeted therapies evaluated in phase 3 trials in HCC. Cabozantinib is not presented in this table as the first result came from a clinical trial published in July 2018. Extracted from Forner A et al., The Lancet, 2018.

2.3.5.3. Future of HBV-related HCC treatments

Although Sorafenib and Regorafenib are available for HCC treatment, outcomes remain poor as they allow a survival gain of few months only. Moreover, the management of HCC is mostly the same regarding etiology, thus it would be full of interest to identify key mechanisms of HBV-related carcinogenesis in order to develop efficient and well-tolerated therapies.

Chekmate for hepatocellular carcinoma may come from immune therapies. Past years, a better comprehension of the mechanisms underlying the ability of tumor to escape immune surveillance has led to impressive results with immunotherapy in several cancers, and this strategy is currently being developed for HCC (da Motta Girardi et al., 2018). Nivolumab, an anti-PD1 monoclonal antibody has received an accelerated approval from the FDA for the treatment of HCC. The approval is based on a phase 1/2 clinical trial in which patients showed a better overall rate response, over Sorafenib. Moreover, Nivolumab showed no lower response in patients with chronic viral hepatitis (El-Khoueiry et al., 2017; Ray, 2017).

The second promising axis reside in the development of chimeric antigen receptor-engineered T-cell (CAR-T) and T-cell receptor (TCR-T) immune cell therapies. LioCyx, the first engineered TCR-T-cell therapy for treatment of liver cancer has received the approval for a phase 1/2 clinical trial. These strategies are at the dawn but results are encouraging and should offer a new approach for the treatment of HBV-related liver cancers (Koh et al., 2016)(Figure 28).

Finally, investigating multi-kinases inhibitors acting as Sorafenib with a spectrum extended to other molecular pathways is still a trend. The polo-like kinase 1, a cell-cycle regulating kinase which is also involved in DNA damage responses appears to be a first-choice target for HBV-related HCC. On the one hand, PLK1 was extensively studied and reported as a key driver of liver carcinogenesis and associated with poor prognosis (He et al., 2009; Mok et al., 2012). Inhibition of PLK1 was described to control and limit the progression of established HCC by inhibiting cell proliferation and promoting apoptosis (Deng et al., 2011; Wang et al., 2017a). Recently PLK1 siRNA encapsulated in proprietary lipo nanoparticles (LNPs) were clinically trialed in patients with advanced HCC (NCT02191878); collected data showed an overall partial response according to Choi's criteria in 22% of subjects and tumour density reduction of up to 59%. On the other hand, our studies have highlighted PLK1 as a potent proviral factor for HBV and a specific inhibition of this kinase results in antiviral activity (confer First and Second studies). Thus, the use of specific PLK1 inhibitors or multi-kinases inhibitor such as rigosertib could be of merit for HBV-related HCC treatment (Dietrich et al., 2017).

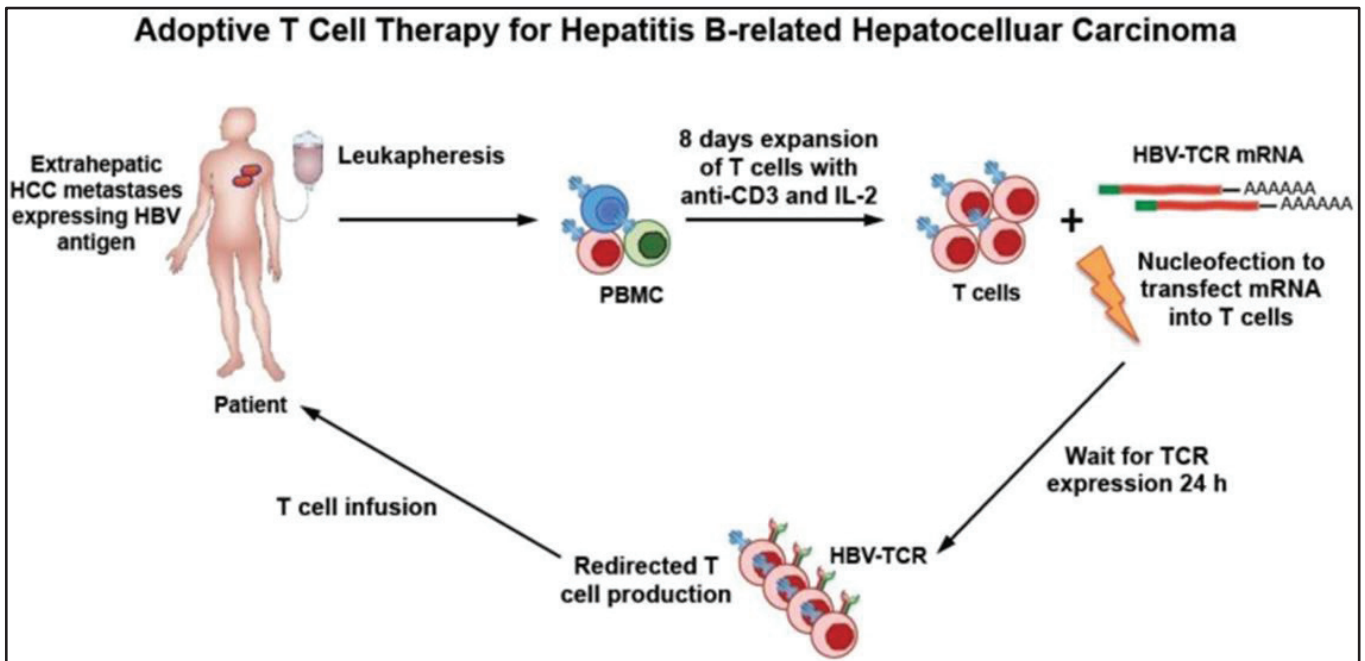


Figure 28: Schematic illustration of production of HBV-TCR redirected T cells. The primary T cells are electroporated with mRNA encoding HBV-specific TCR to transiently redirect their specificity towards HBV. The redirected T cells can be re-infused into the patient, whereby they will recognize and lyse HCC cells expressing HBV antigen within 3–4 days. Extracted from Koh S *et al.*, Diseases, 2016.

Chapter 3: Polo-like kinase 1, a new target for anti-HBV and anti-HCC therapies?

3.1. Polo-like kinases: an extremely well-conserved family from Yeast to Human

In 1988, Sunkel and Glover reported in the *Journal of cell science* that *Drosophila* neuroblast cells with mutant alleles of the locus *polo* showed abnormal mitotic and meiotic divisions. Chromosomes were randomly oriented, with a circular arrangement, leading to the production of polyploid cells. Moreover, a specific mutant named *polo*² was found to be lethal, highlighting the essential roles of the polo kinase in larvae cell division and development (Sunkel and Glover, 1988). Latter, homologous genes were found in *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, encoding CDC5 and Plo1 proteins respectively (Kitada et al., 1993; Ohkura et al., 1995). Subsequent research showed that PLKs were present in all branches of the Eukarya, and that humans had five paralogues; PLK1-5 (de Cárcer et al., 2011)(Figure 29). All these related proteins share a specific motif called Polo-box (PB), which can be organized to dictate PLKs activity, localization and functions. Although the original mutants were characterized by the presence of abnormal spindles formation, later studies indicated that PLKs are involved at multiple stages during mitosis (Archambault and Glover, 2009).

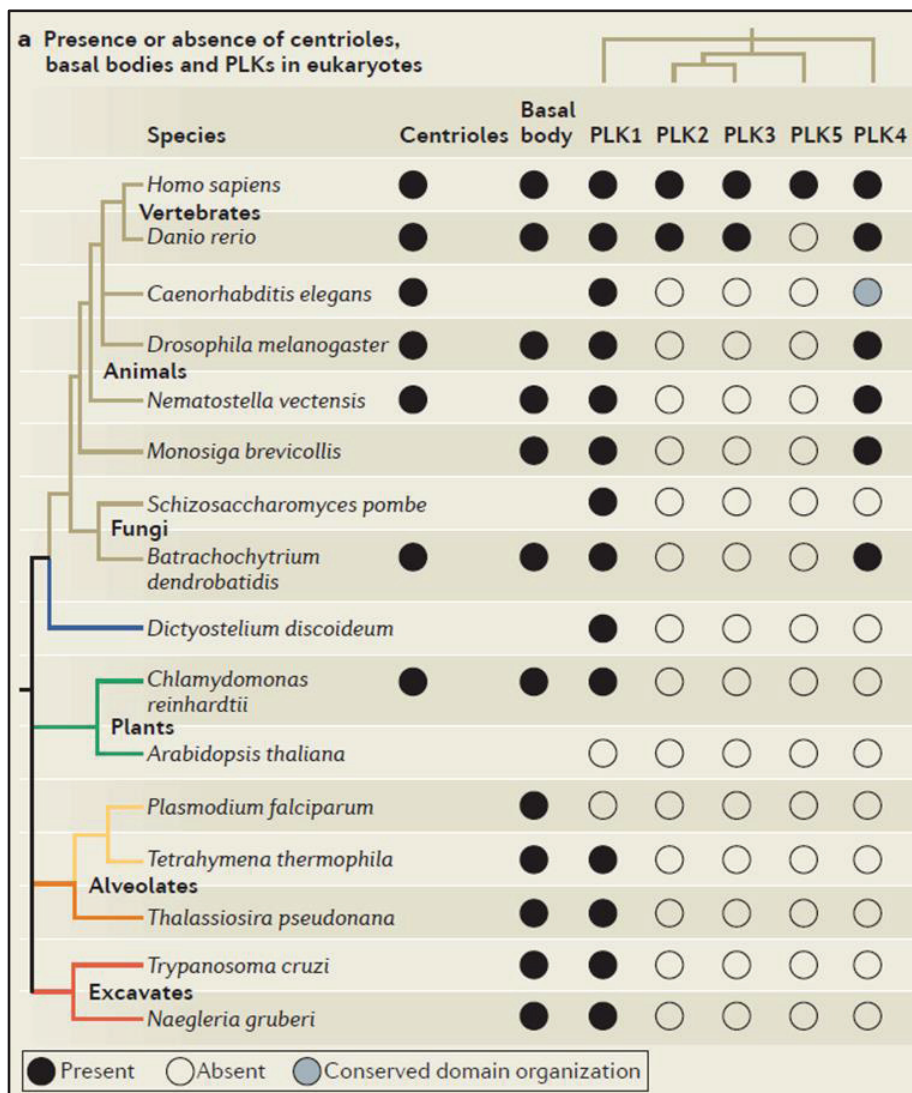


Figure 29: Phylogenetic distribution of PLKs. PLKs are present in all branches of Eukarya with PLK1 being the founder member. PLK4 is the most divergent of the family and results probably from an early *PLK1* duplication event. The *PLK2* subfamily contains *PLK2*, *PLK3* and *PLK5* and arises from *PLK1* duplication. However, *PLK5* is an inactive kinase that lacks important residues for substrate recognition in the PBD. Extracted from S. Zitouni et al., Nature Reviews, 2014.

3.2. Structural organization and features of human PLKs

PLKs members derived from the ancestral PLK1 and shared similar architecture. The N-ter contains a Ser/Thr catalytic kinase domain while in the C-ter region the Polo box domain (PBD) is located, this latter being composed by two or more PBs. PLK1-3 share a strong homology while PLK4 is divergent in the primary sequence and PLK5 is considered as a pseudo kinase as it lacks of residues for substrate recognition in the PBD (Carvalho-Santos et al., 2010)(Figure 30).

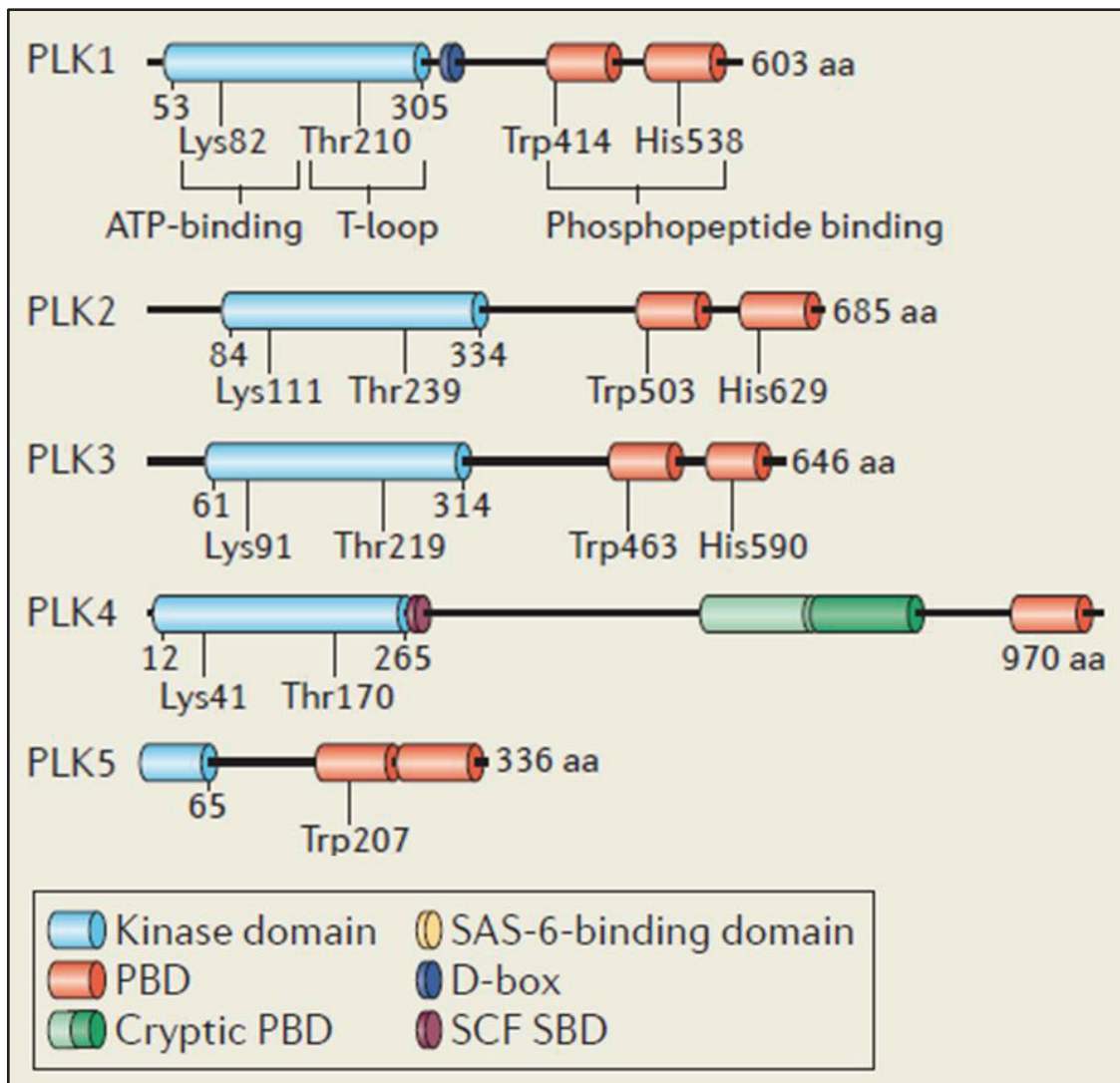


Figure 30: Structural domains of human PLKs. The Ser/Thr kinase activity is held in the kinase domain. PLK5 is described as a pseudokinase as it lacks important residues to fulfil the enzymatic activity. The D-box of PLK1 is important for its degradation through the APC/c complex in anaphase. Degradation of PLK4 is mediated by autophosphorylation of the SCF SBD (S phase kinase-associated protein 1 (SKP1)–cullin–F-box Slimb-binding domain). The SAS-6 binding domain is not found in human PLKs but is important for recruitment of SAS-6 and subsequent appropriate centriole assembly in the *C.elegans* PLK1 homolog's ZYG-1. PDB means Polo box domain and is involved in interaction with phosphopeptides. Extracted from S. Zitouni *et al.*, Nature Reviews, 2014.

3.3. Focus on the human PLK1

3.3.1. General structure of PLK1

PLK1 is a 603 aa, 66kDa Ser/Thr kinase. It holds a kinase domain in C-ter, aa 53-305, followed by a linker where is located a D-box domain, of importance for APC/c mediated degradation of PLK1. This interdomain linker (IDL) is involved in the inactivation of PLK1 as it maintains inaccessible the activation loop located in C-ter due to steric hindrance. The IDL is followed by a PBD containing two distinct polo boxes, which serve as phospho-binding motifs (**Figure 31**). The region between the two PBs recognizes peptides containing phosphorylated Ser and Thr residues with the consensus motif Ser-[pSer/pThr]-[Pro/X] where X stand for any residue. As for the others PLKs members, the PBD of PLK1 located at the C-ter regulates the catalytic activity of PLK1, its localization and substrate binding (Cheng et al., 2003; Elia et al., 2003).

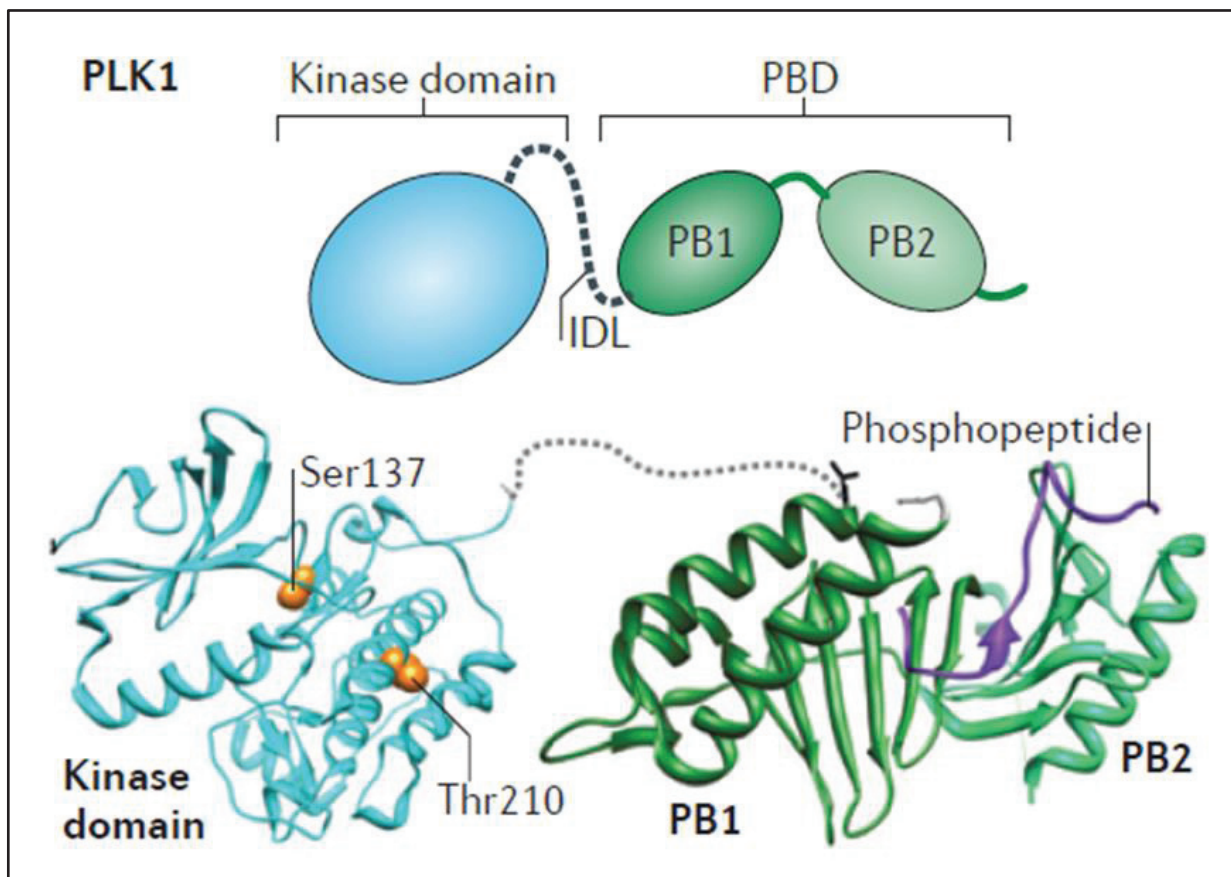


Figure 31: Structural organization of human PLK1. Model representation (**Top**) and quaternary structure (**bottom**) based on PDB accession identifications 2OU7 for the kinase domain (**blue**) and 1UMW for the Polo-box domain (**green**). Thr210 and Ser137 are important for PLK1 activation (**orange**). A groove between PB1 and PB2 allows the recognition of peptides (**purple**) containing phosphorylated Ser and Thr residues. IDL = inter-domain linker. Adapted from S. Zitouni *et al.*, Nature Reviews, 2014.

3.3.2. PLK1 activation: a multi-step and dynamic process

The activation of PLK1 is mediated through two phosphorylations within the C-ter kinase domain; the first is located at the Thr210 in the T-loop (also known as the activation loop) and the second at the Ser137 located near the end of the hinge. Thus, antibodies recognizing either pPLK1_{T210} or pPLK1_{S137} are useful to evidence PLK1 activation level.

Starting from the inactive conformation of PLK1, the kinase domain is inhibited by three different ways:

- The interdomain linker sequesters the T-loop where is located the Thr210 of the kinase domain
- The PBD reduces the flexibility of the hinge where is located the Ser137
- Binding of the PBD to Map205 protein, thereby stabilizing the autoinhibited state

These inhibitions can be release partially and/or totally, allowing a very fine control of PLK1 spatiotemporal activity (Zitouni et al., 2014). The phosphorylation of the Ser137 is known to disrupt the intramolecular interaction between the PBD and the kinase domain. The interaction with the protein Bora is the most described regarding the freeing of the T-loop, allowing activating kinases such as Aurora A to phosphorylate the Thr210 of PLK1 (Xu et al., 2013). Furthermore, phosphopeptides interacting with the hinge between the two PBs is another mechanism showed to disrupt the inhibitory interaction between the PBD and the kinase domain. Finally, phosphorylation of Map205 by CDK1 results in destabilization of the inhibited stage of PLK1 (Figure 32). However, the regulation of PLK1 activation model is based on studies conducted mainly in *Drosophila* and although being partially confirmed in human cells, structural studies of full-length PLK1 are needed to further clarify its regulation and mechanism of action.

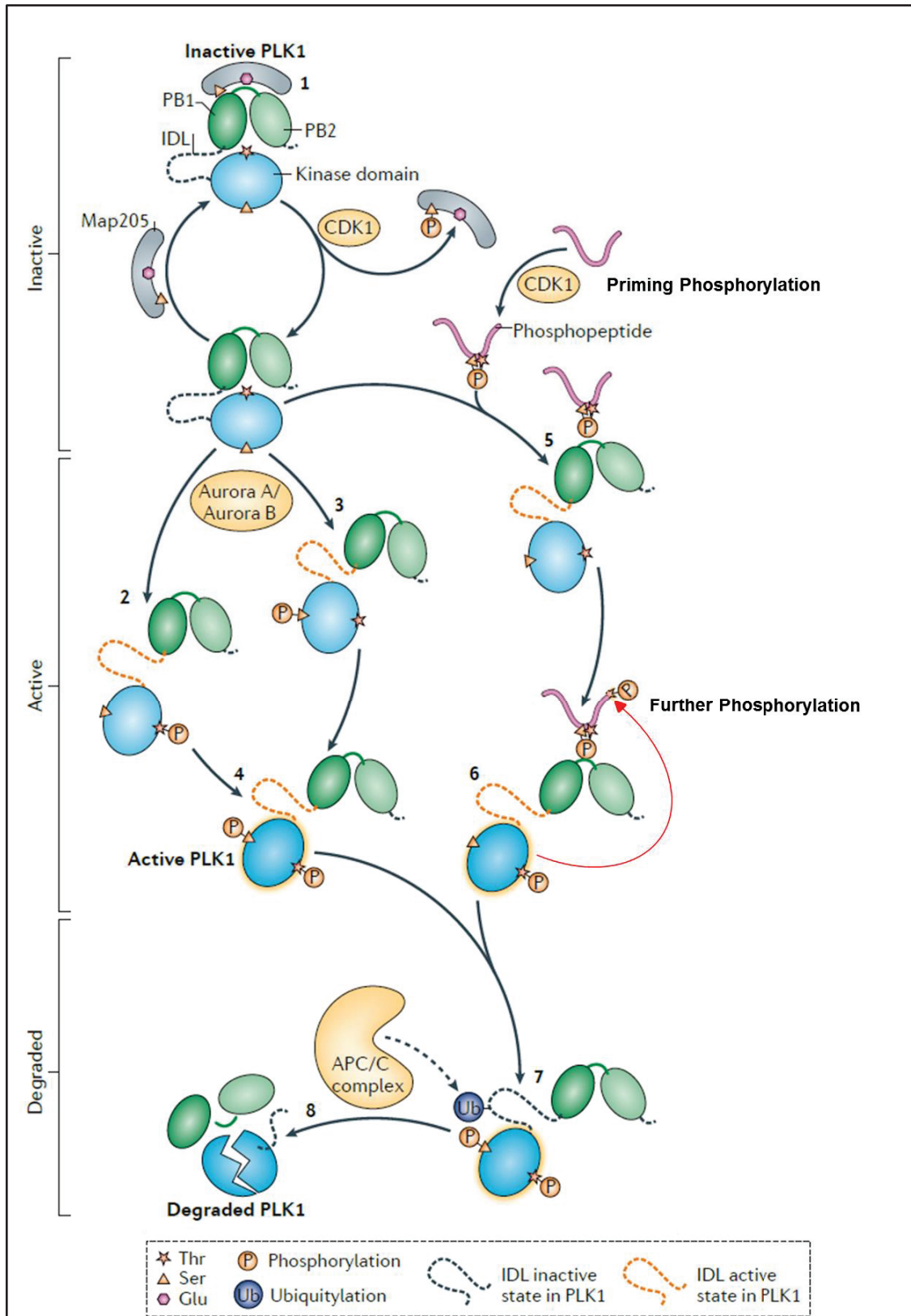


Figure 32: Schematic representation of multileveled regulation of PLK1. 1) PLK1 is autoinhibited by the PBD and the IDL. Map205 stabilizes this resting state. CDK1 can phosphorylate Map205 at Ser283 to release this inhibition. 2, 3, 4) PLK1 full activation is dependent of activating kinases such as Aurora A. 5) Binding of phosphopeptide to the hinge between the two PBs results in the activation of PLK1. 6) Activated PLK1 could further phosphorylate phosphopeptides. 7) APC/c complex recognizes the D-box within PLK1 IDL, mediating its degradation through ubiquitylation (8).

All the steps described here occur possibly at different localizations and cell-cycle stages, explaining the multimodal way of activating PLK1. Adapted from S. Zitouni *et al.*, Nature Reviews, 2014.

3.3.3. Spatiotemporal regulation of PLK1 activity

Among PLKs members, PLK1 has the most dynamic localization, mostly due to its involvement in cell-cycle progression, which requires a timely-fashion regulation of its activity. In quiescent cells, PLK1 was not extensively studied albeit this kinase is found to be mainly cytosolic during the interphase with localization near the microtubules and centrosomes. During mitosis, PLK1 is present at kinetochores, central spindle, centrosomes, microtubules and midbody, depending the stage of cellular division (Petronczki et al., 2008).

This spatial organization of PLK1 is thought to be dependent on phosphopeptides, harbouring the consensus motif Ser-[pSer/pThr]-[Pro/X], binding to the PBD. Moreover, PLK1 can also bind to nonphosphorylated peptides and phosphopeptides previously phosphorylated by PLK1 itself, which is known as self-priming. All these mechanisms highlight the complexity of PLK1 regulation and indicate the presence of a local feedback loop. As example, its localization to central spindle is mediated through PLK1-phosphorylated PRC1 while the localization to the kinetochores is dependent on CDK1-phosphorylated INCENP (Goto et al., 2006; Hu et al., 2012). As explained before, the protein Map205 binds PLK1 in a phosphor-independent manner and allows relocalization of inactive PLK1 to microtubules, suggesting kinase-independent activity of PLK1.

Finally, other post-translational modifications seem to play a role in PLK1 localization. Among them, the non-proteolytic ubiquitylation of PLK1 through the CUL3/KLHL22 complex is likely to promote the release of PLK1 from kinetochores. Indeed, in the absence of KLHL22, PLK1 accumulates to kinetochores, leading to the activation of the spindle assembly checkpoint. The CUL3/KLHL22 complex, which ubiquitylates Lys492, located within the PBD, results in PLK1 dissociation from kinetochores, which is mandatory for mitosis (Beck et al., 2013)(Figure 33).

3.3.4. Regulation of PLK1 level

PLK1 being mainly involved in the regulation of key steps of the cell-cycle, its expression is tightly regulated a both mRNA and protein levels. However, the post-transcriptional modifications of PLK1 seem to be crucial as they allow a fast and transient activation/inhibition of PLK1 kinase activity. Regarding PLK1 protein level, it is maintained low in interphase and peaks during mitosis (Winkles and Alberts, 2005). In G1, the *PLK1* gene is under the control of transcriptional repressors such as the CDE-CHR (cell-cycle-dependent element - cell-cycle gene homology region) sequences within the *PLK1* promoter, which are involved in *PLK1* repression in coordination with p53/p21. During the G2 phase, the DREAM complex increases PLK1 transcription through the action of FoxM1 protein (Reichert et al., 2010).

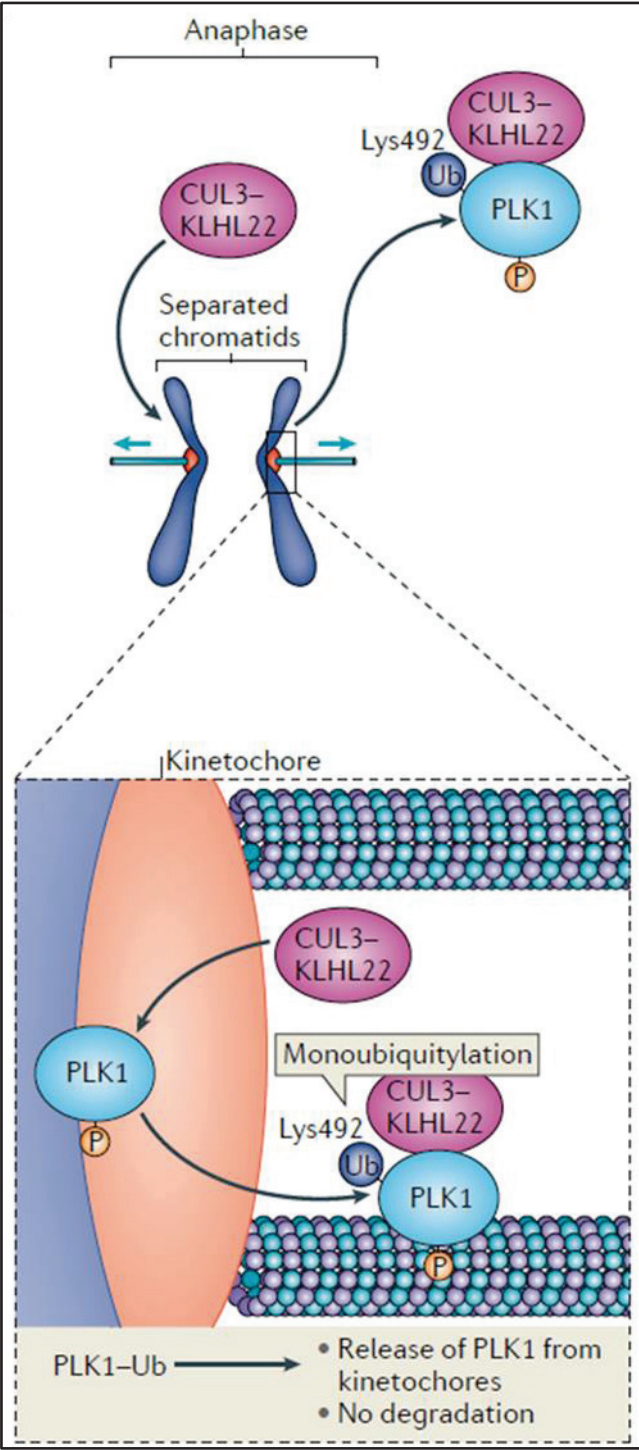


Figure 33: Model of ubiquitylation-dependent release of PLK1. During anaphase, PLK1 is located to the kinetochores. Ubiquitylation of its Lys492 by the CUL3-KLHL22 complex leads to PLK1 release from kinetochores and relocalization to microtubules, resulting in normal sister chromatids separation. Adapted from S. Zitouni *et al.*, Nature Reviews, 2014.

3.3.5. PLK1 is involved in nearly every steps of the cell-cycle

"For an overview of PLK1 involvement in the cell-cycle, please refer to Figure 34."

3.3.5.1. G2/M transition and the mitotic entry

PLK1 is an early trigger for G2/M transition. In the absence of PLK1 kinase functions, several proteins fail to be recruited in early mitosis, as shown with *polo* mutant in *D.melanogaster* (Sunkel and Glover, 1988). The G2/M transition is allowed by the removal of inhibitory phosphates on CDK1, resulting in the activation of the M phase-promoting factor (MPF; also known as CDK1-CyclinB) complex. This state is regulated by the activity of two kinases, MYT1 (Myelin transcription factor 1) and WEE1 and one phosphatase, CDC25 (Cell-division cycle protein 25). Both WEE1 and CDC25 are phosphorylated on several sites, which could be attributed to CDK1, highlighting positive and negative-feedback loops. Data indicates that PLK1 can phosphorylate and thereby regulate both, CDC25 and MYT1.

MPF activity results in cyclin B translocation to the nucleus, which is followed by further activation of MPF and nuclear envelope breakdown. However, PLK1 is not mandatory for CDK1 activation, which seems to occur with no, or low level of PLK1 (Toyoshima-Morimoto et al., 2002). Furthermore, inhibition of PLK1 in several organisms generally causes aberrations and/or delays during mitosis rather than a G2 arrest, indicating a regulatory action of PLK1 during this step (van Vugt and Medema, 2005). On the other hand, PLK1 acts as a trigger kinase and controls CDC25 after a release from G2 arrest. This indicates that PLK1 has a key role for the entry in mitosis in this case. In summary, PLK1 activates MPF by activating CDC25 and by inhibiting both MYT1 and WEE1 (Kumagai and Dunphy, 1996).

3.3.5.2. PLK1 and the centrosome machinery

The centrosome is the main microtubule-organizing centre (MTOC) and is found to be very conserved in term of structures in Eukarya (Figure 29). It is composed of two centrioles, which are surrounded by the pericentriolar material. The centrosomes follow a cycle in parallel of the cell-cycle, with duplication occurring in G1/S while assembly occurs during the S phase. They undergo maturation in late G2, and then separate during mitosis. In human, PLK1 was shown to be involved in the centrosomal localization of γ -tubulin during centrosome maturation (Teixidó-Travesa et al., 2012). The aim of this process is to increase the microtubule nucleation activity, which is required for spindle formation. A recently identified substrate of PLK1, Nlp (Ninein-like protein), interacts with γ -tubulin ring complexes (Casenghi et al., 2003). At the onset of mitosis, the Nlp phosphorylation by

PLK1 results in its dissociation from centrosome, allowing the recruitment of other proteins. This leads to increase the γ -tubulin level, required for mitosis.

The mitotic entry is a step being regulated by the association of PLK1 with the centrosome. As discussed earlier, the complex CDK1-CyclinB, is a key regulator of M phase in eukaryotes and evidence showed that the activation of the MPF initially occurs at the centrosome (Jackman et al., 2003). In accordance with the cell-cycle clock, PLK1 coordinates centrosome cycle so that the right number of functional structures is assembled at the right time.

3.3.5.3. PLK1 is involved in kinetochore microtubules attachment and chromosomes segregation

During mitosis, PLK1 localizes to both spindles and kinetochores (van Vugt and Medema, 2005). These centromere associations support two important related processes. First, they are the anchorage site for spindles and secondly, centromeres play a crucial role for the spindle checkpoint, including a control ensuring that all chromosomes have undergone bipolar attachment, before sister chromatids separation (van Vugt and Medema, 2005). Aurora B is a kinase with well-established functions in destabilizing attachments and its substrates are highly phosphorylated in the low-tension state (Liu et al., 2012). There is still a debate on how the attachment initially occurs, however, evidence has shown that PLK1 expression level is high during prometaphase to balance the destabilizing activity of Aurora B and that PLK1 is required for the initial stable attachment of kinetochore microtubules. Indeed, in metaphase, PLK1 phosphorylates BubR1, which stabilizes microtubule-kinetochore attachment. The non-proteolytic ubiquitylation of PLK1 PBD by CUL3–KLHL22 at Lys492 is a crucial step in promoting the release of PLK1 from kinetochores, which is mandatory for the separation of sister chromatids (Beck et al., 2013).

3.3.5.4. PLK1 and sister-chromatids separation

The separation of sister-chromatids occurs through the APC/c-dependent ubiquitylation and is highly dependent on correct chromosome alignment and on attachment of spindle microtubules to the kinetochores. The ubiquitylation of Securin leads to its degradation and releases the Separase, a protease that cleaves Cohesins, responsible of the sister-chromatids cohesion. Moreover, the APC/c degrades CyclinB, resulting in the inactivation of M-CDK (mitotic cyclin-dependent kinase) complexes and promoting exit from mitosis (Eckerdt and Strebhardt, 2006). In this context, PLK1 is able to phosphorylate and activate components of the APC/c, thus, regulating sister-chromatids separation.

3.3.5.5. PLK1, centriole disengagement and cytokinesis; the end of a journey

Following the cell-cycle kinetic, the next step regards the centriole disengagement. It was thought that PLK1 alone was essential and necessary for this process (van Vugt and Medema, 2005). However, the centriole disengagement is delayed without the activity of APC/c-Cdh1 and PLK1 was shown to be not mandatory. These two ways to complete the process allow the cell to control the kinetic of centriole disengagement. This ensures that centrioles will not prematurely separate in late G2 or in early mitosis, thereby risking multipolar spindle assembly, but rather disengage in a timely fashion. PLK1 and CDK1 activities on the APC/c allow a smooth regulation of this process (Zitouni et al., 2014).

Cytokinesis is known as the final step of the mitosis, resulting on the formation of two daughter cells. It involves rearrangement of the cytoskeleton to form a midzone that keeps sister chromatids apart, leading *in fine* to abscission of daughter cells. In eukaryotes, this process requires ingression of the actomyosin-based contractile ring between segregated sister genomes. The activity and relocalization to spindle the spindle midzone of PLK1 during late anaphase allows recruitment of RhoGEF Ect2 to the central spindle, which promotes the activation of the RhoA GTPase, inducing assembly and ingression of the contractile ring (Ebrahimi et al., 2010).

This overview of PLK1 roles in cell-cycle highlights its importance and enables a better understanding on its potential involvement in carcinogenesis processes. Another key role of PLK1 is its involvement in DNA repair mechanisms, which is also a process relevant for carcinogenesis. This point is developed in the part below.

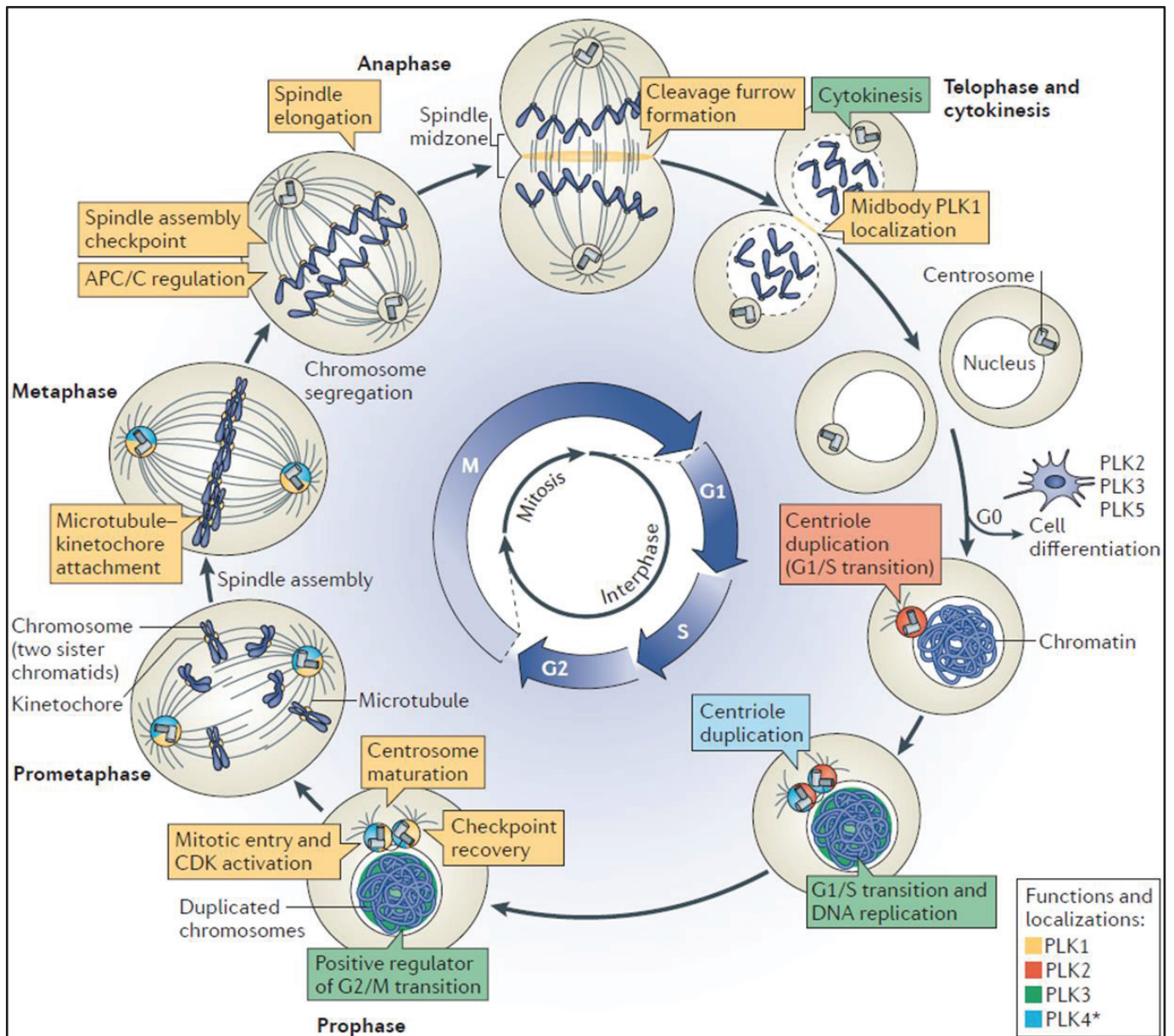


Figure 34: Schematic representation of PLK1 functions and localizations during the cell-cycle. As mentioned in the text, PLK1 is involved in nearly every step of the mitosis while other PLKs members are restricted to specific stages and localizations. Starting from G2, PLK1 is found near the centrosome. In prophase and metaphase, PLK1 localizes at kinetochores and at the spindle pole while in anaphase and telophase it relocates to central spindles. Extracted from S. Zitouni *et al.*, Nature Reviews, 2014.

3.3.6. PLK1 in DNA damage response

Cells are continually threatened with stresses conditions, either endogenous such as ER stress, replicative stresses, or exogenous factors; mainly UV exposure and genotoxic reagents. When DNA is damaged, a checkpoint mechanism is activated, allowing cells enough time to repair before going further into the cell-cycle. If damages are too severe, an apoptotic pathway takes place. At the onset of mitotic entry, a cooperation between Aurora B and Bora leads to the activation of PLK1 (van Vugt and Medema, 2005). When double-strand breaks (DSB) occur, especially in G2 phase, the ATM (Ataxia telangiectasia mutated) protein is recruited by the MRN (Mre11/Rad50/Nbs1) complex (Figure 35). ATM directly phosphorylates Bora, leading to its degradation by the E3 ubiquitin ligase SCF- β -TRCP (Skp, Cullin, F-box containing complex). In this case, a G2 arrest occurs due to the non-activation of PLK1. However, the mechanism described above could not explain the complete inhibition of PLK1. Until lately, it was unclear how PLK1 was totally shut down, allowing the G2 DNA damage checkpoint establishment. A recent paper uncovered the role of B55 α in PLK1 dephosphorylation by mediating PP2A/PLK1 (Protein phosphatase 2A) association (Wang et al., 2015). Moreover, it has been reported that PLK1 directly phosphorylates the Rad51 recombinase and facilitates the homologous recombination (HR) (Yata et al., 2012).

PLK1 also plays a role when DNA damages occur during mitosis. This is a very short period comparing to other stages of cell-cycle but critical in term of genomic distribution. DNA damages at this step often result in mitotic arrest. In mitosis, PLK1 is activated and the cell has to shut down its activity in order to set up a mitotic arrest. The down-regulation of PLK1 occurs through the intervention of PP2A. PP2A activity on PLK1 dephosphorylation is dependent on ATM-Chk1 (ATM-Checkpoint kinase 1) pathway in mitotic DNA damage response (Cholewa et al., 2013).

Finally, evidences showed relationship between PLK1 and p53, responding together to fix DNA damages. The p53 protein is known as a key tumor suppressor, a genome guardian, by inducing cycle arrest when cells are damaged due to stress conditions. DNA damages leads to p53 activation through its phosphorylation by ATM/ATR and Chk1/Chk2. The p53 protein has opposite functions to PLK1 regarding its involvement in the cell-cycle. After DNA damages, the aim is to stop cell-cycle progression, mainly by upregulating p53 and downregulating PLK1. The accumulation of phospho-p53 allows the activation of downstream effectors, such as p21, which inhibits CDKs and induces DNA damage regulating proteins. PLK1 is inhibited by an ATM/ATR-dependent fashion, as seen before and p53 can transcriptionally regulate PLK1 by binding with E2F1 at PLK1 promoter. This complex p53-E2F1 suppresses the expression of PLK1 (Zhou et al., 2013). Moreover, p53 can act indirectly to repress PLK1. Indeed, p21/waf1, during DNA damage response, targets a CDE/CHR (Cell-cycle-dependent element/Cell-cycle genes homology region) within PLK1 promoter, resulting in its downregulation. At last, p53 acts as a downregulator on FoxM1, which stimulates PLK1 expression (Pandit et al., 2009).

Here different mechanisms, occurring in DNA damage responses, have been presented. This allowed defining and understanding the pivotal role of PLK1. Cell has to act at genomic and proteomic levels to shut down drastically the activity of PLK1. Two main points are concerned by this inhibition. On one hand, this allows the cell-cycle arrest. On the other hand, avoiding the recovery of cell-cycle arrest during repairing damaged DNA is mandatory. In the part below, the involvement of PLK1 in carcinogenesis is discussed, in the light of actual knowledges and overviews done before.

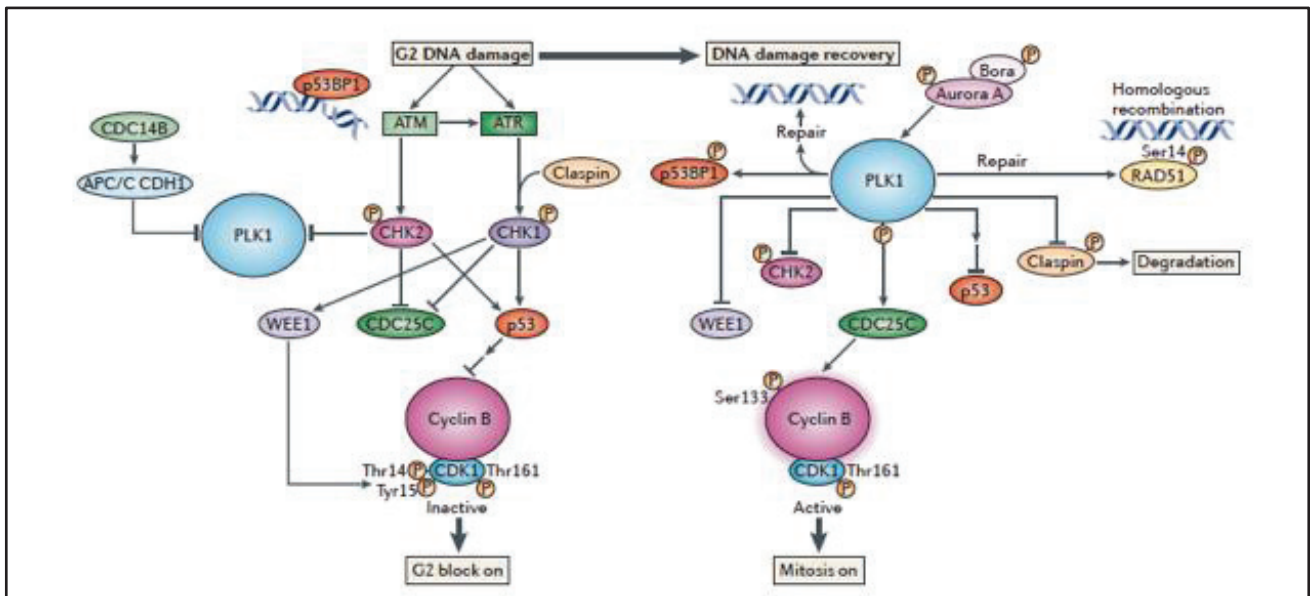


Figure 35: PLK1 and the DNA damage response. CDC14B activates the APC/c complex component CDH1, which promotes the degradation of PLK1 and cycle arrest. Consequently, WEE1 is activated and CDC25 is inactivated through a signalling cascade that involves the Ser protein kinase ATM and the Ser/Thr protein kinase ATR, and the dual function checkpoint kinases Ser/Thr protein kinase Chk1 and Chk2. ATM and ATR, which are closely related kinases that are activated by DNA damage, initiate the DNA damage checkpoint by phosphorylating several key proteins. Once activated, the checkpoint leads to cell-cycle arrest and DNA repair or apoptosis. ATM is activated by double-strand breaks and phosphorylates Chk2, whereas ATR is activated by single-strand breaks and phosphorylates Chk1. PLK1 indirectly inactivates p53 function to control the DNA damage response. During DNA repair, CDK1 is reactivated to enable cell-cycle progression. PLK1 initiates repair by phosphorylating DNA repair protein RAD51 homologue 1 (RAD51), which triggers the degradation of WEE1 and claspin, leads to the inactivation of Chk1 and Chk2 and promotes the phosphorylation of tumour suppressor p53 binding protein 1 (p53BP1), which triggers its dissociation from DNA. Dashed arrows indicate non-functional pathways. P, phosphate; Ub, ubiquitin. Extracted from S. Zitouni *et al.*, Nature Reviews, 2014.

3.3.7. Involvement of PLK1 in oncogenesis

PLK1 is considered as a proto-oncogene, overexpressed in up to 80% of malignancies, and has been associated with poor prognosis (**Table V**). As seen before, the expression of PLK1 is directly linked with the progression of the cell-cycle and its function goes even beyond this. In physiological conditions, PLK1 starts accumulating during S-phase, shows a peak at G2-M transition, stabilizes through mitosis and decreases upon mitotic exit. Of course, this pattern of expression is radically altered in cancer cells. As example, PLK1 can localize to nucleus before G2-M, and can even be detectable in the G1-S phase. These observations suggest that PLK1 must have cancer-cells specific functions. This is supported by the fact that PLK1 is involved in the G1-S transition and DNA replication in cancer cells, by phosphorylating Orc2, a major component of the pre-replicative complex. On the other hand, the overexpression of PLK1 leads cells to go through the cell-cycle, representing a high mitotic potential. Both, overexpression/deregulation of PLK1 activity and PLK1 cancer-cells specific functions are involved in oncogenesis phenomena.

Cancer Type	Fold Change
Cholangiocarcinoma	24.3
Uterine corpus endometrial carcinoma	21.3
Lung squamous cell carcinoma	20.8
Glioblastoma	12.4
Hepatocellular carcinoma	11.7
Breast invasive carcinoma	11.3
Esophageal carcinoma	10.2

Table V: Comparison of PLK1 gene expression between cancers and normal tissue. Mean PLK1 expression in cancers/mean PLK1 expression in normal tissue. Adapted from Liu *et al.*, Translational Oncology, 2016.

3.3.7.1. PLK1 interconnects with several cancer-associated pathways

3.3.7.1.1. PLK1 inhibits tumour suppressors

PLK1 has been found interacting with a wide range of tumour suppressors. Among them, proteins involved in DNA damage repair pathways and progression through the cell-cycle such as BRCA1/2 (Lee et al., 2004; Chabaliier-Taste et al., 2016), ATM (Lee et al., 2010), ATR (Deming et al., 2002), CYLD (Stegmeier et al., 2007), BubR1 (Izumi et al., 2009). The imbalance between these interactions, resulting in deregulation of important pathways may contribute to cancer development. In the two next subparts, the focus is set on two majors' tumour suppressors that have been shown to be downregulated by PLK1; p53 and PTEN.

3.3.7.1.1.1. PLK1, a major foe for the "guardian of the cell"

The tumour suppressor p53, known as "the guardian of the cell", is involved in antiproliferation by inducing apoptosis of cells harboring uncontrolled, abnormal growth (Fu and Wen, 2017). For instance, PLK1 has been shown to regulate p53 activity through four different pathways:

- PLK1 inhibits p53 apoptotic activity as well as p53-dependent transcriptional activation by direct phosphorylation.
- PLK1 phosphorylates MDM2 at Ser260, an E3 ubiquitin ligase for p53, and stimulates MDM2-mediated p53 turnover, thus inhibiting p53 activity.
- Topors is an ubiquitin and SUMO E3 ligase involved in p53 stability. PLK1 has been shown to activate Topors by phosphorylating it at Ser178, leading to an inhibition of Topors-mediated SUMOylation of p53, reducing its stability in association with an increased ubiquitin-mediated degradation of p53.
- PLK1 is also able to phosphorylate G2 and S-phase-expressed 1 protein (GTSE1), leading to its nuclear translocation where it can bind to p53, resulting in p53 shuttling out of the nucleus for degradation.

3.3.7.1.1.2. PLK1 and the tumour-suppressor PTEN

Phosphatase and tensin homologue (PTEN) governs a plethora of processes including survival, proliferation and energy metabolism, mostly by suppressing the PI3K-AKT-mTOR pathway through its lipid phosphatase activity. In addition, it has been shown that PTEN could act in a phosphatase-independent manner, thereby increasing its spectrum of activities. Among with p53,

PTEN is one of the best-characterized and commonly misregulated tumour suppressors in cancers (Song et al., 2012). In 2014, Choi and colleagues have shown that PLK1 was able to phosphorylate PTEN at the Ser380 *in vivo*, which was associated with the accumulation of PTEN on the chromatin, thus, increasing mitotic activity (Choi et al., 2014). Moreover, Li and colleagues showed that PLK1 enhanced aerobic glycolysis through the inactivation of PTEN and the activation of the PI3K pathway. Indeed, PLK1 phosphorylates PTEN and Nedd4-1, an E3 ubiquitin ligase of PTEN, resulting in PTEN inactivation and promoting tumorigenesis (Li et al., 2014).

3.3.7.1.2. PLK1 interplays with several oncogenes

On the one hand, as discussed before with p53 and PTEN, PLK1 is able to negatively regulate tumour suppressors. On the other hand, PLK1 also activates intensively oncogenes. Both mechanisms are known to be mandatory for cancer initiation. As for the section “**3.3.7.1.1. PLK1 inhibits tumour suppressors**”, PLK1 is found to be associated with several oncogenes but we decided to focus on PLK1 interaction with FoxM1 and the Myc family members, as they are the best documented and characterized regarding involvement in oncogenesis process.

3.3.7.1.2.1. PLK1 and FoxM1: a cell-cycle story

The Forkhead box protein M1 (FoxM1) transcription factor is required for normal cell proliferation and is found elevated in a wide range of cancers (Halasi and Gartel, 2013). Interestingly, FoxM1 plays a role far beyond the cell-cycle progression and is found involved in all major hallmarks of cancer depicted by Weinberg (Hanahan and Weinberg, 2011). The transcriptional activity of FoxM1 correlates with its phosphorylation level, which is at a basal level in G1 and fully phosphorylated at the G2/M transition. In this context, PLK1-dependent phosphorylation of FoxM1 is required to fully activate FoxM1 (Fu et al., 2008). In counterpart, activated FoxM1 is in capacity to increase PLK1 expression, thereby creating a positive loop, amplifying PLK1 levels and FoxM1 activity. Using mouse neural progenitor cells, the overexpression of FoxM1 accelerated neurosphere formation, while FoxM1 expression increased as cells progressed to tumorigenic progenitors and glioma-like cells. This finding was correlated with PLK1-dependent activation of FoxM1 (Joshi et al., 2013).

3.3.7.1.2.2. PLK1 and the Myc family

The MYC family contains three members that are described as oncogenes: c-Myc, L-Myc and N-Myc. These three proteins are mainly involved in the cell-cycle regulation albeit a wealth of data has shed new light on the functions of Myc and how their deregulations could contribute to cellular transformation (Adhikary and Eilers, 2005). Overexpression of c-Myc is sufficient for the induction of cell-cycle progression while a conditional knockout in mice implies that c-Myc function is mandatory for proliferation in most cell types (Trumpp et al., 2001). PLK1 is known to directly phosphorylate c-Myc, leading to its accumulation and *in fine* to oncogenic transformation, tumor-initiating cell activation and resistance to mTOR-targeted therapy (Tan et al., 2013).

N-Myc overexpression is associated with poor prognosis and resistance to therapy in human cancers. Moreover, pharmacological strategies targeting directly N-Myc are still elusive. In 2016, Xiao *et al.*, identified a reciprocal activation between PLK1 and N-Myc. PLK1 promotes ubiquitin-dependent degradation of Fbw7 by directly phosphorylating it, thus counteracting Fbw7-dependent degradation of N-Myc. In turn, through its transcription factor activity, N-Myc enhances PLK1 transcription, resulting in a positive feed-forward that strengthens N-Myc oncogenic programs (Xiao et al., 2016).

Recently, a study using integrated network analysis and machine learning approach unveiled 12 upregulated genes in triple-negative breast cancer, amongst which *PLK1* and *Myc* were found. Due to aggressiveness of this breast cancer subtype and the lack of approved targeted therapies, the identification of key hub genes and pathways may serve to the development of potential targeted drugs (Naorem et al., 2018).

3.3.8. Interplays between PLK1 and viruses

PLK1, as seen before, is a key protein for the cell-cycle and could be considered as a *bona fide* oncogene. Frequently, viral infections and oncogenesis affect common cellular mechanisms and pathways, aiming to enhance cell division and escapement from immune system to spread. Interestingly, studies showed deregulation of PLK1 expression and/or activity during viral infections. In this part, examples from hepatitis C virus (HCV), human papillomavirus (HPV), influenza A virus and parainfluenza virus 5 (PIV5) are detailed (Figure 36).

3.3.8.1. Interplays between PLK1 and HCV

HCV is a positive strand RNA virus. It encodes a unique polyprotein, further processed into four structural proteins (Core, E1, E2 and p7) and six non-structural proteins (NS2, NS3, NS4A, NS4B, NS5A, and NS5B)(Chen et al., 2010). NS5A exists in basal phosphorylated form (p56) and in

hyperphosphorylated form (p58) and these post-translational modifications are of importance for HCV replication (Ross-Thriepland and Harris, 2015). In cells transfected with PLK1-specific shRNA, HCV replication and NS5A hyperphosphorylation were significantly reduced, indicating that PLK1 kinase activity is required for this process. In adequation with these results, overexpression of PLK1 in the knockdown cells rescued HCV replication. Moreover, evidences indicated that PLK1 immunoprecipitates and colocalizes with NS5A. At last, it has been shown that PLK1 induces both phosphorylated and hyperphosphorylated forms of NS5A, highlighting further the importance of this kinase for HCV replication (Chen et al., 2010).

3.3.8.2. Interplays between PLK1 and HPV

HPV infection often causes no symptoms and resolve spontaneously, however the infection may persist causing warts and precancerous lesions. Up to 40 types of HPV are typically transmitted through sexual contact but HPV-16 and HPV-18 are involved in nearly all cervical cancers (Castle et al., 2018). The E2 protein of HPV is essential for viral life cycle. Through a yeast two-hybrid screening, Wang and colleagues have shown that PLK1 interacts and phosphorylates HPV-5 E2 (Wang et al., 2009). However, HPV-16 E2 does not interact with PLK1, suggesting a E2 variability between HPV subtypes. Moreover, both HPV-5 E2 and PLK1 are able to interact with Brd4, this latter being known to promote G1 and S phases' progression (Mochizuki et al., 2008). During HPV infection, Brd4 acts as a transcriptional cofactor of E2. HPV-5 E2 may form a stable complex with Brd4, preventing its phosphorylation by PLK1, leading *in fine* to deregulated cellular functions of Brd4.

3.3.8.3. Interplays between PLK1 and influenza A virus

Influenza is a respiratory disease caused by influenza A and B viruses. Every winter, season epidemics result in high economical losses due to sick days. Currently, vaccines and antiviral drugs are available, but both come with limitations. In recent years, several genome-wide RNAi screenings have unveiled cellular factors required for influenza virus infections in human, in order to establish some of them as drug targets. PLK1 was found to be one of these hits in four independent screens and in 2017, Pohl and colleagues reported that knockdown of PLK1 as well as kinase activity inhibition by four different compounds resulted in a loss of influenza A replication in infected human lung cells. This phenotype was reported to be specific of PLK1 activity with no measurable toxicity and thus established PLK1 as potential drug target (Pohl et al., 2017).

3.3.8.4. Interplays between PLK1 and PIV5

Paramyxoviruses are negative-sense, single-stranded RNA viruses. The replication is dependent on the viral phosphoprotein (P) and the large (L) protein, with phosphorylation of P being known to regulate viral gene expression. The PIV5 is a prototypical paramyxovirus and by contrast to others examples presented before, PLK1 was shown to downregulate viral replication by phosphorylating the P protein. However, increased viral gene expression correlates with higher cell death and cytokine production, thus, authors hypothesized that PIV5 limits its viral gene expression through PLK1-dependent phosphorylation of P, in order to avoid these host effects (Sun et al., 2009).

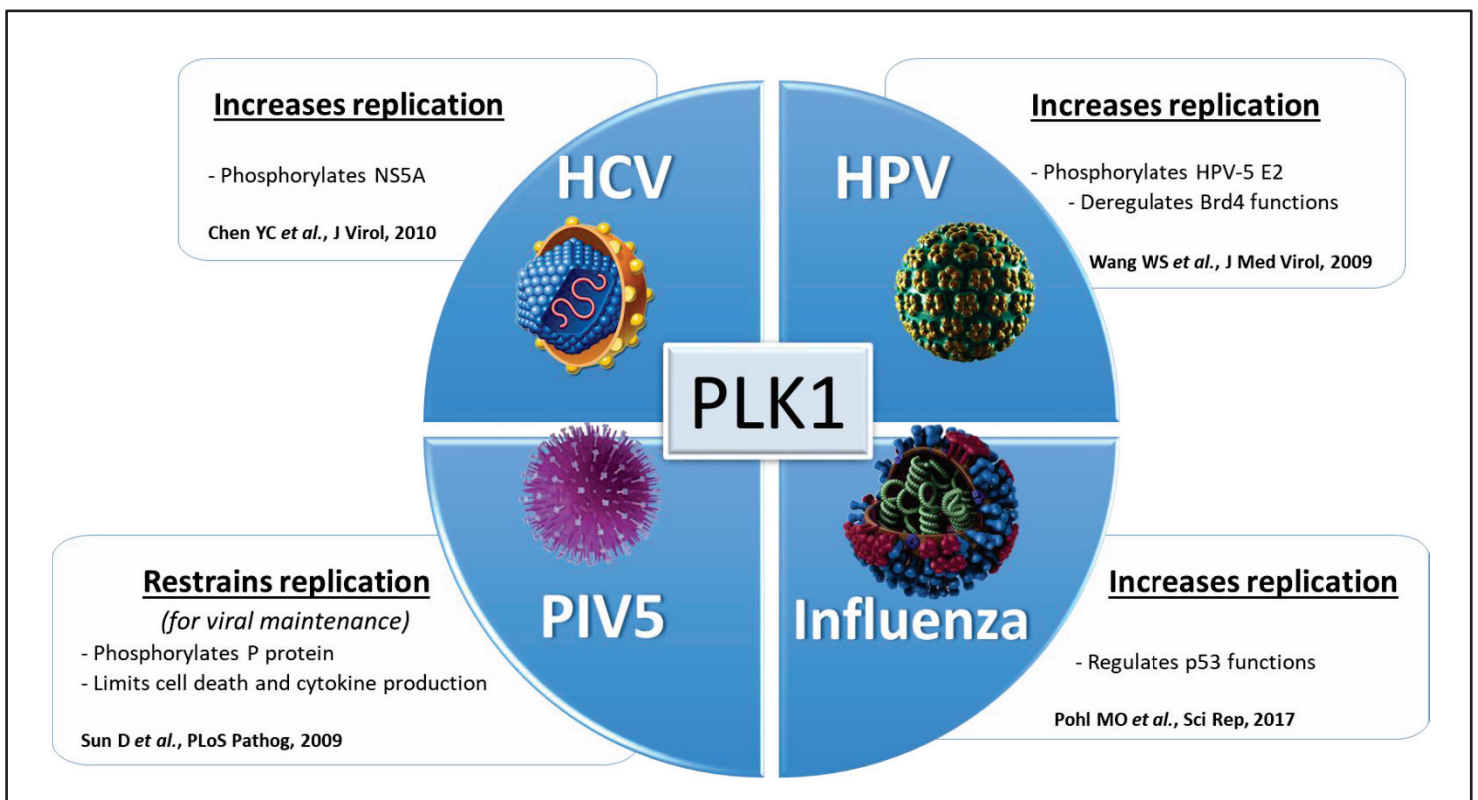


Figure 36: PLK1 interplays with several viruses, mostly increasing their replication.

3.3.9. PLK1 as a keystone in virus-induced oncogenesis? The example of HBV-related HCC

As discussed in the sections “**3.3.7. Involvement of PLK1 in oncogenesis**” and “**3.3.8. Interplays between PLK1 and viruses**”, PLK1 is largely involved in both carcinogenesis process and virus replication, mainly by endorsing proviral roles for this latter. HBV being responsible for an estimated >50% of HCC globally, the community went naturally to study the involvement of PLK1 in this virus-induced oncogenic transformation. In the late 2000s, there was still debate on the exact implication of PLK1 regarding cellular transformation. On the one hand, PLK1 could be a key driver of carcinogenesis, mostly by promoting cell proliferation and genetic instabilities. On the other hand, the overexpression of PLK1 could be just a side effect of other key driver events and reflect solely the high proliferative rate observed in tumours (Cholewa et al., 2013). However, recently, several studies have pointed out PLK1 to be a key driver of hepatocarcinogenesis. In a study from Wang and colleagues aiming at deciphering the key signaling pathways, genes and transcription factors associated with hepatocellular carcinoma, PLK1 was defined as a key gene involved in HCC development (Wang et al., 2018). Furthermore, in 2017, a paper published in *Cancer Cell* reported that PLK1 is a key driver of HCC subtype C1, this latter being mainly associated to HBV (Chaisaingmongkol et al., 2017).

3.3.9.1. PLK1 and the mechanisms behind HBV-induced cellular transformation

In an *in vitro* model of murine hepatocyte transformation featuring the overexpression of the HBx protein and selection of polyploidy events, it was shown that PLK1 was the main driver of the process (Studach et al., 2010). Some of the PLK1-mediated oncogenic mechanisms were elucidated. The group of Dr Andrisani has shown that PLK1 induces the degradation of SUZ12 and ZNF198, two components of the polycomb repressive complex (PRC2) and LSD1-CoREST-HDAC1 complex, which are involved in gene repression. This is associated with the re-expression of stemness genes (e.g., BAMBI, EpCAM, Myc...), which are also found overexpressed in HBV-related HCC (Wang et al., 2011). Moreover, a link with the long noncoding RNA HOTAIR, which is found overexpressed in many cancers, was established. HOTAIR holds together the two repressive complexes PRC2 and LSD1-CoREST-HDAC1 and it was shown that PLK1-dependent ubiquitination of SUZ12 and ZNF198 was enhanced by expression of HOTAIR. Clinically, the overexpression of HOTAIR and PLK1 was correlated with aggressiveness of the tumours that are more prone to develop satellite nodules (Zhang et al., 2015).

In addition to the mechanisms described above which linked HBV-induced carcinogenesis and PLK1, mostly by modifying the epigenetic landscape, the L-HBsAg was also unveiled as potent

HCC-inducer and related to PLK1. In *The Journal of Pathology*, Li and colleagues reported that expression of L-HBsAg was a priming event in HCC development by inducing cytokinesis failure and consequent aneuploidy through induction of DNA damage and overexpression of PLK1 in hepatocytes. Interestingly this PLK1 overexpression was associated with G2/M checkpoint overriding, promoting cell-cycle progression and genomic instabilities. Inhibiting PLK1 resulted in G2/M checkpoint restoration and in a decrease of tumour formation incidence, tumour volume and tumour weight *in vivo* (Li et al., 2018).

In the section “**2.3.4. Mechanisms of HBV-induced HCC**”, several mechanisms involved in HBV-induced oncogenesis were reviewed. HBV acts as a weak oncovirus and cannot lead to one-hit cellular transformation, thus several interplays are more likely coopting to induce oncogenesis. Moreover, the hepatocellular carcinoma is well known as a multifactorial originating cancer and could not be defined by a single event. In this respect, several studies have identified PLK1 to be part of this HBV-induced transformation and inhibiting PLK1 could be of benefit for anti HBV-related HCC therapy.

3.3.10. PLK1 as a target for anticancer drugs

Due to its large involvement in the cell-cycle progression and in pathways that are related to carcinogenesis, PLK1 has been considered as a druggable target for the development of new therapies in a wide variety of cancers. In this respect, remarkable efforts from academics and private companies are underway. Two major class of chemical inhibitors of PLK1 are available, either acting in an ATP-competitive manner or by interfering with the PBD to suppress protein-protein interactions. Furthermore, genomic tools such as siRNA targeting PLK1 are under development/investigation. In the part below, the principal anti-PLK1 molecules are overviewed. For a more detailed review, please refer to “**Plk1 inhibitors in cancer therapy: From laboratory to clinics**” from Gutteridge and colleagues (Gutteridge et al., 2016).

BI-2536 is one of the earliest PLK1 inhibitors and was developed by Boehringer Ingelheim Company. In 2007, Steegmaier and colleagues reported that the ATP-competitive BI-2536 inhibits tumor growth *in vitro* as well as *in vivo* (Steedmaier et al., 2007). Furthermore, BI-2536 was found to exhibit a relatively safe profile in clinical trials (Frost et al., 2012). However, this molecule is no longer used in monotherapy, mostly due to modest response rates observed in phase 2 trial for the treatment of unresectable exocrine adenocarcinoma of the pancreas (Mross et al., 2012). BI-2536 showed excellent results when combined to other targeted therapies such as synergistic effects observed with Vincristine in neuroblastoma (Czaplinski et al., 2016) or with Eribulin in rhabdomyosarcoma (Stehle et al., 2015).

Volasertib, known as BI-6727, is a second generation of PLK1 inhibitors and was also developed by Boehringer Ingelheim by tailoring the dihydropteridinone structure of BI-2536 (Rudolph et al., 2009). Volasertib is a potent molecule in inhibiting mitosis with very low IC₅₀ of 0.87 μmol/L. First studies using Volasertib were shown to impede proliferation in preclinical models of acute myeloid leukemia (AML) (Rudolph et al., 2015) and to induce cell mortality in bladder cancer (Brassasco et al., 2013). In order to improve the efficacy, Volasertib was combined to other therapies and has shown synergistic effects with the MEK inhibitor Trematinib (Posch et al., 2015) and with histone deacetylase (HDAC) inhibitors *in vitro* (Wissing et al., 2013). Finally, Volasertib inhibits the subcutaneous xenograft growth of HCC in nude mice, mostly by inducing apoptosis, thereby providing new therapeutic potential of Volasertib on hepatocellular carcinoma (Zheng et al., 2016). Like many others targeted therapies, Volasertib may become less effective as cancers develop resistances. In 2015, Wu and colleagues reported that overexpression of the ATP-binding cassette (ABC) drug transporter ABCB1 confers resistance to Volasertib (Wu et al., 2015). One may suggest that the use of ABCB1 modulators combined to Volasertib could prevent this mechanism and delay resistance phenomenon. Regarding clinical trials, several have tested Volasertib, in mono or combined therapies, in recent years. Notably, dose escalations to determine the safety profile of Volasertib were done in solid tumours and this drug was considered being well-tolerated (Lin et al., 2014). Volasertib appears to have better response rates in some cancers, thus combination therapy trials are being actively pursued. The most advanced study was a phase III trial of Volasertib plus low dose cytarabine LDAC for the treatment of acute myeloid leukaemia, but unfortunately, did not meet the primary endpoint of objective response. The first results were published in 2016 and the primary analysis showed a higher number of patients responded to volasertib plus LDAC (25.2%) than placebo plus LDAC (16.8%) but the overall result was not statistically significant. The occurrence of fatal infection frequency was considered as the major reason for a negative overall survival trend. This trial is still ongoing and updates will be presented in 2019 (NCT01721876).

The Poloxin is the major representative of PLK1 inhibitors shown to block the PBD-dependent protein-protein interactions (Reindl et al., 2008). Poloxin leads to mitotic arrest and apoptosis of several cancer cell lines, mostly through modulating Kizuna, a key substrate of PLK1 at centrosomes. Furthermore, in xenograft mouse models, Poloxin was shown to suppress tumor growth (Yuan et al., 2011). In 2015, in order to improve Poloxin efficacy, Scharow and colleagues reported the identification of Poloxin-2, an optimized analog of its predecessor with improved potency and selectivity (Scharow et al., 2015). Further studies are still needed to document the anti-cancer activity of Poloxin-2 in mono or combined therapies as well as to define its toxicity.

Rigosertib, also known as ON 01910 acts as a Ras mimetic and as a non-ATP-competitive small molecule inhibitor of PLK1 and PI3K (Gumireddy et al., 2005). Rigosertib has been largely trialed in clinic with increased overall response rates in several cancers (Gutteridge et al., 2016). In some cases, PLK1 inhibitors have been employed successfully to overcome acquired Gemcitabine

resistance in certain cancer lines. In this respect, a phase 3 clinical trial was conducted to determine the efficacy of Rigosertib plus Gemcitabine compared to Gemcitabine alone in previously untreated metastatic pancreatic cancer. The combination increased the partial response in patients by 6%, however, due to an overall survival decreased by 3 months compared to Gemcitabine alone, the trial was deemed unsuccessful (NCT01360853).

An innovative approach to inhibit PLK1 tends to emerge and resides in the genomic knockdown of PLK1 using siRNA. In 2002, Spänkuch-Schmitt and colleagues first demonstrated the potential of inhibiting PLK1 by a siRNA approach in order to induce apoptosis in cancer cells (Spänkuch-Schmitt et al., 2002). In 2016, Arbutus Biopharma published final results from a phase 1/2 clinical trial regarding the anti-tumor activity of intravenous siRNA targeting PLK1, TKM-080301 in subjects with advanced hepatocellular carcinoma. More specifically, TKM-080301 is a type of LNP formulation, referred to as SNALP (Stable Nucleic Acid Lipid Particles) containing siRNA targeting PLK1. In this so-called chemorefractory cancer, administration of siPLK1 resulted in increased partial response and inhibition of tumor growth. However, due to adverse effects, TKM-080301 was considered to have suboptimal efficacy (NCT02191878). In another phase 1 clinical trial, TKM-080301 safety and effectiveness were evaluated for primary or secondary liver cancer and was administered by hepatic arterial infusion (NCT01437007). Although some issues are still matters of concern (delivery, toxicity, cost, and biological barriers), siRNA definitively opens new perspectives for drug development.

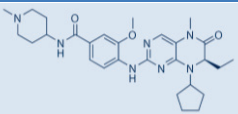
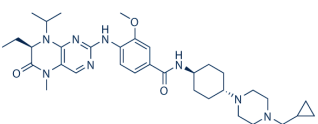
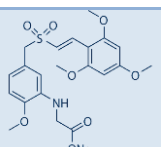
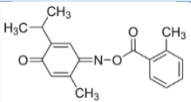
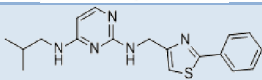
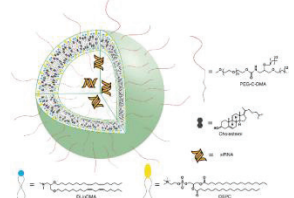
Compound	Structure	Mode of Action	Most advanced trial	Ref.
BI-2536		ATP-competitive	Phase II	NCT00710710 NCT00701766
BI-6727 (Volasertib)		ATP-competitive	Phase III	NCT01721876
ON 01910 (Rigosertib)		Non-ATP-competitive	Phase III	NCT01928537 NCT01241500 NCT01360853
Poloxin		PBD interaction	N/A	Yuan et al., 2011
Poloxin-2		PBD interaction	N/A	Scharow et al., 2015
TKM-080301 (LNP siPLK1)		Genomic KO of PLK1 mRNA	Phase I/II	NCT02191878 NCT01262235 NCT01437007

Table VI: Several PLK1 inhibitors being clinically trialed or in early development for anti-cancer therapies.

PART II: RESEARCH PROJECT

Chapter 1: Hypothesis and Objectives

In highly HBV endemic regions, 70-80% of hepatocellular carcinoma cases are HBV-attributable. Despite the HBV vaccine, the World Health Organization reports 240 million people are chronically infected with HBV. Current treatments to control chronic HBV infection for reducing the incidence of liver cancer are ineffective, thus new and effective therapies are needed.

Polo-like-kinase 1 (PLK1), which plays pivotal roles in mitosis and is over-expressed in many human cancers, represents a promising druggable target in oncology. Focusing on HBV, it was shown that the X protein (HBx) could activate PLK1 in murine cell transformation models. Our and collaborators recent studies have identified a positive link between PLK1 activation and HBV replication. The interplay between PLK1 and HBV replication was described using the HepAD38 cellular model of HBV replication. In this context, the HBV DNA is stably integrated into the host genome, under control of a Tet-off expression system. Transcription of HBV pregenomic RNA (pgRNA), the template of viral replication, is initiated by tetracycline removal. It has been shown that in HBV-replicating HepAD38 cells, increased PLK1 expression correlates with down-regulation of two proteins that are components of chromatin modifying complexes; SUZ12 protein of the PRC2 complex, and ZNF198 of the LSD1-CoREST-HDAC1 complex. PLK1 inhibition was described to inhibit HBV replication by reducing viral transcription. How PLK1 regulates HBV transcription remains unknown. Yet it remained to be determined whether PLK1 could also play a role for HBV replication in non-dividing hepatocytes. The goals of my thesis project were to investigate if:

- 1) PLK1 was activated upon HBV infection in non-dividing hepatocytes**
- 2) PLK1 plays proviral role towards HBV**
- 3) PLK1 inhibition could serve as new antiviral strategy**

Ancillary studies: **1) Decipher the mechanisms of PLK1-HBV interplays**

2) Study the potential link between PLK1/HBV and PLK1/HBV-related HCC

To this extent, I used relevant models that resemble physiologic HBV infection, such as of Primary Human Hepatocytes (PHH), dHepaRG and liver-humanized FRG mice, where HBV replicates in non-transformed and non-dividing cells, thus enabling the study of the interphasic role of PLK1, irrespective of its well-established cell division implication.

To inhibit PLK1 activity, I have used chemical approach with several ATP and non-ATP competitive inhibitors (i.e., BI-2536, BI-6727, Poloxin) as well as genomic approach to knockdown PLK1 at mRNA level. Regarding this latter, I had access to an innovative technology developed by Arbutus Biopharma; siRNA embedded into lipid nanoparticles that allow a specific delivery of siRNA to hepatocytes, thus increasing the bioavailability of drugs *in vivo*.

Chapter 2: Results obtained

2.1. First study: Polo-like-kinase 1 is a proviral host factor for hepatitis B virus replication

Polo-like-kinase 1 is a proviral host factor for hepatitis B virus replication.

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Polo-Like-Kinase 1 Is a Proviral Host Factor for Hepatitis B Virus Replication

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Chronic hepatitis B virus (HBV) infection is a major risk factor for hepatocellular carcinoma (HCC) and current treatments for chronic hepatitis B and HCC are suboptimal. Herein, we identified cellular serine/threonine Polo-like-kinase 1 (PLK1) as a positive effector of HBV replication. The aim of this study was to demonstrate the proviral role of PLK1 in HBV biosynthesis and validate PLK1 inhibition a potential antiviral strategy. To this end, we employed physiologically relevant HBV infection models of primary human hepatocytes (PHHs) and differentiated HepaRG cells in conjunction with pharmacologic PLK1 inhibitors, small interfering RNA (siRNA)-mediated knockdown, and overexpression of constitutively active PLK1 (PLK1^{CA}). In addition, a humanized liver Fah^{2/2}/Rag2^{2/2}/Il2rg^{2/2} (FRG) mouse model was used to determine the antiviral effect of PLK1 inhibitor BI-2536 on HBV infection *in vivo*. Finally, *in vitro* PLK1 kinase assays and site-directed mutagenesis were employed to demonstrate that HBV core protein (HBc) is a PLK1 substrate. We demonstrated that HBV infection activated cellular PLK1 in PHHs and differentiated HepaRG cells. PLK1 inhibition by BI-2536 or siRNA-mediated knockdown suppressed HBV DNA biosynthesis, whereas overexpression of PLK1^{CA} increased it, suggesting that the PLK1 effects on viral biosynthesis are specific and that PLK1 is a proviral cellular factor. Significantly, BI-2536 administration to HBV-infected humanized liver FRG mice strongly inhibited HBV infection, validating PLK1 as an antiviral target *in vivo*. The proviral action of PLK1 is associated with the biogenesis of the nucleocapsid, as BI-2536 leads to its decreased intracellular formation/accumulation. In this respect, our studies identified HBc as a PLK1 substrate *in vitro*, and mapped PLK1 phosphorylation sites on this protein. **Conclusion:** PLK1 is a proviral host factor that could be envisaged as a target for combined antiviral and antimetabolic strategies against HBV infection and HBV-mediated carcinogenesis. (HEPATOLOGY 2017;66:1750-1765).

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Chronic hepatitis B virus (HBV) infection is an independent risk factor for development of hepatocellular carcinoma (HCC).^(1,2) Liver

cancer is the fifth and seventh most common cancer worldwide according to sex and the third most common in terms of death. With approximately 250 million people chronically infected with HBV, according to the latest World Health Organization estimation,

Abbreviations: ATP, adenosine triphosphate; cccDNA, covalently closed circular DNA; CDK2, cyclin dependent kinase 2; dHepaRG, differentiated HepaRG; DMSO, dimethyl sulfoxide; EC₅₀, effective concentration at 50%; ELISA, enzyme-linked immunosorbent assay; FRG, Fah^{2/2}/Rag2^{2/2}/Il2rg^{2/2}; HBc, HBV core protein; HBeAg, hepatitis B e antigen; HBsAg, hepatitis surface antigen; HBV, hepatitis B virus; HBx, HBV X protein; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; IFN- α , interferon- α ; IHC, immunohistochemistry; mRNA, messenger RNA; PHH, primary human hepatocyte; PLK1, Polo-like-kinase 1; PLK1^{CA}, constitutively active PLK1; pgRNA, pregenomic RNA; qPCR, quantitative polymerase chain

reaction; rcDNA, relaxed circular DNA; RNAi, RNA interference; RT-qPCR, reverse transcription qPCR; siHBV, siRNA-targeting HBV transcripts; siHCV, siRNA against HCV; siPLK1, siRNA-mediated knockdown of PLK1; siRNA, small interfering RNA; SP, serine/proline; S/T, serine/threonine; vge, virus genome equivalents.

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many patients with chronic hepatitis B are expected to progress to HCC, despite existing therapies.⁽¹⁾ Although a prophylactic vaccine is available, new infections still happen because vaccination campaigns are not always well-implemented, and the vaccine is not always 100% protective.⁽²⁾ Furthermore, children of infected mothers still become chronically infected in endemic areas.⁽²⁾ Current treatments include use of pegylated interferon-*a* (IFN-*a*) and/or antiviral nucleoside analogs; the latter require life-long administration, which can result in viral resistance when drugs with a low genetic barrier are used.⁽³⁾ New and effective therapies are urgently needed.⁽³⁾ In particular, drugs that target both viral replication and prevent neoplastic transformation could represent a valuable addition to the therapeutic arsenal.

Replication of HBV within hepatocytes involves both nuclear and cytoplasmic events.⁽⁴⁾ After viral entry, nucleocapsids are transported to the nucleus, releasing the viral DNA and core/capsid HBV core protein (HBc) into the nucleoplasm. There, the viral genome, initially a relaxed circular partially double-stranded DNA (rcDNA), is converted into covalently closed circular double-stranded DNA (cccDNA), which is responsible for viral persistence. This nuclear form of the genome serves as a template for viral RNA transcription, including pregenomic RNA (pgRNA).⁽⁴⁾ In the cytoplasm, pgRNA interacts with HBV polymerase and is packaged into capsids, where it is reverse-transcribed to generate newly synthesized rcDNA.⁽⁴⁾ HBc plays an essential and dynamic role in HBV biosynthesis, including selective binding to pgRNA in association with viral polymerase, encapsidation, and reverse-transcription of pgRNA, transport of mature capsids to the site of viral envelopment for

virion generation, and transport to the nucleus for recycling.⁽⁵⁾ Although the mechanism by which HBc and capsid assembly regulate viral DNA synthesis is not fully understood, it is well established that phosphorylation of HBc has an important role in various aspects of pgRNA reverse-transcription.^(5,6) Kinases that mediate HBc phosphorylations include cyclin-dependent kinase 2 (CDK2), which phosphorylates serine/proline (SP) sites located at the C-terminus of HBc, including S155, S162, and S170.^(7,8) Another study has identified additional serine/threonine (S/T) sites as having a role in regulating all aspects of HBV replication, and serine/arginine protein kinase 1 as another kinase that phosphorylates HBc *in vitro*.⁽⁹⁾

Our earlier studies, aiming at understanding the mechanism by which the HBV X protein (HBx) mediates oncogenic transformation of hepatocytes,⁽¹⁰⁻¹²⁾ led us to the study of Polo-like-kinase 1 (PLK1), an S/T kinase involved in cell-cycle regulation.^(13,14) In physiologic conditions, PLK1 is required for checkpoint recovery, mitotic entry, and progression, whereas it is overexpressed in many human cancers, including HBV-mediated liver cancer.^(13,14) We have shown that HBx activates PLK1 prematurely, allowing propagation of DNA damage to daughter cells by attenuating both DNA repair and p53 apoptosis.^(11,12) In a cellular model of HBx-mediated transformation and in liver tumors of animals modeling HBx- and HBV-mediated hepatocarcinogenesis, expression of PLK1 is elevated.^(11,12,15,16) However, whether PLK1 is activated during physiologic HBV infection of normal hepatocytes, and whether it has a role in the establishment and maintenance of infection is unknown. To address this issue, in this study, we used HBV infection models of nontransformed primary human

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hepatocytes (PHHs) and differentiated HepaRG (dHepaRG) cells, a bipotential human dedifferentiated hepatocyte cell line that upon differentiation supports HBV infection.^(17,18) In this study, we demonstrate that the cellular PLK1 enzyme acts as a proviral host factor for HBV replication *in vitro* by favoring nucleocapsid formation and subsequent reverse transcription. In addition, we identified the viral core protein HBc as a PLK1 substrate *in vitro* and mapped the sites of PLK1 phosphorylation. Finally, we provide evidence that PLK1 inhibitors serve as a novel class of anti-HBV molecules *in vitro* and validate the antiviral effect of PLK1 inhibitor BI-2536 in HBV-infected liver humanized mice *in vivo*.

Materials and Methods

CHEMICALS, ANTIBODIES, AND OTHERS REAGENTS

All chemicals were purchased from Sigma-Aldrich unless specified otherwise. PLK1 inhibitors (BI-2536, BI 6727, MLN 0905, and ON 01910) were from Selleckchem. Polyclonal HBc⁽¹⁹⁾ and commercially available HBc antibodies were used for immunofluorescent staining and immunoblots. Rabbit polyclonal anti-HBc was from Dako (B0586) or Abcam (ab140243) and monoclonal anti-HBc from Abcam (ab8637; Clone C1). PLK1 antibodies used: rabbit polyclonal (ab21738) for detection of phospho-PLK1-S₁₃₇ in western blotting and immunofluorescence; rabbit monoclonal (ab115095) for detection of phospho-PLK1-T₂₁₀ in western blotting and immunofluorescence, and rabbit polyclonal (ab109777) for detection of PLK1. Small interfering RNA (siRNA; SmartPool or sequence designed) were purchased from Dharmacon and transfected into dHepaRG cells or PHHs using Dharmafect-1 reagent according to the manufacturer's instructions. Tenofovir (TFV) and HBV capsid assembly inhibitors Bay41-4109⁽²⁰⁾ and AT130⁽²¹⁾ were kindly provided by Gilead Sciences and Novira Pharmaceuticals, respectively. IFN- α (i.e., Roferon) was obtained from Hoffmann-La-Roche.

HepaRG CELLS AND PRIMARY HUMAN HEPATOCYTES AND INFECTION BY HBV

Human liver progenitor HepaRG cells⁽¹⁸⁾ were cultured for 2 weeks in complete William's medium (Gibco) supplemented with 10% Fetal Clone II serum (Thermo Scientific), 50 U/mL penicillin/streptomycin (Gibco), 2 mM glutaMax

(Gibco), 5 μ g/mL human insulin (Sigma), and 5 $\times 10^{-25}$ M hydrocortisone hemisuccinate (UpJohn, SERB) at 37°C in humidified CO₂ (5%) incubators and differentiated into hepatocyte-like cells by way of treatment with 1.8% dimethyl sulfoxide (DMSO; Hybrid-max, Sigma) for 2 weeks as described previously.⁽²²⁾ Engineered HepaRG cell lines were also used. The HepaRG-TR-PLK1^{CA} cell line enabling tetracycline-inducible expression of a constitutively active form of PLK1 (i.e., T210D mutant⁽²³⁾) was constructed as described for the HepaRG-TR-HBc cell line.⁽²⁴⁾ PHHs were obtained from surgical liver resections after informed consent of patients (kindly provided by Prof. Michel Rivoire [Centre Léon Bérard, Lyon, France]; agreement numbers DC-2008-99 and DC-2008-101). PHHs were prepared as described previously⁽²⁵⁾ and were cultured in complete William's medium supplemented with 1.8% DMSO.

HBV INFECTION AND ANALYSIS OF VIRAL PARAMETERS DURING REPLICATION

dHepaRG cells and PHHs were infected as described previously,⁽²⁶⁾ with HBV inoculum prepared either from HepG2.2.15 or HepaAD38 cells. Intracellular accumulation of viral RNA and DNA, secretion of hepatitis B e antigen (HBeAg) and hepatitis B surface antigen (HBsAg) were monitored by quantitative polymerase chain reaction (qPCR), reverse transcription qPCR (RT-qPCR), southern blotting, and enzyme-linked immunosorbent assay (ELISA), as described.⁽²⁶⁾ Briefly, HBeAg and HBsAg were quantified in culture medium by way of ELISA, using a chemiluminescence immunoassay kit (Autobio, China) according to the manufacturer's instructions. Total DNA was purified from infected cells using MasterPure Complete DNA Purification Kit (Epicentre). Southern blot analysis of HBV DNA was performed using equal amounts of total DNA, electrophoresed on 1.1% agarose gels, hybridized to HBV DNA radiolabeled with [α -³²P]CTP⁽²²⁾ and analyzed by autoradiography. Total RNA was extracted from infected cells using NucleoSpin RNA (Macherey-Nagel) and transcribed into complementary DNA using SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA). Real-time PCR for total HBV DNA and cccDNA was performed using LightCycler 96 (Roche) as described previously.^(26,27)

TRANSFECTIONS, PLASMIDS, AND SMALL INTERFERING RNAs

Transient transfections in HEK293T cells were performed by employing Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. HBc-WT (EL43), HBc-3A (EL113), and HBc-3D (EL114) were kindly provided by Dr. Daniel Loeb, derived from EL43 plasmid.⁽²⁸⁾ The CTD-GST fusion protein plasmids GST-HCTD141 (CTD WT) and GST-HCTD141-AAAAAAA (CTD 7A)⁽⁷⁾ were a kind gift from Dr. Jianming Hu. siRNAs (Smart pool grade; Dharmacon) for PLK1, HBV, and hepatitis C virus (HCV) (5-25 nM) were transfected in HBV-infected HepaRG or PHHs using Dharmafect I (Dharmacon) following the manufacturer's protocol.

HUMANIZED FRG MOUSE MODEL AND HBV INFECTION

All animal studies were reviewed and approved by our local ethics committee. Highly immunosuppressed FRG mice deficient in T cells, B cells, and natural killer cells were used⁽²⁹⁾ and maintained in a pathogen-free facility. High-quality cryopreserved human hepatocytes were purchased (BD, Biosciences) and injected through an intrasplenic route into 2- to 3-month old mice as described previously.⁽³⁰⁾ Liver humanized FRG mice featuring serum production of human albumin, at least 5 mg/mL, were infected with 200 μ L of HBV inoculums (1.10^8 virus genome equivalents [veg] to 1.10^9 veg in phosphate-buffered saline) through an intraperitoneal route.⁽³⁰⁾

Mice were treated with intraperitoneal injection of BI-2536 (10 mg/kg twice a week) for 1 month. Serum was harvested every week by retro-orbital bleeding and stored at -80°C in aliquots for further antigenemia and viremia analysis. Mice were sacrificed at week 8 postinfection and hepatic tissues were frozen and processed for virologic parameter analyses or fixed in formalin and embedded in paraffin for immune-staining.

CAPSID MIGRATION ASSAY

The intracellular formation/accumulation of HBV nucleocapsids in *in vitro* infected hepatocytes or in mouse-derived liver resection was accessed from cell or liver lysate by way of native agarose gel electrophoresis followed by transfer onto the enhanced chemiluminescence membrane and western blot analysis, as described previously.⁽⁶³¹⁾

IN VITRO PLK1 KINASE ASSAYS

Assays were performed as described previously⁽¹⁰⁾ using recombinant PLK1 (BPS Bioscience, Protein One). Core protein was immunopurified from the HepaRG-TR-HBc cell line or purchased from Meridian Life Science, Inc. Site-directed mutagenesis of putative PLK1 phosphorylation sites in HBc-WT and HBc-3D was performed employing the Quick-Change Lightning site-directed mutagenesis Kit (Agilent). Point mutations in the GST-CTD-WT and GST-CTD-7A plasmids were introduced following the same procedure. Mutations were confirmed by DNA sequencing. For protein staining, PageBlue Protein Staining Solution (ThermoFisher) was used according to the manufacturer's protocol.

STATISTICAL ANALYSIS

Statistical analysis was performed using two-way analysis of variance, *t* tests, or nonparametric Mann-Whitney tests using GraphPad Prism software. For all tests, $P \leq 0.05$, $P < 0.01$, and $P < 0.001$ were considered statistically significant.

Results

PLK1 IS ACTIVATED BY HBV INFECTION IN NON-DIVIDING/DIFFERENTIATED HEPATOCYTES

Our earlier studies demonstrated that (1) HBx activates the mitotic S/T kinase PLK1, in a conditional HBx-expressing cell line,⁽¹¹⁾ (2) PLK1 activation initiates proteasomal degradation of chromatin modifying nuclear proteins SUZ12 and ZNF198,⁽¹⁰⁾ and (3) SUZ12 down-regulation in HBV replicating hepatocytes results in expression of hepatic cancer stem cell markers and pluripotency genes.⁽³²⁾ We have also shown activation of PLK1 in HBV-replicating HepAD38 cells,⁽¹⁰⁾ further suggesting a link between HBV infection and PLK1 activation. However, it has yet to be determined whether PLK1 activation occurs in the context of physiologic infection of nondividing, differentiated, and nontransformed hepatocytes. To this end, PHHs and dHepaRG cells were infected with HBV and expression and activation of PLK1 was quantified. Upon infection of dHepaRG cells, PLK1 messenger RNA (mRNA) increased by 15-fold 24 hours postinfection, followed by a constant level of expression of 3

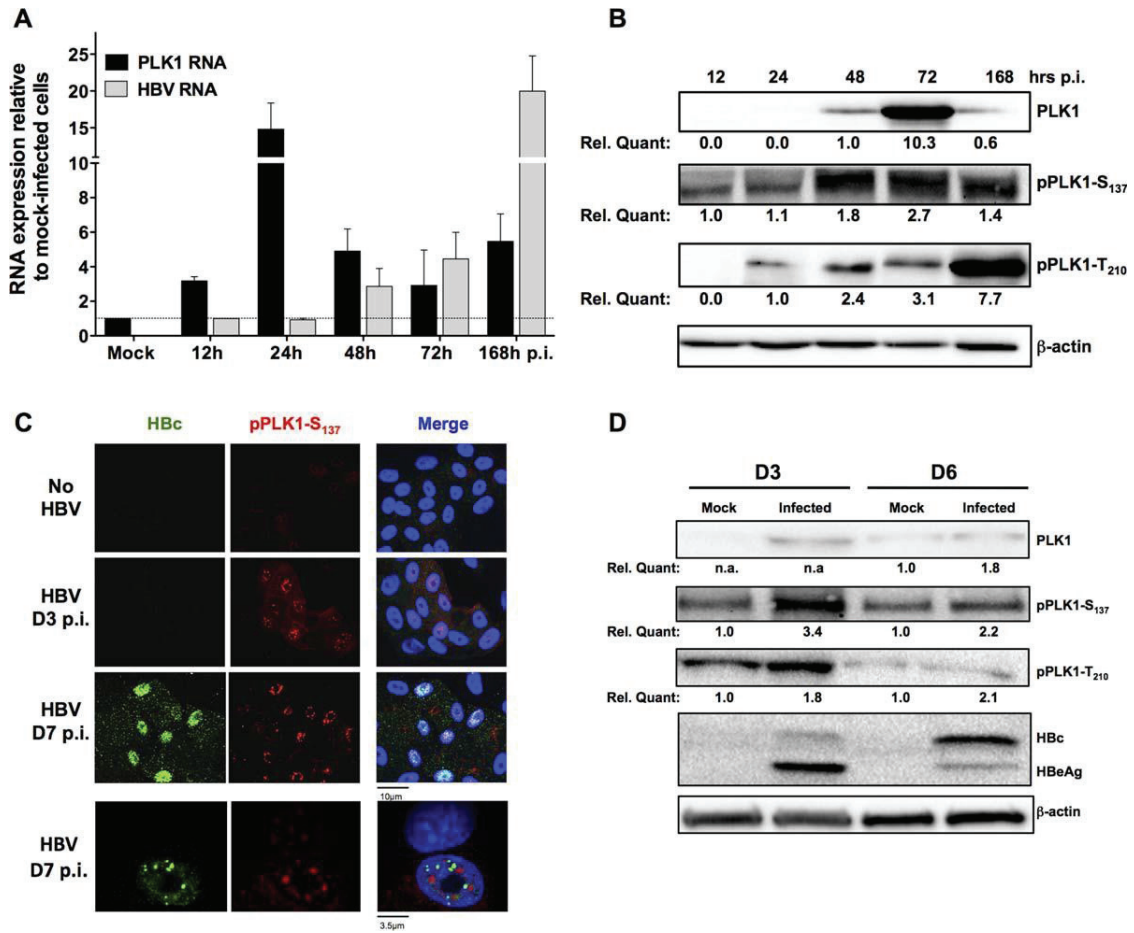


FIG. 1. HBV infection activates PLK1. dHepaRG cells (A-C) or PHHs (D) were infected with a low dose (100 vge/cell) or high dose (1000 vge/cell) of HBV. (A) Cells were harvested at the indicated time points, and RNA was extracted and subjected to RT-qPCR. Fold induction of the mRNA expression level of PLK1 and HBV was normalized to housekeeping genes, compared with mock infection. (B) Immunoblotting of PLK1 and phosphorylated PLK1 (pPLK1-S₁₃₇ and pPLK1-T₂₁₀) using whole cell extract of mock- or HBV-infected dHepaRG cells isolated at the indicated time points postinfection. Quantification by chemiluminescence was performed with a ChemiDoc XRS1 system (Biorad). (C) Immunofluorescence microscopy of the indicated proteins with and without HBV infection in dHepaRG cells at different times postinfection. Cells were fixed with 2% paraformaldehyde and stained with the indicated antibodies. (D) Immunoblots of PLK1 and phosphorylated PLK1 using whole cell extract from mock- or HBV-infected PHHs.

to 5- fold from 48 to 168 hours postinfection (Fig. 1A). This resulted in a transient increase in PLK1 protein levels (Fig. 1B). More interestingly, an increase in PLK1 phosphorylation on S137 and/or T210, indicative of PLK1 activation, was detected as a function of HBV infection by immunoblotting (Fig. 1B) and immunofluorescence microscopy (Fig. 1C) employing phospho-specific PLK1 antibodies. Remarkably, this activation of PLK1 by HBV infection was also detected by immunoblots of lysates from various preparations of PHHs (representative blots are shown in Fig. 1D).

PLK1 INHIBITORS, INCLUDING BI-2536, SUPPRESS HBV DNA ACCUMULATION IN PERSISTENTLY HBV-INFECTED HEPATOCYTES

To test whether PLK1 activation has a proviral effect, dHepaRG cells were infected with HBV virions and on day 7 postinfection, when infection had reached a plateau in terms of viral replication, cells were treated with various PLK1 inhibitors. Specifically, increasing concentration of adenosine triphosphate (ATP)-competitive (i.e., BI-2536 and BI 6727) or non-ATP-competitive (i.e., MLN 0905 and ON 01910) PLK1 inhibitors were added for 3 days; accumulation of intracellular HBV DNA was measured by way of Southern blotting and qPCR (Supporting Fig. S1A,B). Among all drugs tested, BI-2536 was the most active and selected for further studies, after monitoring batch-to-batch stability (Supporting Fig. S2). HBV-infected dHepaRG treated with BI-2536 on day 7 postinfection exhibited no effect on levels of secreted HBeAg and HBsAg, or intracellular viral RNA (Fig. 2A). Interestingly, BI-2536 significantly inhibited synthesis of total HBV DNA, with an effective concentration at 50% (EC_{50}) of approximately 4 nM (Fig. 2A), in the absence of any toxicity (cytotoxic concentration at 50%) of approximately 10 μ M (Fig. 2B). Interestingly, BI-2536 reduced the level of capsid (i.e., assembled HBc/core protein) detected by immunofluorescence microscopy (Fig. 2C) with an antibody recognizing HBc assembled into capsid, and not monomeric forms of HBc (Supporting Fig. S3). Finally, BI-2536 was shown to strongly (up to 95%) and dose-dependently reduce the formation/accumulation of HBV nucleocapsids in infected cells (Fig. 2D). Taken together, these results demonstrate that PLK1 inhibition by BI-2536 affects the assembly of HBV capsids, as specific HBc assembly inhibitors (such as

BAY41-4109 or AT130) do, thereby inhibiting subsequent rcDNA synthesis. Similar results were also obtained in HBV-infected PHHs (Fig. 3), with an EC_{50} at approximately 50 nM. Interestingly, in this model, a trend toward an increase in intracellular viral RNA was observed (Fig. 3A), thus inversely correlating with total DNA decrease. This corroborates the hypothesis that rcDNA synthesis is likely inhibited by an impaired assembly of capsids around pgRNA.

We also analyzed the effect of BI-2536 on the establishment of HBV infection and found that the antiviral effect was even greater with not only an effect on neosynthesis of HBV DNA but also effect on other measured viral parameters (Supporting Fig. S4).

LOSS-OF-FUNCTION OF PLK1 SUPPRESSES HBV DNA ACCUMULATION IN INFECTED HEPATOCYTES

To conclusively establish that PLK1 is a proviral factor, we investigated effect of loss of function of PLK1 on HBV replication by siRNA-mediated knockdown of PLK1 (siPLK1). As controls, we employed siRNA-targeting HBV transcripts (siHBV; positive control), and siRNA against HCV (siHCV) serving as negative control. The effectiveness of PLK1 knockdown by siRNA transfection was determined by quantification of PLK1 mRNA levels as a function of time after transfection (data not shown). Transfection of siPLK1 reduced by nearly 90% endogenous level of PLK1 mRNA at 72 hours after transfection, whereas all nontargeting siRNAs had no effect on PLK1 mRNA levels (Fig. 4A). Transfection of increasing amounts of siPLK1, ranging from 5 to 25 nM in HBV-infected dHepaRG cells, resulted in a progressive reduction of 50% to nearly 70% in the accumulation of intracellular HBV DNA (Fig. 4A). By contrast, transfection of 25 nM siHCV had a minimal effect, whereas transfection of 25 nM siHBV reduced HBV DNA by nearly 80% (Fig. 4A). With siPLK1, we again observed a decreased accumulation of assembled HBc within infected cells by way of immunofluorescence using an antibody recognizing assembled capsids (Fig. 4B) and by way of native capsid migration assay (Fig. 2D), thus confirming the potential relationship between proviral action of PLK1, formation of HBV capsids, and decrease in newly synthesized rcDNA. Similar results were obtained in HBV-infected PHH cultures transfected for 72 hours with siRNAs targeting PLK1, HBV, or an irrelevant target (Fig. 4C). Specifically, Southern blot analyses demonstrated a 50% reduction in HBV DNA by transfection of siPLK1, a 10% reduction with siHCV, and a 65% reduction with siHBV (Fig. 4D).

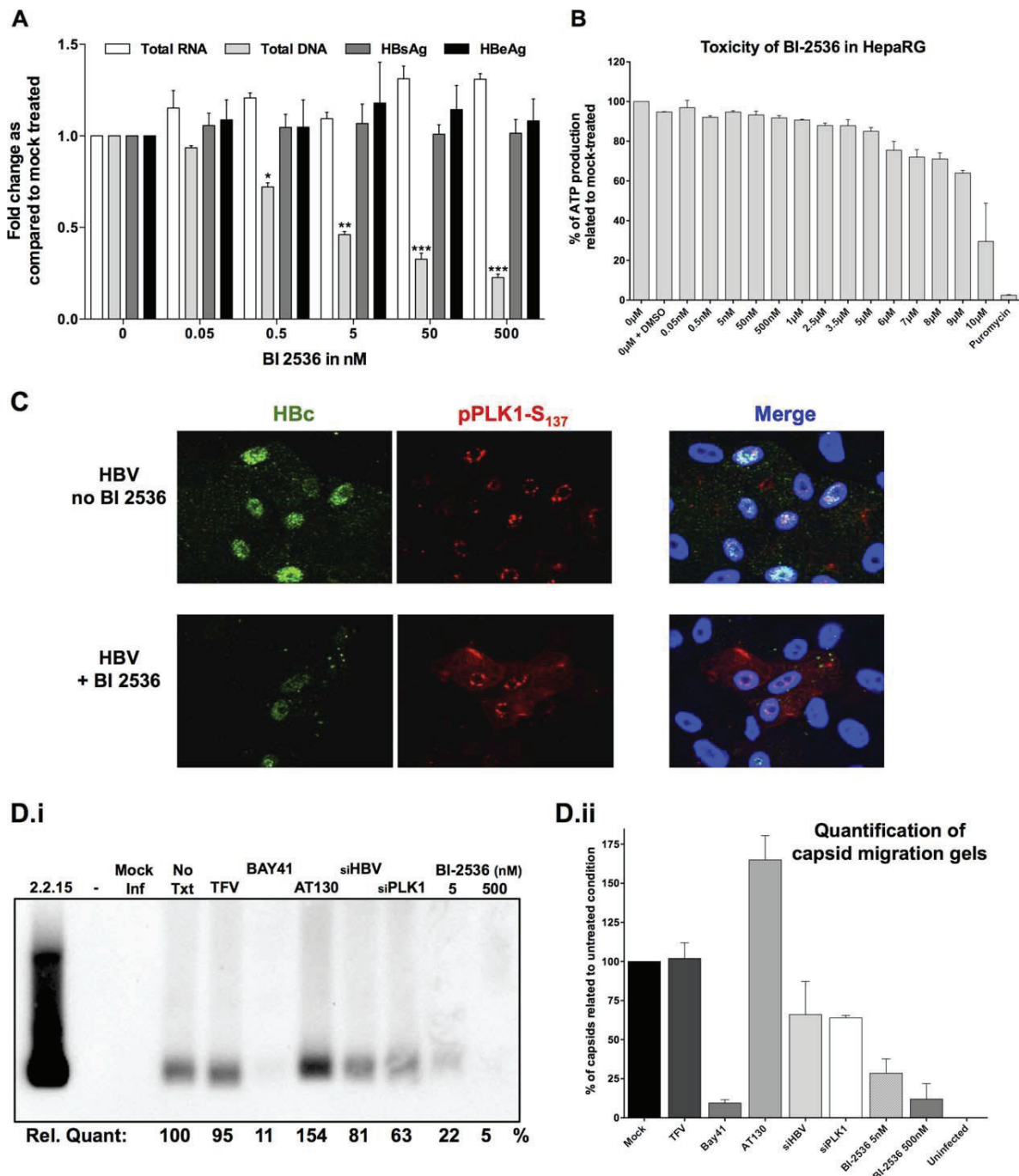


FIG. 2. Effect of BI-2536 on HBV replication in nondividing and nontransformed dHepaRG cells. dHepaRG cells were infected with 100 vge/cell of HBV for 7 days followed by treatment with increasing concentration of PLK1 inhibitor BI-2536, as indicated, for 3 days. (A) Secreted HBsAg and HBeAg quantified by ELISA from supernatants of HBV-infected dHepaRG cells, day 10 post-infection. Total RNA and DNA extracted from infected cells, day 10 postinfection, were analyzed by way of HBV-specific RT-qPCR or qPCR, normalized to housekeeping genes, and compared with mock-treated cells. The results are presented as a fold change in expression or secretion compared with untreated controls and are expressed as the mean \pm standard error of the mean of at least three independent experiments. (B) Cell viability of HepaRG cells measured using Cell Titer Glo One Solution Assay, with increasing concentration of BI-2536, as indicated. DMSO and puroMyacin were used as controls. (C) Immunofluorescence microscopy of the indicated proteins with and without BI-2536 in infected dHepaRG cells. (D) Mock and infected dHepaRG cells were treated with biochemicals or transfected by siRNA (25 nM) targeting either HBV (siHBV) or PLK1 (siPLK1) for 3 days. Cell lysates were analyzed by way of native agarose gel electrophoresis, transferred onto enhanced chemiluminescence membrane, and immunoblotted with Hbc antibody. (D.i) Immunoblotting with quantification below. No Txt 5 no treatment; TFV 5 tenofovir at 10 μ M; Bay41 5 Bay41-4109 at 10 μ M; AT130 5 AT130 at 10 μ M; BI-2536 at the indicated concentration. (D.ii) Quantification of two independent experiments by chemiluminescence with a ChemiDoc XRS1 system (Biorad).

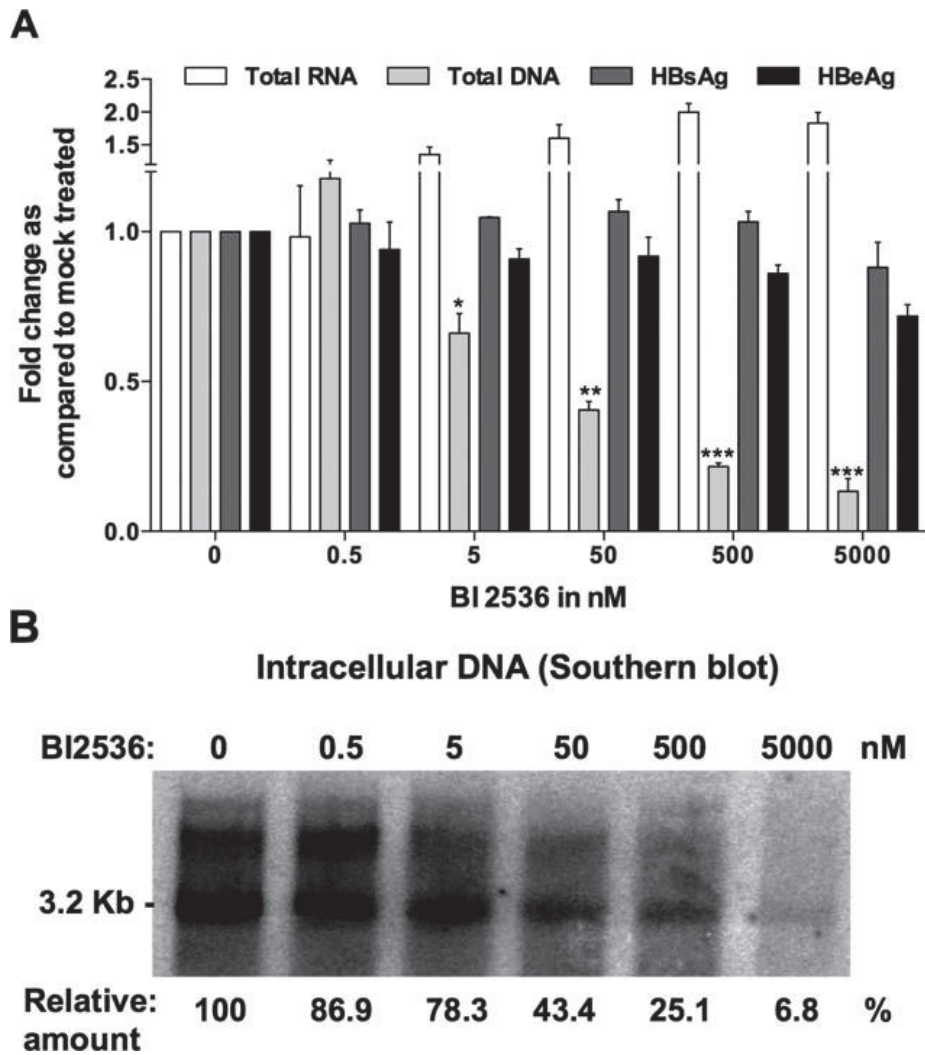


FIG. 3. Effect of BI-2536 on HBV replication in nondividing and nontransformed PHHs. PHHs were infected with 100 vge/cell of HBV for 4 days followed by treatment with increasing concentration of PLK1 inhibitor BI-2536, as indicated, for 3 days. (A) Quantification of secreted HBsAg and HBeAg by ELISA using supernatants of HBV-infected PHHs on day 7 postinfection. Total RNA and DNA extracted from HBV infected cells, day 7 postinfection, were analyzed by HBV-specific RT-qPCR or qPCR, normalized to housekeeping genes, and compared with mock-treated cells. Fold change in expression or secretion quantified relative to untreated controls are expressed as the mean \pm standard error of the mean of at least three independent experiments. (B) Representative Southern blotting (n = 3). Total DNA extracted from cells on day 7 postinfection was analyzed by way of agarose gel electrophoresis, transferred onto a nylon membrane, and hybridized to a radioactive HBV probe as described previously.⁽²²⁾

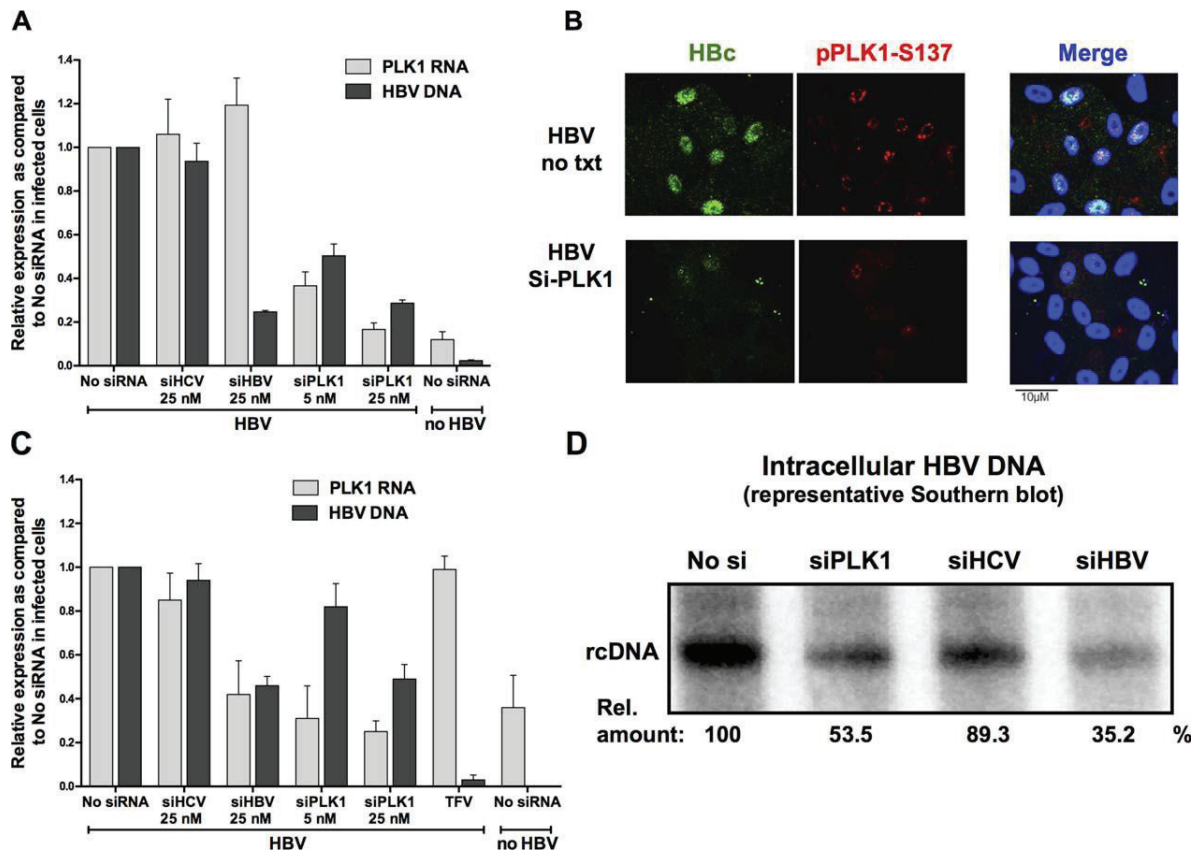


FIG. 4. Effect of PLK1 knockdown on HBV replication in dHepaRG cells and PHHs. PHHs or dHepaRG cells infected with 100 vge/ cell of HBV for 7 days were transfected with siRNA (5 or 25 nM) targeting PLK1 (siPLK1) or HBV (siHBV) or HCV (siHCV), as indicated. (A, C) Total RNA and DNA were extracted and subjected to RT-qPCR or qPCR with PLK1 or HBV primers. Quantification of PLK1 mRNA and HBV DNA is relative to absence of siRNA transfection (no siRNA). Results represent the mean \pm standard error of the mean of at least three independent experiments. (B) Immunofluorescence microscopy of the indicated proteins, with and without siPLK1 transfection in HBV-infected dHepaRG cells. Cells were fixed with 2% paraformaldehyde at day 10 postinfection, and stained with the indicated antibodies. (D) Representative Southern blotting. DNA was extracted from infected PHHs transfected by the indicated siRNA.

A MODEST GAIN-OF-FUNCTION OF PLK1 IS ASSOCIATED WITH INCREASED HBV REPLICATION

To investigate the effect of gain-of-function of activated PLK1 on HBV replication, we generated an HepaRG cell line expressing a constitutively active form of PLK1 (PLK1^{CA}) in a tetracycline inducible manner (i.e., HepaRG-TR-PLK1^{CA}) (Supporting Fig. S5A). HBV-infected dHepaRG-TR-PLK1^{CA} cells were induced to express PLK1^{CA} on day 7 postinfection by addition of increasing amounts of tetracycline (0, 16, 80, and 400 ng/mL) for 3 days. Quantification of viral DNA and secreted antigens was performed day 10 postinfection. Under these conditions of established infection, a modest expression of PLK1^{CA} enhanced HBV replication by 1.5- to 2- fold, whereas no effect on HBeAg secretion was observed (Supporting Fig. S5B). This result suggests that active PLK1 enhances the formation of new viral genomes through reverse transcription of pgRNA; that is, it exerts a positive effect on capsid-assisted reverse transcription of pgRNA.

REDUCTION OF HBV VIREMIA IN LIVER-HUMANIZED INFECTED FRG MICE TREATED WITH BI-2536

To demonstrate the effect of BI-2536 on inhibition of HBV replication *in vivo*, we employed liver-humanized FRG mice⁽³⁰⁾ infected with HBV (5.10^8 vge/mouse). Once serum viremia reached $7 \log_{10}$ vge/mL, antigenemia $5 \log_{10}$ IU/mL for HBsAg, and 2500 net control unit (NCU)/mL for HBeAg at week 4 postinfection, treatment was administered by way of intraperitoneal injection of 10 mg/kg of BI-2536 (or same concentration of DMSO diluted in saline) biweekly for 4 weeks. Serum viremia and antigenemia were monitored at the end of weeks 1, 2, 4, 6, 7, and 8, whereas weeks 6, 7, and 8 were those under treatment. Treated mice exhibited at week 8 a mean reduction of $1.406 \log_{10} \pm 0.26$ in viremia (Fig. 5A), whereas a minimal effect was observed on antigenemia (Fig. 5B,C). Intraliver analyses using samples collected at euthanasia confirmed the reduction of total HBV DNA while showing no change in HBV RNA and cccDNA, further confirming the antiviral phenotype of BI-2536 (Fig. 5D). If immunohistochemistry staining with a polyclonal anti-HBc antibody, which recognize all forms of HBc (Supporting Fig. S3), revealed a slight decrease in accumulation of this protein at the end of the treatment period (representative immuno-

histochemistry [IHC] shown in Fig. 6A), an analysis of the accumulation of assembled capsid by native capsid migration assay confirmed the inhibitory effect of BI-2536 (representative IHC is shown in Fig. 6B,C). This antiviral effect was obtained in the absence of toxicity as measured by monitoring mouse weight, secretion of human albumin in serum, and level of alanine aminotransferase (Supporting Fig. S6). It is worth noting that similar duration of treatment with IFN- α (25 lg/kg twice a week; n = 5 mice treated) or entecavir (2.5 lg/mouse per day in drinking water; n = 3 mice treated) in liver-humanized FRG mice led to a viremia reduction of $0.668 \log_{10} \pm 0.18$ or $1.919 \log_{10} \pm 0.23$, respectively (Supporting Fig. S7). These results indicate that a mean reduction of $1.406 \log_{10} \pm 0.26$ in monotherapy obtained with BI-2536 was relevant and in between performance of IFN- α and entecavir in this model.

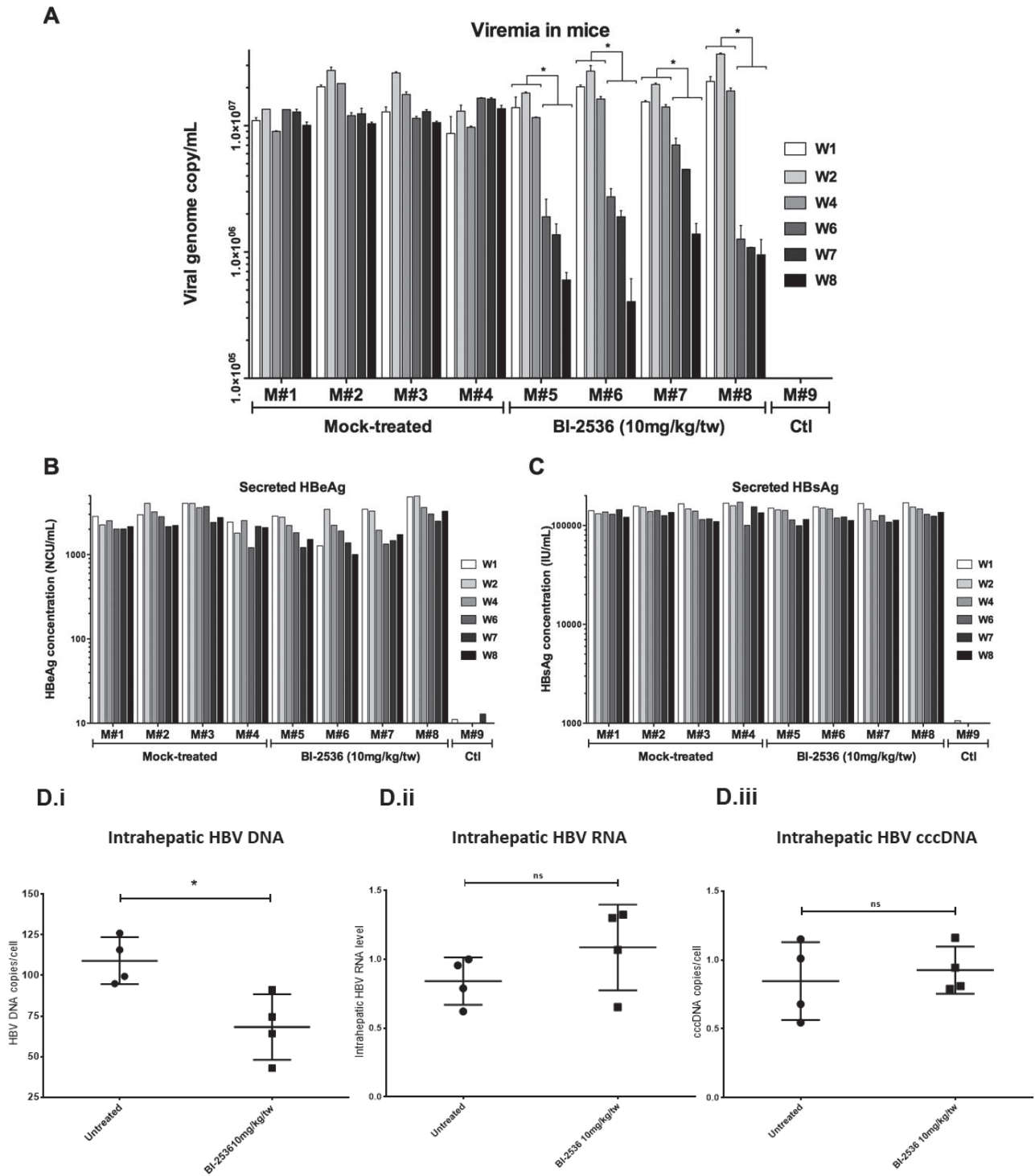


FIG. 5. Effect of BI-2536 on HBV replication in liver humanized HBV-infected FRG mice. Eight mice engrafted with human PHHs for 2 months were infected with HBV ($5.10e8$ vge/mouse). At week 4 postinfection, treatment was initiated as indicated. BI-2536 (10 mg/kg) was injected twice per week intraperitoneally for 4 weeks. Blood was collected at the end of weeks 1, 2, 4, 6, 7, and 8 and viremia (A) and antigenemia (B, C) were monitored by way of qPCR and ELISA. From liver pieces, intrahepatic HBV DNA (D.i), RNA (D.ii), and cccDNA (D.iii) were quantified respectively by qPCR, RT-qPCR, and cccDNA-specific qPCR.⁽²⁷⁾

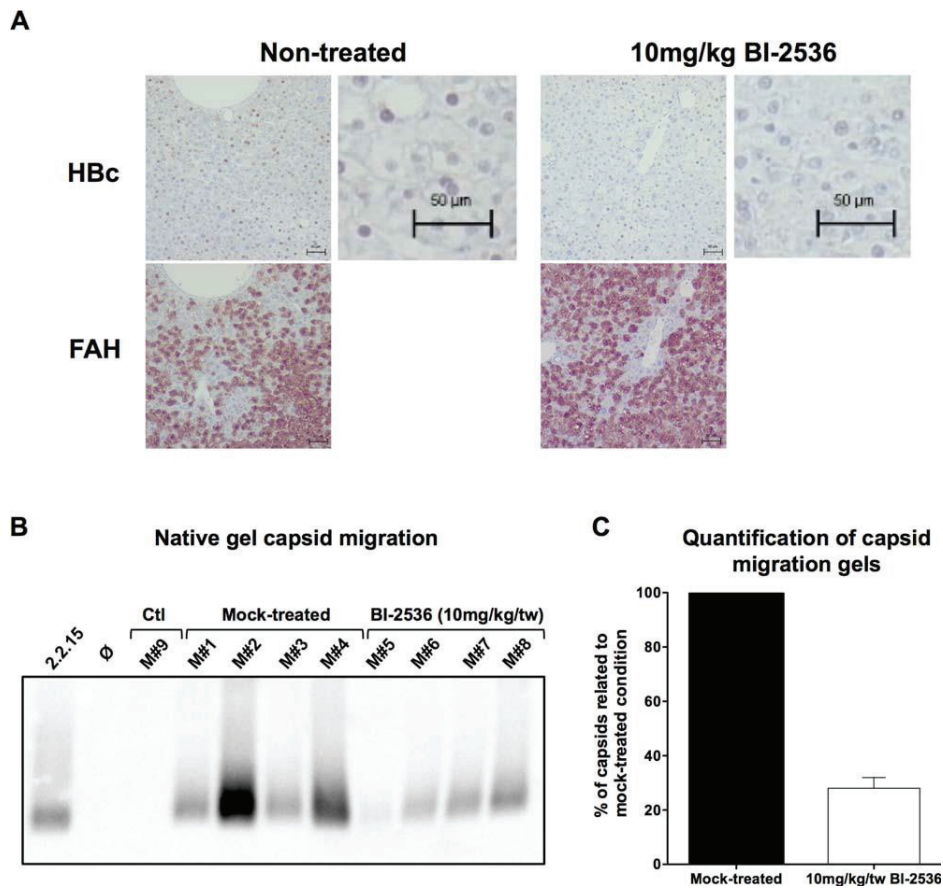


FIG. 6. Effect of BI-2536 on capsid formation in liver-humanized HBV-infected FRG mice. Immunohistochemistry staining (A) and native capsid migration assays (B) employing liver samples from the same mice as in Figure 5. (A) Representative immunohistochemistry staining with anti-HBc antibody (Dako) and FAH antibody for non-treated and BI-2536 treated livers. (B) Capsid migration assay employing extracts from mock-treated FRG mice (mouse numbers M#1- M#4) and BI-2536 treated animals (mouse numbers M#5-M#38). (C) Cumulative quantification of capsid migration signals from panel B.

COMBINATION OF BI-2536 WITH APPROVED OR INVESTIGATIONAL ANTI-HBV DRUGS

Our *in vitro* and *in vivo* data demonstrated that PLK1 inhibitors could be used as efficient and safe anti-HBV drugs. PLK1 inhibitor, including BI-2536, seems to target the capsid-associated reverse transcription of pgRNA into rcDNA. Other drugs also target this step of the viral life cycle, including nucleoside/nucleotide analogs and core inhibitors (also called core protein allosteric modulators). However, all of these drugs have different molecular targets and therefore could be used in combination to enhance the inhibitory phenotype. To establish whether BI-2536 could be used in combination with nucleoside/nucleotide analogs or core protein allosteric modulators without leading to antagonistic effect, we combined these molecules to treat infected dHepaRG cells. A combination of BI-2536 and IFN- α was also included. As expected, only BI-2536 + IFN- α treatment led to a reduction in HBeAg secretion (Fig. 7A), whereas all other combinations led to a sharp reduction of intracellular HBV DNA (Fig. 7B). No obvious antagonism was evidenced for all combinations tested, thus suggesting that these drugs could be further considered for combination studies.

HBc IS A SUBSTRATE FOR PLK1 *IN VITRO*

During established infection, inhibition of PLK1 by treatment with BI-2536 suppressed only viral DNA synthesis (Figs. 2A, and 3A,B), suggesting that PLK1 may have a role in capsid-associated reverse transcription of pgRNA into rcDNA. Conversely, inhibition of PLK1 could prevent these processes, based on capsid assembly. Because HBc has an essential and dynamic role in this process of capsid assembly, pgRNA encapsidation, and rcDNA biosynthesis, we investigated whether HBc is a PLK1 phosphorylation substrate.

To determine whether HBc is a direct target of PLK1, we performed *in vitro* kinase assays with purified recombinant HBc and PLK1 (Fig. 8). We observed *in vitro* PLK1 phosphorylation of HBc using either recombinant HBc or HBc isolated from HepaRG-TR-HBc cells (Fig. 8A); furthermore, addition of 500nM BI-2536 inhibited this phosphorylation (Fig. 8A, left panel). The C-terminal domain of HBc (CTD), spanning amino acids 143 to 183 (Fig. 8B), contains 8 S/T residues.

We performed *in vitro* PLK1 kinase assays with GST-CTD fusion peptide isolated from transfected cells and featuring either wild-type or mutated CTD

sequences at all S/T sites. This approach was validated by showing that fusion protein with seven (not including T147) or all eight S/T positions mutated exhibited absence of PLK1 phosphorylation (Fig. 8B). PLK1 requires a priming phosphorylation of its substrate, usually by CDK1 or CDK2 at SP sites. Interestingly, HBc is known to be phosphorylated by CDK2 at three SP phosphorylation sites (Fig. 8B). Accordingly, we tested whether HBc proteins containing S to A or S to D substitutions at all three SP positions (labeled as 3A or 3D) could serve as PLK1 substrates. Interestingly, only the phosphomimetic substitutions—S155D, S162D, and S170D (referred to as 3D)—exhibited phosphorylation by PLK1 (Fig. 8C). Next, we observed that S168A exhibited significantly reduced phosphorylation in the context of the phosphomimetic substitutions at SP sites, whereas no difference in the phosphorylation by PLK1 was detected by T146A and T147A substitutions (Fig. 8D). Single point mutations at residues 168, 176, and 178, again in the context of the phosphomimetic substitutions at SP sites, resulted in significant reduction of HBc phosphorylation by PLK1 (Fig. 8E). Therefore, we conclude that S168, S176, and S178 are PLK1 phosphorylation sites *in vitro*.

Discussion

The hepatitis B virus (HBV) is one of the most important human oncogenic viruses.^(1,2) Approximately 250 million individuals worldwide are chronically infected with HBV, having increased risk for developing HCC.^(1,2) If clinicians dispose of several anti-HBV drugs, they have limited chemotherapeutic options for HCC. It is thus crucial to identify novel molecules that target both virus biosynthesis and neoplastic transformation. In this study, we provide evidence that PLK1, a kinase already demonstrated to be involved in HCC, could represent a potential target to tackle both HBV replication and liver oncogenesis. Earlier studies demonstrated that HBx activates PLK1 in a hepatocyte model cell line⁽¹¹⁾ and that PLK1 was activated in the HepAD38 cellular model of HBV replication.⁽¹⁰⁾ However, this cellular model of viral replication does not model physiologic infection. In this study, using physiologically relevant HBV infection models comprising PHHs and HepaRG cells, we show that HBV infection leads to early activation of PLK1. Here, we show that PLK1 phosphorylation occurs upon HBV infection in nondividing and differentiated cells; the mechanism of PLK1 activation during HBV infection is yet to be determined. HBx protein, remains the main candidate for this activation likely mediating PLK1 activation via the

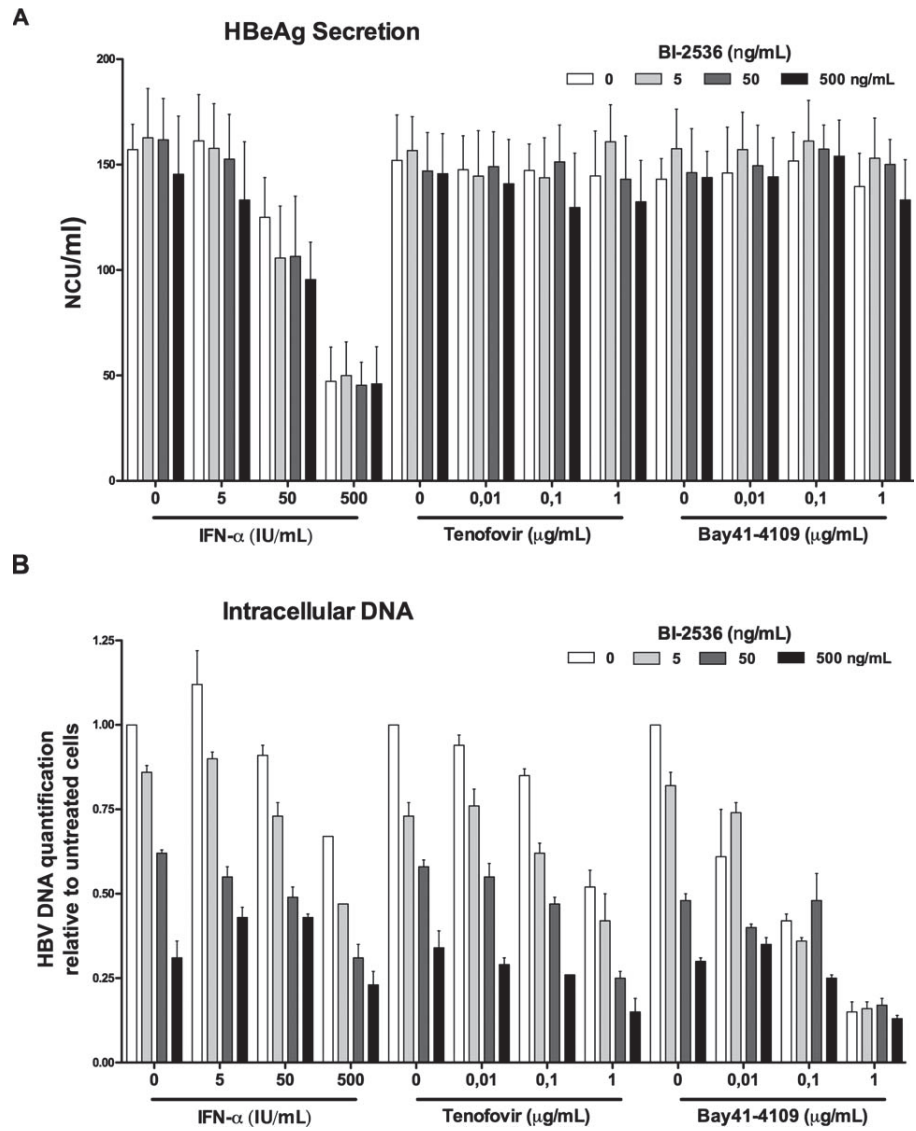


FIG. 7. Combination treatment of BI-2536 and tenofovir, IFN- α , or Bay41-4109. dHepaRG cells were infected with 100 vge/cell of HBV for 7 days, followed by treatment with the indicated concentration of drugs for 3 days. (A) HBeAg secretion in supernatant was monitored by way of ELISA. (B) Intracellular HBV DNA was quantified by way of qPCR.

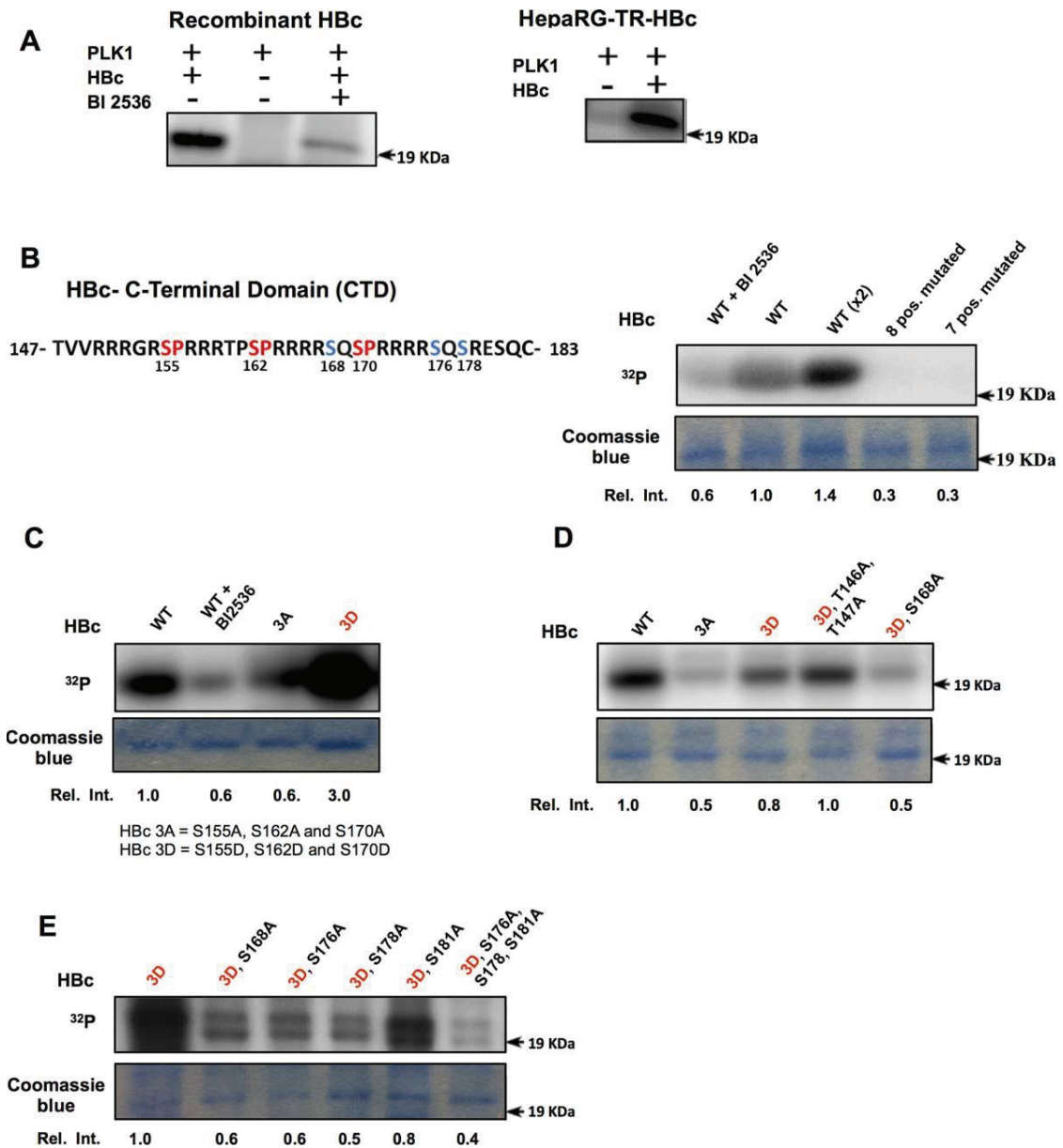


FIG. 8. HBc is a phosphorylation substrate of PLK1 *in vitro*. *In vitro* PLK1 kinase assays of wild-type (WT) HBc and site-directed mutants. (A) Recombinant HBc or immunoaffinity purified HBc isolated from HepaRG-TR-HBc cells and PLK1 were used in *in vitro* PLK1 kinase assays. Reactions were performed with (1) or without (2) PLK1 inhibitor BI-2536 (500 nM), as indicated, in the presence of c^{32} P-ATP, and analyzed using sodium dodecyl sulfate–polyacrylamide gel electrophoresis and autoradiography. (B) Amino acid sequence of C-terminal domain (CTD) of HBc. Eight S/T residues are present in HBc CTD. CDK2 SP sites are indicated in red color, and putative PLK1 phosphorylation sites in blue. (B-E) *In vitro* kinase assays of WT HBc and site-directed mutants with (1) or without (2) addition of BI-2536, as indicated. HBc 3A contains serine to alanine substitutions of residues 155, 162, and 170. HBc 3D contains serine to aspartate substitutions of the same residues. Coomassie Blue staining of the same gel used for autoradiography is shown. Relative intensity quantified by ImageJ software is the ratio of signal from *in vitro* kinase reaction signal versus the corresponding signal of Coomassie Blue–stained band, expressed relative to WT (n = 3).

p38-MAPK.^(11,33) Here, we provide evidence for the functional advantage to the viral life cycle of this early activation of PLK1 by HBV infection.

Employing PLK1 pharmacological inhibitors and an RNA interference approach, we have shown that PLK1 enzymatic activity is crucial for maintenance of HBV replication in nondividing hepatocytes. Indeed, BI-2536, an ATP-dependent PLK1 inhibitor, drastically inhibited HBV replication *in vitro* in both HepaRG cells and PHHs, as well as *in vivo* in liver humanized FRG mice, without toxicity. Interestingly, the antiviral activity of BI-2536 was potent, with an EC₅₀ ranging from 5 to 50 nM, depending on the cell type used; such an antiviral efficacy is rarely obtained with host targeting agents and corresponds to the efficacy of direct acting agents including nucleoside analogs or assembly inhibitors. Accordingly, an *in vivo* antiviral activity was also obtained with BI-2536 in HBV-infected liver humanized FRG mice, and this antiviral activity was comparable to that obtained with entecavir or IFN-*α* in the same model. The RNA interference (RNAi) approach confirmed the PLK1 specificity, as kinase inhibitors are often nonspecific. The antiviral phenotype obtained by RNAi targeting PLK1 was also very strong *in vitro*. The use of such an approach *in vivo* using siRNA that could be specifically targeted to infected hepatocytes could represent an interesting alternative to pharmacological inhibitors, which may be quite toxic when administered systemically. Recently, it has been shown that RNAi nanoparticles in complex with PLK1 siRNA, administered systemically, efficiently delivers PLK1 siRNA to the liver with no observable toxicity.⁽³⁴⁾ Finally, we have also shown that BI-2536 could be combined *in vitro* and presented no antagonism to currently approved (i.e., IFN-*α* and tenofovir) anti-HBV drugs and Bay41-4109,⁽²⁰⁾ a representative core inhibitor, the latter being a novel class of inhibitors in current clinical development.⁽⁵⁾

The molecular mechanism underlying the proviral role of PLK1 has not been completely determined in this study. Nevertheless, we have shown that a target of PLK1 is the capsid/core protein (HBc), which is responsible for capsid assembly around the pgRNA, and indirectly reverse-transcribed pgRNA, as the latter occurs only within capsids. PLK1-mediated phosphorylation requires prior phosphorylated primed substrates. Here, we present evidence that HBc is a direct target of PLK1 *in vitro*, and importantly, efficient phosphorylation of HBc required prior phosphorylation at positions S155/S162/S170, sites shown to be phosphorylated by CDK2.^(7,8) Our studies identified S168, S176, and S178 as PLK1 phosphorylation sites *in vitro*,

dependent on prior phosphorylation of S155, S162, and S170. Thus, PLK1-mediated phosphorylation of HBc in a sequential phosphorylation process could directly mediate the dynamic events associated with capsid formation, pgRNA encapsidation, and reverse transcription. Further mechanistic studies and molecular tools (phospho-specific antibodies) are required to fully delineate this mechanism of PLK1 involvement.

Many other viral proteins are directly phosphorylated by PLK1, including the nonstructural 5A protein from HCV,⁽³⁵⁾ the mumps virus nucleoprotein,⁽³⁶⁾ and the parainfluenza virus 5 protein.⁽³⁷⁾ PLK1-mediated phosphorylation of the target can either lead to its degradation or change in function; therefore, PLK1 can either act as a proviral or antiviral factor. For HCV, PLK1 seems to be a proviral factor, as it contributes to the hyperphosphorylation of nonstructural 5A,⁽³⁵⁾ which is a crucial event to switch from a replicative cycle (i.e., amplification of viral RNA) to a productive one (i.e., generation and secretion of novel virions). Nevertheless, the use of anti-PLK1 drugs has only limited value to inhibit HCV replication, because the virus replicates better *in vitro* in dividing cells, thus precluding nontoxic PLK1 inhibition. However, in the case of HBV, this framework for a potential use of PLK1 targeting drugs does exist. Indeed, HBV only replicates in nondividing and highly differentiated cells. Although we have shown here that an anti-HBV phenotype could be obtained *in vivo*, more experiments are warranted to further demonstrate the safety of such a host targeting agent-based approach. Many PLK1 pharmacological inhibitors as well as strategies based on PLK1 silencing are in current development for treating several cancers⁽³⁸⁾; a repositioning of such drugs for the treatment of chronic hepatitis B patients could be foreseen. The fact that PLK1 inhibitors could reinforce the inhibition of the neosynthesis of encapsidated rcDNA, as nucleoside analogs already do represent an excellent framework for the potential development of combination therapies.

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Supporting Information

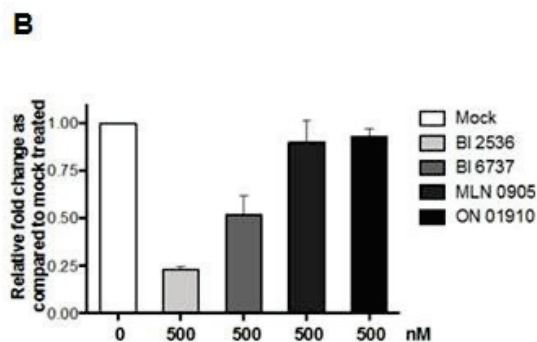
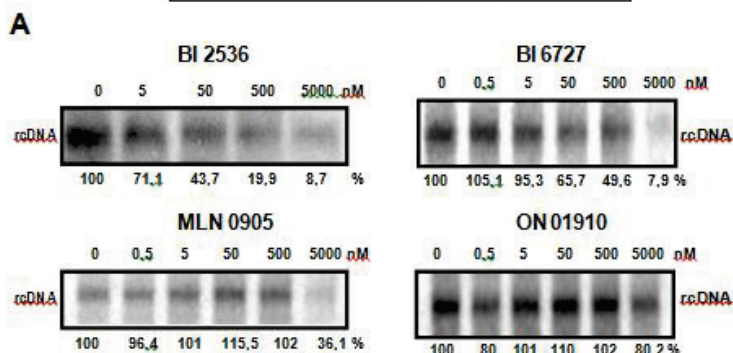
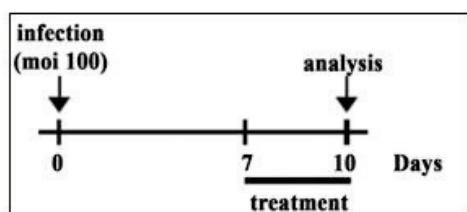
Polo-like-kinase 1 is a proviral host-factor for hepatitis B virus replication

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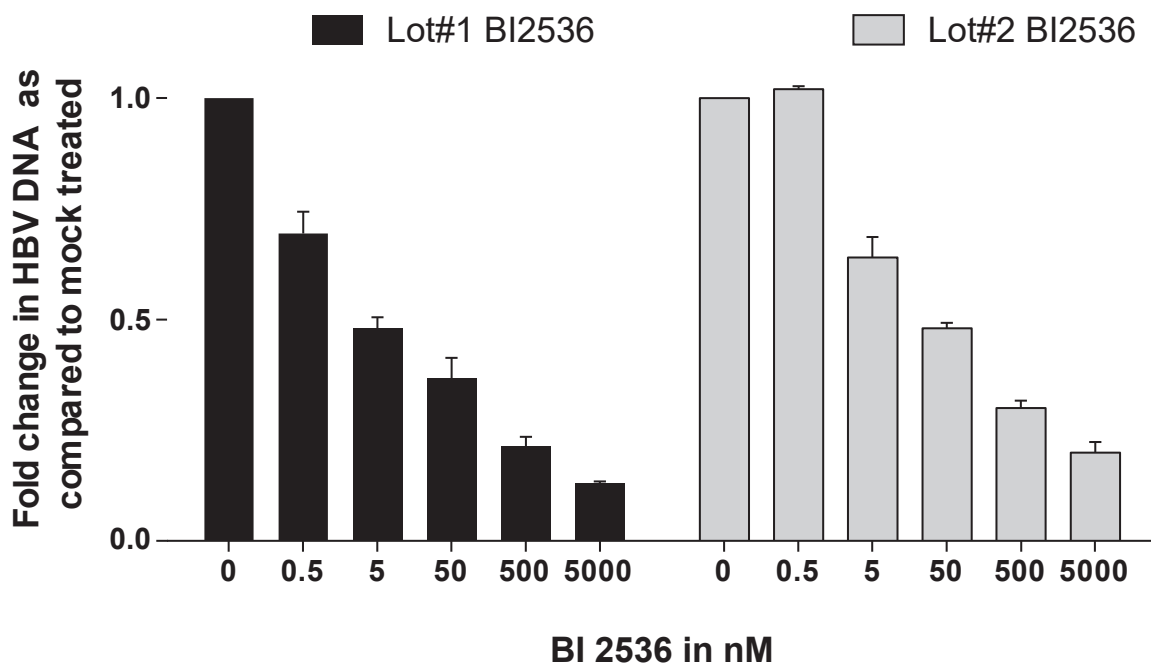
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Supplementary figures and figure legends



21 **Supplementary Figure 1: Dose response effect of competitive and non-competitive PLK1**
 22 **inhibitors on HBV replication in non-dividing hepatocytes.** PHH were infected with 100 vge/cell of
 23 HBV for 7 days followed by treatment with different concentration (0, 5, 50, 500 and 5000 nM) of
 24 indicated PLK1 inhibitors for 3 days. A) Total intracellular HBV DNA was extracted and analyzed by
 25 Southern blot. B) Infected dHepaRG cells on day-7 p.i. were treated with 500 nM of different PLK1
 26 inhibitors for 3 days; total HBV DNA was extracted and quantified by qPCR. Results are expressed as
 27 fold change normalized to housekeeping genes and are from 3 independent experiments.

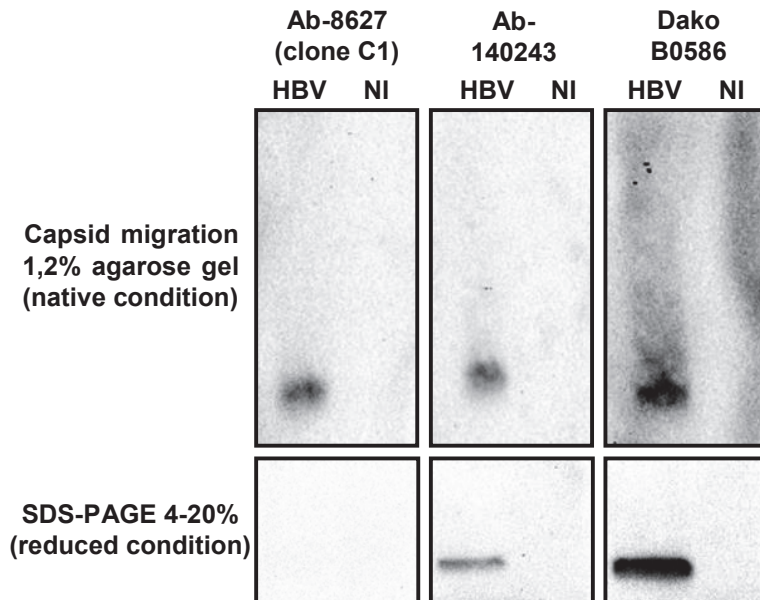


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30 **Supplementary Figure 2: Comparison of batches of BI 2536.** Differentiated HepaRG cells infected
 31 with HBV (100 vge/cell) were treated with increasing concentration of BI 2536 (0, 0.5, 5, 50, 500 and
 32 5000 nM) on day-7 p.i., for 3 days. Total DNA was extracted and HBV DNA was quantified by qPCR
 33 relative to housekeeping genes. Results are shown as fold change in HBV DNA, compared to mock-
 34 treated. Two different batches of BI 2536, from different providers were used as indicated. Results are
 represented as the mean \pm SEM from at least 3 independent experiments.

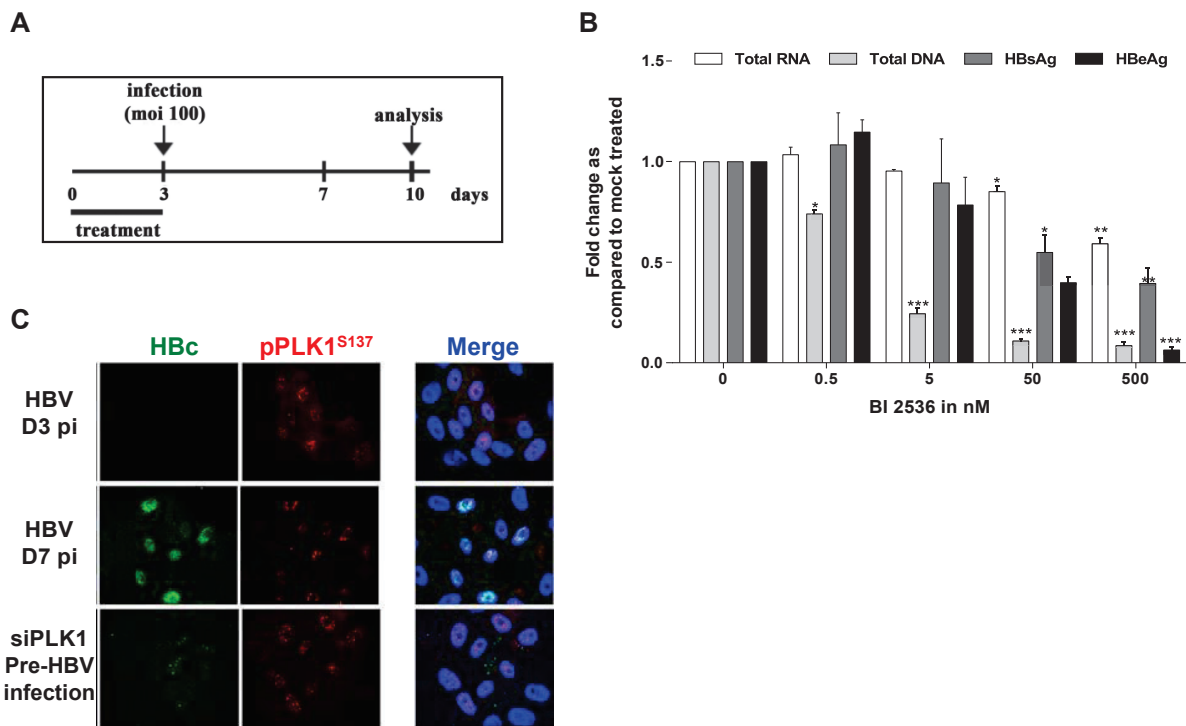
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39 **Supplementary Figure 3: Specificity of anti-HBc antibodies.** Three antibodies (Ab8637 (mouse
 40 monoclonal), Ab140243 (rabbit polyclonal), and Dako-B0586 (rabbit polyclonal)) were tested in
 41 western blotting against assembled HBV nucleocapsid (obtained from a cell lysate of HepG2215 cells,
 42 which contain an HBV transgene and feature an intracellular HBV life cycle; NI means lysate from
 43 non infected cells (i.e; HepG2 cells)) run into agarose gel or denatured HBcAg run in SDS-PAGE.
 44 Ab8637 is the only antibody tested capable to recognize only assembled nucleocapsids. The 2
 45 polyclonal antibodies recognized both from of HBcAg.



47

48 **Supplementary Figure 4: Effect of BI 2536 on establishment of HBV infection in dHepaRG. A)**

49 Diagram of infection and treatment protocol. B) Differentiated HepaRG cell were treated with

50 increasing concentration of BI 2536 for 3 days, then infected with 100 vge/cell of HBV for 7 days.

51 Secreted antigens HBsAg and HBeAg were quantified by ELISA from supernatants of HBV infected

52 dHepaRG cells at day-10 p.i. Total RNA and DNA extracted from infected cells on day-10 p.i. were

53 analyzed by HBV-specific RT-qPCR or qPCR, normalized to housekeeping genes, and compared to

54 mock-treated cells. Fold change in expression or secretion was quantified relative to untreated

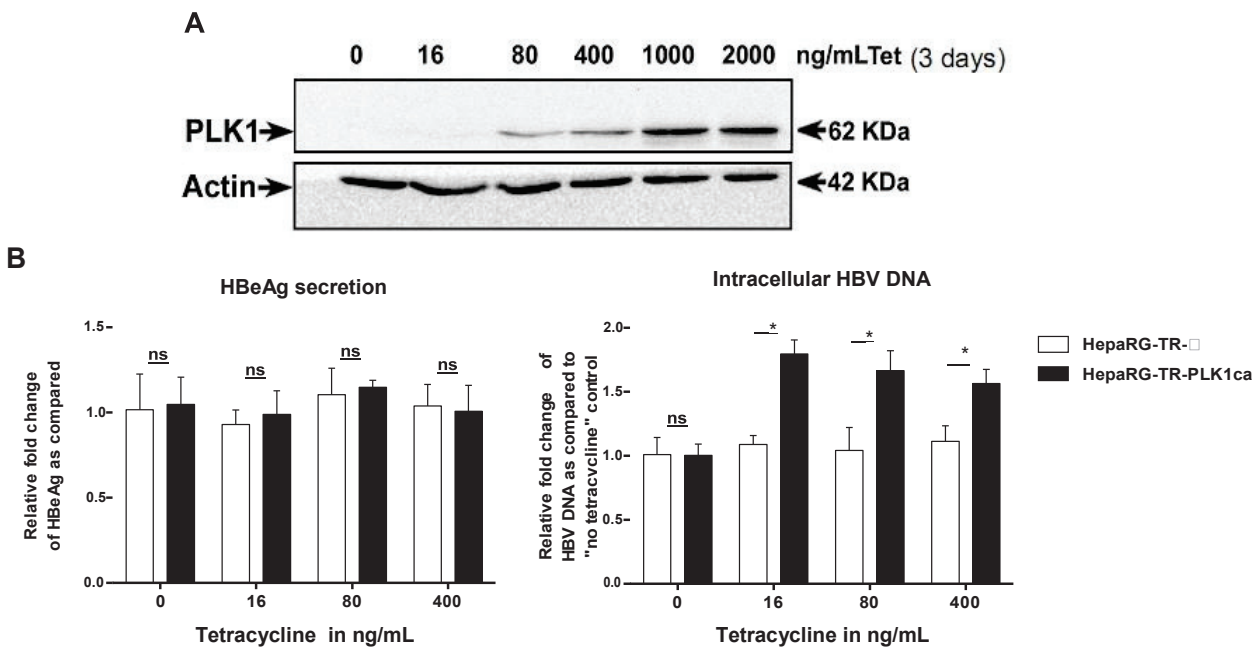
55 controls, and are mean \pm SEM of at least 3 independent experiments. C) Immunofluorescence

56 microscopy of indicated proteins, +/- transfection of siPLK1 in HepaRG cells. siRNA was transfected

57 for 3 days prior to infection and cells were fixed on days-3 or 7 p.i.

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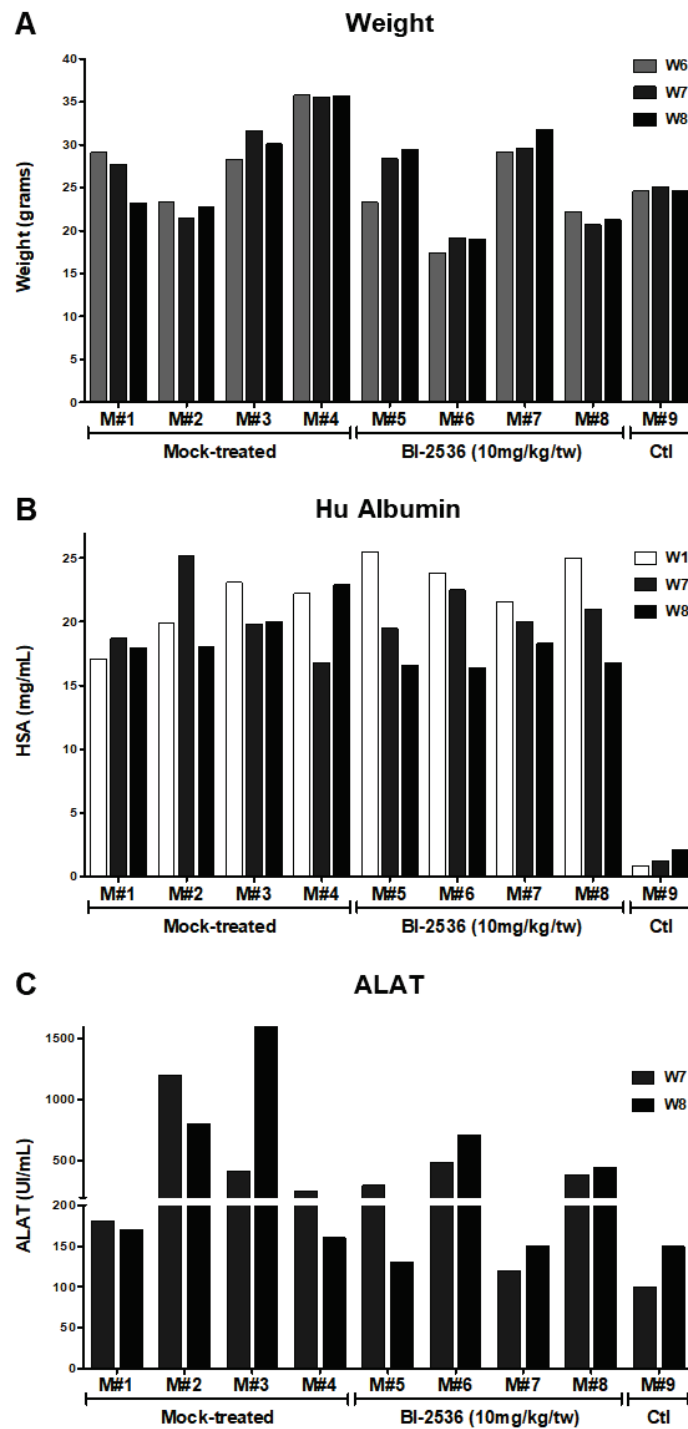
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62 **Supplementary Figure 5: Limited over-expression of PLK1 leads to an increase of HBV**
 63 **replication in dHepaRG.** A) Differentiated HepaRG-TR-PLK1^{CA} cells were induced by the addition
 64 of tetracycline (0, 16, 80 400, 1000 or 2000 ng/mL) for 72 hours and PLK1 levels analysed by
 65 immunoblotting. B) dHepaRG-TR-PLK1^{CA} cells were infected with 100 vge/cell of HBV for 7 days
 66 followed by induction of PLK1 by the addition of indicated amount of tetracycline for 3 days. HBeAg
 67 secretion was analysed by ELISA. Total DNA was extracted and HBV DNA was measured by qPCR.
 68 Results are shown as fold change in HBV DNA as compared to control cell line. Results are
 69 represented as the mean ± SEM of 2 independent experiments.

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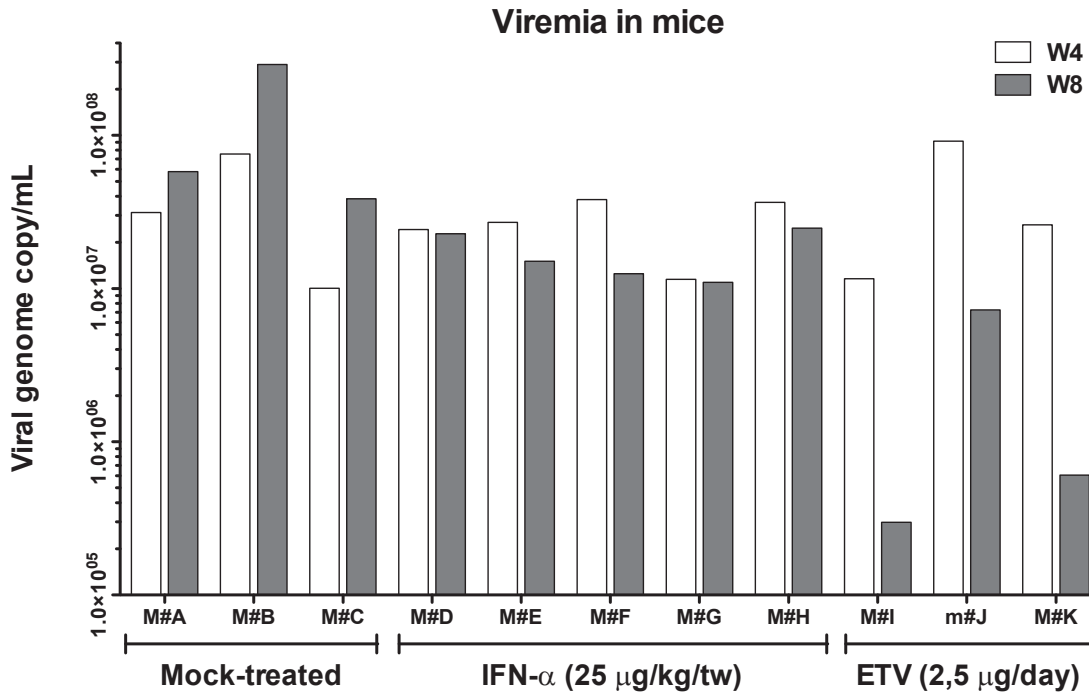
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74 **Supplementary Figure 6: Monitoring of physiologic parameters in liver-humanized HBV-**
 75 **infected FRG mice treated or not with BI 2536. A) Weight of mice during treatment are shown. B)**
 76 **Human albumin was dosed in sera of non-treated and treated mice at week-1 post-infection, and at the**

77 end of treatment (or mock-treatment) (i.e. weeks 7 and 8). C) ALAT was dosed in sera of
 78 mice at weeks 7 and 8.

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80



82 **Supplementary Figure 7: Effect of IFN-α and entecavir on HBV replication in liver humanized**

83 **HBV-infected FRG mice.** Eleven mice, engrafted with human PHH for two months, were infected

84 with HBV (5.10e8 vge/mouse). At week-4 post infection treatment was started in 8 mice and 3 other

85 mice were mock treated. IFN-α (25 µg/kg) was injected twice a week by IP injections, for 4 weeks.

86 ETV (2.5 µg/day) was administrated in drinking water. Blood was taken at the end of weeks 4 and 8 and viremia monitored by qPCR.

2.1.1. Comment on the first study: Polo-Like-Kinase 1: A key cellular target for anti-HBV therapy?

Comment in: "HEPATOLOGY 2017;66:1719-1721":

Polo-Like-Kinase 1: A Key Cellular Target for Anti-HBV Therapy?

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HEPATOLOGY, VOL. 66, NO. 6, 2017

Polo-Like-Kinase 1: A Key Cellular Target for Anti-HBV Therapy?

SEE ARTICLE ON PAGE 1750

More than 240 million individuals are estimated to be chronically infected with hepatitis B virus (HBV)⁽¹⁾ and face a lifetime risk of developing hepatocellular carcinoma (HCC). Current treatments using nucleoside analogues and interferon are effective in inhibiting HBV replication, but unable to clear virus from all hepatocytes. Thus, an alternative strategy of targeting host factors indispensable for HBV replication began to attract serious thought. In this issue of HEPATOLOGY, Diab et al. show that Polo-like-kinase 1 (PLK1) fulfils the role of such a host factor and can serve as an anti-HBV target.⁽²⁾ To mimic the living cell condition for HBV infection, the investigators utilized various models to highlight the role of PLK1 in HBV replication, including nontransformed primary human hepatocytes, differentiated HepaRG cells, and a humanized liver mouse model.

Abbreviations: CDK2, cyclin-dependent kinase 2; CTD, C-terminal domain; HBC, hepatitis B core protein; HBV, hepatitis B virus; HBx, HBV X protein; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; NSSA, nonstructural protein 5A; PBD, Polo-Box domain; pgRNA, pregenomic RNA; PLK1, Polo-like-kinase 1; rcDNA, relaxed circular DNA; si, small interfering; SRPK, serine-arginine protein kinase.

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PLK1 is a serine/threonine-protein kinase. The investigators demonstrated that HBV infection transiently elevates PLK1 expression and activates PLK1 kinase activity, which, in turn, phosphorylates hepatitis B core protein (HBc), thereby facilitating capsid formation and DNA synthesis of HBV. HBc has long been known to be essential for multiple steps in HBV biosynthesis, including capsid assembly, encapsidation, and reverse transcription of pregenomic RNA (pgRNA), transport of DNA into the nucleus, virus maturation, and release.⁽³⁾ HBc performs specific functions by different domains. N-terminal domain is sufficient to form the capsid shell, and the C-terminal domain (CTD) is essential for viral replication. The CTD, covering residues 150-183, of HBc is highly basic and rich in arginine. CTD contains eight potential serine/threonine phosphorylation sites, and some phosphoacceptor residues have been demonstrated to be critical for viral replication.⁽⁴⁾

PLK1 is composed of a kinase domain and a Polo-Box domain (PBD). In the current publication, Diab et al.⁽²⁾ demonstrated that, upon HBV infection, PLK1 could be activated by phosphorylation at S137 and T210, which, in turn, phosphorylates HBc at S168, S176, and S178. How PLK1 phosphorylates HBc remains an interesting question. In general, the PBD of PLK1 can accommodate phosphoserine or phosphothreonine residues to mediate PLK1 interaction with targets. In other words, phosphor-priming by other kinases is required for most PLK1 targets. Several other kinases, including cyclin-dependent kinase 2 (CDK2), protein kinase C, SRPK, serine-arginine protein kinase (SRPK) 1, and SRPK2, have previously been known to mediate phosphorylation on HBc.⁽³⁾ In this study, the investigators showed that mutation of three putative CDK2 phosphorylation sites (S155, S162, and S170) to alanine residues strikingly reduced phosphorylation of HBc by PLK1, implying that HBc may undergo hierarchical phosphorylation. How these sequential phosphorylations proceed is worthy of continued investigation. Interestingly, PLK1 is also involved in hepatitis C virus (HCV) RNA replication as well as virus production by hyperphosphorylation of viral nonstructural protein

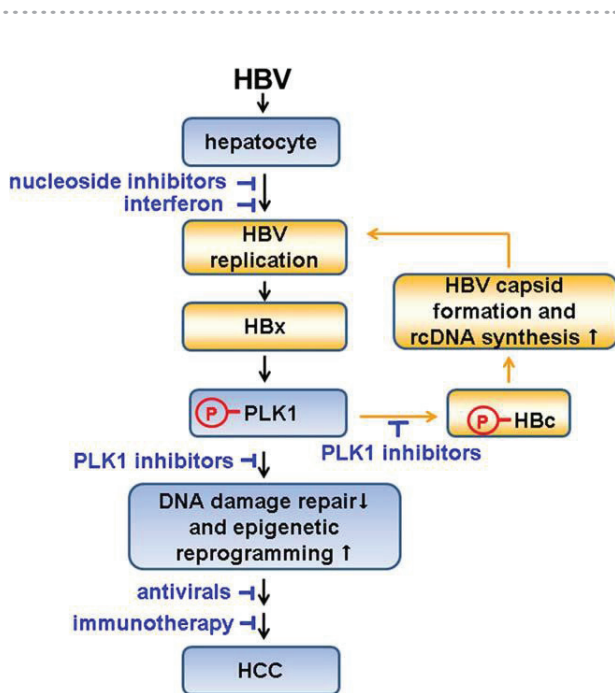


FIG. 1. The role of PLK1 in HBV-mediated HCC. Upon HBV infection, PLK1 is induced and activated by HBx. The phosphorylated PLK1, the active form of PLK1, then phosphorylates HBc to induce HBV replication by facilitating capsid formation and reverse transcription of pgRNA into rcDNA. In addition, the activated PLK1 allows propagation of DNA damage and epigenetic reprogramming, resulting in HBV hepatocarcinogenesis. PLK1 activity can be effectively abolished by PLK1 inhibitor. The boxes colored in blue and orange depict cellular events and viral events, respectively.

5A (NS5A).⁽⁵⁾ Intriguingly, like HBV core protein, HCV NS5A can be phosphorylated by multiple kinases to different degrees of phosphorylation, suggesting that phosphorylation of viral proteins are under exquisite control, with PLK1 providing the last piece of brick. The important roles of PLK1 in both HBV and HCV replication imply that HBV and HCV may share some common features in the mechanism of viral hepatocarcinogenesis.

Moreover, the investigators demonstrated that inhibition of PLK1 activity by PLK1-inhibitor BI-2536 or small interfering (si)RNA reduced the formation of HBV capsid and synthesis of rcDNA (relaxed circular DNA). The decreases in rcDNA synthesis may be caused by reduced pgRNA encapsidation, preferential encapsidation of spliced RNAs, and disruption of reverse transcription. Of note, selectivity in RNA encapsidation has been shown to be regulated by the optimum amount of negative charges from phosphoserines located at the CTD of HBc and encapsidated

nucleic acids.⁽⁶⁾ However, whether PLK1 plays a direct role in RNA encapsidation remains unknown. Further study to delineate the mechanism by which PLK1 affects rcDNA synthesis, for example, pgRNA encapsidation or reverse transcription, will strengthen the key role of PLK1 in regulating HBV replication.

Besides its role in the HBV life cycle, PLK1 is a key player in HBV carcinogenesis.⁽⁷⁾ PLK1 plays multiple roles in the cell-cycle and is essential for precisely regulating the cell division and maintaining genome stability in mitosis, spindle assembly, and DNA damage response. Homozygous loss of PLK1 resulted in early embryonic lethality.⁽⁸⁾ PLK1 expresses ubiquitously in eukaryotic cells and is highly expressed in most of human cancers, including HCC.⁽⁹⁾ PLK1 has been shown to participate in numerous cancer-associated pathways. The role of PLK1 in HBV-related carcinogenesis is focused here. It has been investigated that PLK1 can be activated by HBV X (HBx) protein to allow propagation of DNA damage by simultaneously attenuating the DNA damage checkpoint kinase 1 activity and destabilizing DNA repair protein Mre11.⁽⁷⁾ In addition, PLK1, together with HOX transcript antisense RNA, induces proteasomal degradation of two transcription repression factors, suppressor of zeste 12 homolog and zinc finger protein 198, to alter the chromatin landscape, leading to epigenetic reprogramming associated with oncogenic transformation.⁽¹⁰⁾ Combined with the current finding by Diab et al.,⁽²⁾ the HBV-infected hepatocyte appears to suffer a double whammy by PLK1 through its enhancing effect on HBV replication and its disruptive effects on cellular DNA repair, resulting in HBV-induced carcinogenesis.

The role of PLK1 in both HBV biology and carcinogenesis makes it an ideal therapeutic target for HBV infection and HBV-induced neoplastic transformation or HCC. PLK1 has been proposed as a therapeutic target for HCC. The recent study demonstrated that Huh-7 cells-transplanted nude mice showed significant tumor regression in siPLK1-treated mice as compared to control mice.⁽⁹⁾ Besides, normal human cells are largely unaffected by loss of PLK1, whereas PLK1 depletion promotes apoptosis in cancer cells. These preclinical studies suggest that PLK1 is both a feasible and relevant target for suppressing tumor cell growth. Several PLK1 inhibitors have been tested by clinical trial. However, these small-molecule inhibitors of PLK1 have not achieved a satisfactory therapeutic effect in clinical trials.⁽¹¹⁾ The monotherapy could be supplemented by combined therapy. The investigators

treated HBV-infected dHepaRG cells with the PLK1- specific inhibitor and approved or investigational anti- HBV drugs, and found that most of these combined treatments had additive or synergistic effects on reducing reverse transcription of pgRNA into rcDNA. It suggests the possibility of combined therapies to combat chronic hepatitis B and HBV/HCV-induced HCC by suppression of viral replication and enhancing the immune response with immunotherapeutic interventions. This study not only revealed the function of HBc phosphorylation, but also suggested a new thinking on HBV hepatocarcinogenesis. PLK1 could serve as a promising target for HBV/HCV-induced HCC.

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2.2. Second study: Antiviral activity of PLK1-targeting siRNA delivered by lipid nanoparticles in HBV-infected hepatocytes

Antiviral activity of PLK1-targeting siRNA delivered by lipid nanoparticles in HBV-infected hepatocytes

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Keywords:

Hepatitis B virus; Polo-like-kinase 1; small-interfering RNA, lipid nanoparticle; antiviral; host-targeting agent; hepatocellular carcinoma.

Paper in preparation

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ABSTRACT

A link between HBV and PLK1 has now been clearly evidenced in HBV-driven carcinogenesis, and we have also recently shown that PLK1 is a proviral factor in the early phases of HBV infection. Relative to the latter, we have shown that BI-2536, a chemical PLK1 inhibitor, was very efficient at inhibiting rcDNA neosynthesis, notably by impacting nucleocapsid assembly, *via* HBe phosphorylation regulation. The benefit of a PLK1 inhibitor could therefore be dual, as it could help inhibiting HBV replication, while preventing/modulating carcinogenesis. Yet, as kinase inhibitors often feature poor selectivity, there is a need for a more specific targeting of PLK1, whether this therapeutic strategy is to be developed in chronic HBV infection.

Here, we analysed in primary human hepatocytes, and in dHepaRG the anti-HBV properties of an LNP-encapsulated PLK1-targeting siRNA (related to TKM-080301, which had shown indications of efficacy in a phase-2 clinical trial NCT01262235 for neuroendocrine tumours and adrenocortical carcinoma). Standard assays were used to monitor the effect of LNP-siPLK1, or controls (LNP-siHBV and LNP-siNon-Targeting), on HBV replication and cell viability.

A dose as low as 100ng/mL LNP-siPLK1 resulted in a >75% decrease in HBV DNA release/viremia, which was comparable to that obtained with LNP-siHBV or 10 μ M of Tenofovir (TFV), without affecting the cell viability. Interestingly, and in contrast to that obtained with TFV, a strong inhibition of viral RNA and HBe/HBsAg secretions was also observed under LNP-siPLK1 treatment. This correlated with a significant intracellular decrease of vRNA accumulation, which was independent of any change in cccDNA levels, thus indicating a transcriptional or post-transcriptional modulation. Such an effect was not obtained with a biochemical approach of PLK1 inhibition, unveiling an enzymatic-

independent role of PLK1. The mechanism behind this phenotype is not fully deciphered, yet LNP-siPLK1 treatment leads to increased amount of vRNA in the nucleus compared to the cytosolic compartment, suggesting an involvement in HBV RNA metabolism.

These results warrant additional investigations in preclinical animal model to further determine the usefulness of such a therapeutic strategy.

INTRODUCTION

Hepatitis B virus (HBV) infection represents the main cause of hepatocellular carcinoma (HCC), which is the 2nd leading cause of cancer deaths worldwide (WHO data). An estimated 2 billion people have been exposed to HBV and 257 million remain currently chronically infected (CHB) (WHO data; global hepatitis report, 2017). If left untreated, these patients have a 100 fold increased risk of developing HCC compared to the general population [1]. When patients are successfully treated, using safe nucleos(t)ide analogs (NUCs), their risk of developing HCC is 2 to 5 times reduced compared to the normal risk [2, 3]. The reminiscent risk of HCC development under treatment is likely due to the fact that NUCs do not allow the elimination of an HBV replicative intermediate called cccDNA (i.e., covalently closed circular DNA), which is responsible for viral persistence and rebound when treatment is stopped [4, 5]. In addition to the development of drugs that may target cccDNA [4, 5], the discovery of host-targeting agents (HTA) acting both on HBV replication and HCC initiation and/or progression could increase the efficacy of the current standards of care (SoC) for curing patients from the infection and associated liver diseases.

We previously identified the serine/threonine polo-like-kinase 1 (PLK1) as such a candidate [6]. PLK1 is known to be overexpressed in many human cancers [7], and is thought to be an important driver of HBV-induced HCC [8-11]. Moreover, we found that PLK1 is a pro-HBV factor and small molecules inhibiting PLK1 (SM-PLK1i) (e.g., BI-2536) had a negative impact on HBV replication [6]. The inhibition of PLK1 could therefore be of interest for the treatment of CHB patients with potential effects on both virologic and oncologic sides of the disease.

Due to their poor specificity and overall low safety profile, SM-PLK1i cannot be used to treat CHB patients. Interestingly, small interfering RNAs directed against PLK1 were also shown to

be efficient at targeting PLK1 oncogenic activity [12]. Chemically modified and injectable versions of PLK1 siRNA were also validated in preclinical cancer animal models [13, 14], and one PLK1 siRNA encapsulated in proprietary lipo nanoparticles (LNPs) was clinically trialed in patients with advanced HCC (NCT02191878).

In this study, we found that the use of those LNP-siPLK1 in HBV-infected primary human hepatocytes (PHHs) lead to a decrease in the amount of intracellular HBV RNAs, that subsequently affect the amount of secreted viral particles and HBe/HBs antigens. Knowing that a loss of HBsAg in patients is a clinical end-point goal [3], our finding could have relevant therapeutic implications. Moreover, these data unraveled a novel PLK1 proviral function, which is independent of its kinase activity. Indeed besides being involved in HBV reverse transcription through the regulation of capsid assembly [6], our data suggest that PLK1 is also involved in the regulation of viral RNA accumulation.

MATERIALS and METHODS

Chemicals, antibodies, and others reagents

PLK1 inhibitor BI-2536 was purchased from Selleckchem. All other chemicals were purchased from Sigma-Aldrich. Rabbit polyclonal anti-HBc antibodies were purchased from Dako (B0586) or Abcam (ab115992) and monoclonal anti-HBc antibodies from Abcam (ab8637; Clone C1). Rabbit polyclonal anti-PLK1 antibodies from Abcam (ab21738) were used for detection of phospho-PLK1-S₁₃₇ ; rabbit monoclonal anti-PLK1 antibodies from Abcam (ab115095) were used for detection of phospho-PLK1-T₂₁₀ and rabbit polyclonal anti-PLK1 antibodies from Abcam (ab109777) for detection of total PLK1.

HepaRG and primary human hepatocyte cultures

Human liver progenitor HepaRG cells [15, 16] were cultured and differentiated (dHepaRG) as described previously [17]. Primary human hepatocytes (PHH) were freshly prepared from human liver resection obtained from the Centre Léon Bérard (Lyon) with French ministerial authorizations (AC 2013-1871, DC 2013 – 1870, AFNOR NF 96 900 sept 2011) as previously described [18].

Production of siRNA-containing liponanoparticles production and use in cell culture

LNP-siPLK1 corresponded to TKM-080301, as described in NCT02191878. Information on the equivalence between molarity of siPLK1 and amount of LNP-siPLK1 in nanogram/mL is given in Table 1. Controls LNP-siRNAs were manufactured the same way. The sequences of siRNA against HBV are Fw: 5'-CGACCUUGAGGCAUACUUCUU-3' and Rv: 5'-GAAGUAGCCUCAAGGUCGUU-3'. LNP-siRNAs were administrated to cells by direct dilution into the culture medium. Cells were exposed to LNPs for 24 hours, then washed with culture medium to remove excess of LNPs. Treatment were repeated as indicated in figure legends.

[siPLK1] nM	[LNP siPLK1] ng/mL
0,1	1,3
0,3	4
1	13,3
3	40
7,5	100
10	133,3
15	200
22,5	300
37,5	500

Corresponding concentrations between naked siPLK1 in nM and LNP siPLK1 in ng/mL

HBV infection and analysis of viral replication

Differentiated HepaRG cells (dHepaRG) and PHHs were infected with HBV genotype D (concentrated from HepAD38 cells supernatants) as described previously [19], [20]. After treatment procedures, levels of intracellular and/or extracellular HBV RNAs and DNAs, HBeAg, secretion of HBeAg and HBsAg were monitored by quantitative polymerase chain reaction (qPCR), reverse transcription qPCR (RT-qPCR), immunoblot and enzyme-linked immunosorbent assay (ELISA) respectively, as previously described [6, 19]. Briefly, HBeAg and HBsAg were quantified in culture medium using a chemiluminescence immunoassay kit (Autobio Diagnostics) according to the manufacturer's instructions. Total intracellular DNAs and RNAs as well as secreted viral DNAs/RNAs were purified from infected cells using NucleoSpin® 96 Tissue kit, NucleoSpin® 96 RNA kit, NucleoSpin® 96 virus kit (Macherey-Nagel) respectively, according to the manufacturer's instructions. Reverse-transcription of

RNA into cDNA was performed with the SuperScript® III First-Strand Synthesis kit (Invitrogen) according to the manufacturer's instructions. All qPCR analyses were performed with LightCycler™ 96 and 480 systems (Roche), except for cccDNA and pgRNA specific PCRs that were processed under a QuantStudio™ 7 (Applied), using homemade TaqMan assays [19].

Primary human hepatocytes preparation from chimeric FRG mice and use for antiviral

This study was approved by the WuXi IACUC (Institutional Animal Care and Use Committee). FRG™ (Fah^{-/-}/Rag2^{-/-}/Il2rg^{-/-}) mice were purchased from Yecuris (Tualatin, OR, USA), engrafted with PHH and infected with 1.10⁹ virus genome equivalent (vge) of HBV genotype D. When HBV viremia had reached a maximum (i.e., around 10e9 vge/mL), the liver was digested by *in situ* collagenase perfusion and recovered hepatocytes seeded in DMEM medium into 24-well plates (2.7.10⁵ cells/well). LNP treatments were conducted every 4 days starting at day 0 and BI-2536 treatment was started on day 0 and replenished every two days. DNA in the culture supernatants was isolated using QIAamp 96 DNA Blood Kit (Qiagen) and quantified by qPCR. HBsAg was quantified using specific ELISA kit (Autobio) according to the manual.

Separation of cytoplasm and nucleoplasm

Extractions of nuclear and cytosolic proteins were performed using NE-PER Nuclear and Cytoplasmic kit (Thermo Scientific), according to the manufacturer's instructions. Sodium orthovanadate, cOmplete™ EDTA-free protease inhibitor cocktail (Roche) and sodium fluoride were added freshly to all lysates. Nuclear and cytosolic RNAs were isolated as described in *Weil et al.*, [21], followed by a purification with TRI reagent (Merck) and precipitation with isopropanol. Purity of nuclear vs cytoplasmic extracts, was assessed by RT-qPCR using primers to amplify the nuclear U6 snRNA (Fw: 5'-CTCGCTTCGGCAGCACATATAC-3' / Rv: 5'-GGAACGCTTCACGAATTTGCGTG-3') and the cytoplasmic Human Tyr-tRNA (Fw: 5'-

CCTTCGATAGCTCAGCTGGTAGAGCGGAGG-3' / Rv: 5'-CGGAATTGAACCAGCGACCTAAGGATGTCC-3').

Capsid migration assay

The intracellular HBV nucleocapsids were analyzed by agarose electrophoresis in native condition followed by transfer onto an enhanced chemiluminescence membrane (Amersham) and western blot analysis, as described previously [19].

Southern Blotting

Total DNAs extracted with NucleoSpin® 96 Tissue kit (Macherey-Nagel) were pooled, precipitated using 5M NaAc and ethanol and analyzed by Southern blot as previously described [22]. DNAs from HepG2-NTCP cells extracted using a modified Hirt procedure were used as positive controls [23].

Statistical analysis

Statistical analysis was performed using two-way analysis of variance, t tests, or non-parametric Mann-Whitney tests using GraphPad Prism software, version 7.04. For all tests, $P \leq 0.05^*$, $P \leq 0.01^{**}$, and $P \leq 0.001^{***}$ were considered statistically significant.

RESULTS

Efficacy and safety of LNP siPLK1 in primary human hepatocytes

Compared to control LNP siRNAs, LNP siPLK1 was efficient at inhibiting PLK1 expression, with 70% loss of PLK1-mRNA at 13.3ng/mL without toxicity in proliferating HepG2 cells. Of note, LNP siPLK1 induced 50% mortality in proliferating HepG2 cells at 133ng/mL due to active replication and basal involvement of PLK1 in G2/M checkpoint (**Fig. Sup. 1**). Considering these data, as well as the concentration correspondence table (**Table1**) and our previous work done using naked siRNA targeting PLK1 [6], we tested the effect of LNP-siPLK1 and controls in HBV-infected primary human hepatocytes with a range of concentration from 100 to 500 ng/mL. We observed a strong reduction in the levels of PLK1 mRNA (**Fig. 1A**) and PLK1 phosphoprotein (**Fig. 1B**). As previously reported, LNP-siHBV led to a reduction of PLK1 mRNA to a level closer to that observed in uninfected cells, thus confirming that HBV increase PLK1 expression in PHH. Confirming also our previously published data obtained with a biochemical approach [6], the inhibition of PLK1 expression was not cytotoxic in PHH (3 different batches tested) even when LNP siPLK1 was administrated 3 times at 500ng/mL (**Fig. 1C and 1D**). Moreover PHH remained functional, as measured by the apolipoprotein B (ApoB) secretion (see **Fig. 2C**).

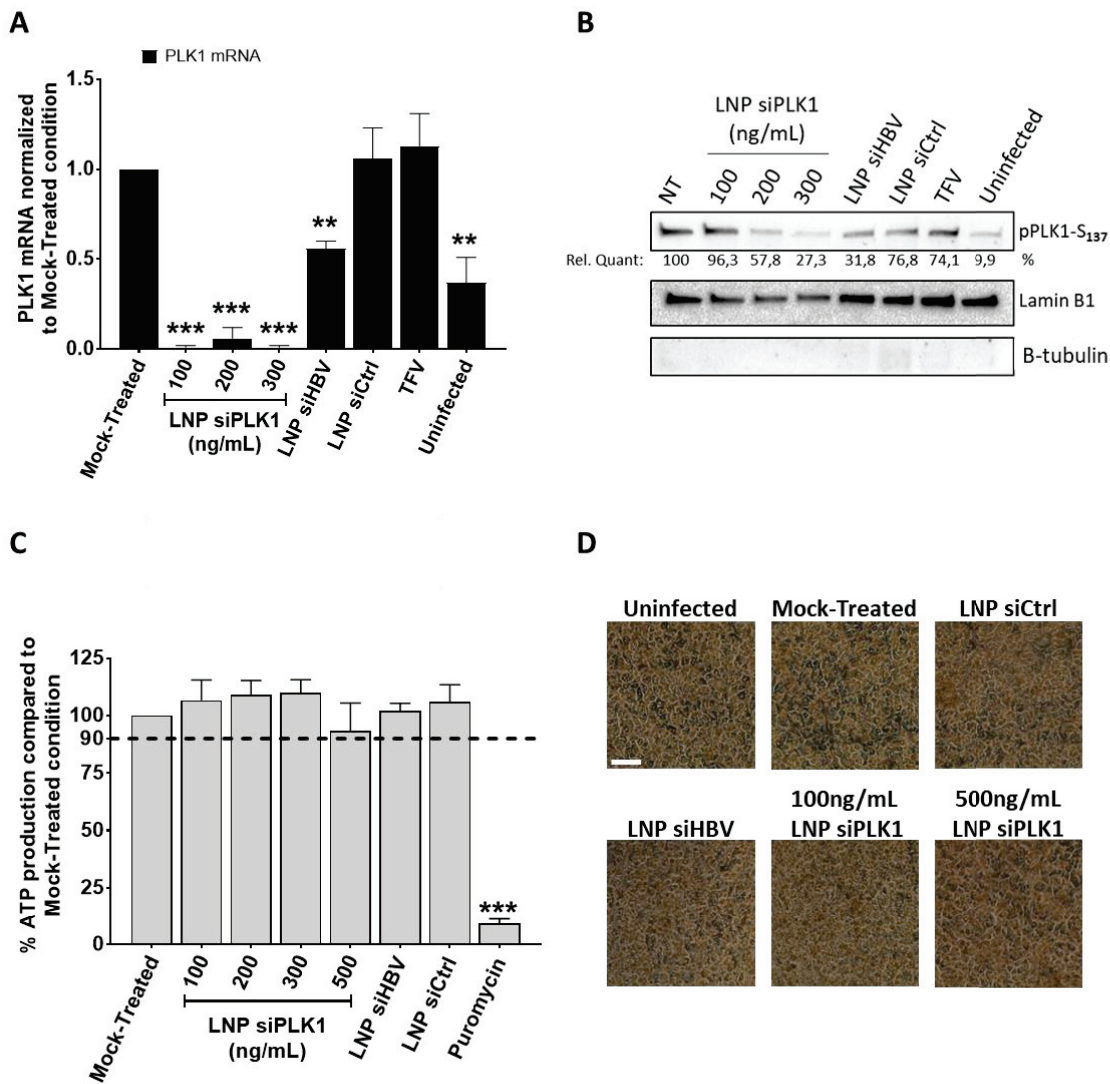
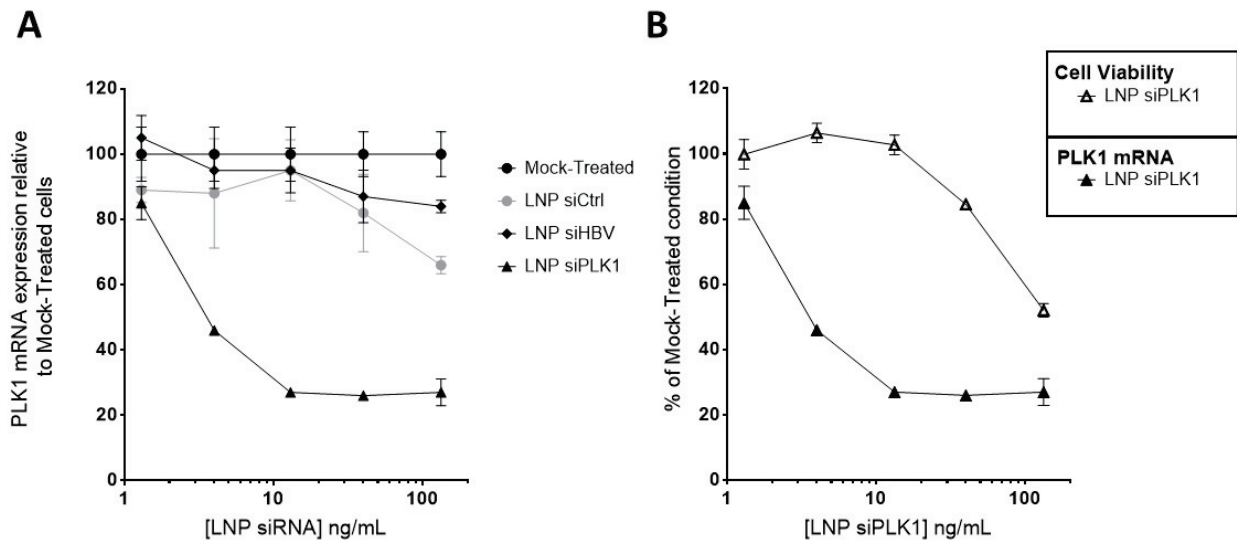


Figure 1. Evaluation of the efficacy and toxicity of LNP siPLK1 in primary human hepatocytes. PHHs were infected with HBV genotype D at a multiplicity of infection of 200vge/cell. From 4 days post-infection (p.i.), cells were treated every 3-4 days with increasing LNP siPLK1 concentrations or the indicated control molecules (LNP siHBV and LNP siCtrl) were used at 200ng/mL and 10µg/mL of puromycin served as positive death control, Tenofovir (TFV) was used at a concentration of 10µM). Cells and supernatants were harvested at day-13 p.i. for subsequent analyses. (A) RNAs were extracted and levels of PLK1 mRNAs (relative to PRNP mRNAs) analyzed by RT-qPCR. Results were normalized to the Mock-Treated condition. (B) Proteins were extracted from cytosol and nuclear compartments with NE-PER Nuclear and Cytoplasmic kit. Thirty µg of nuclear fraction for each condition were analyzed by western blot. Lamin B1 served as loading control for nuclear proteins while β-Tubulin served as control for cytoplasm contamination (a representative blot is shown). (C) ATP production, reflecting cell viability, was measured using Cell TiterGlo One Solution Assay. (D) Phase microscopy pictures of PHH at the end of the experiment acquired on an Olympus IX70 microscope, objective X40. Scale bar (white) corresponds to 500µM. Results presented in graphs are the mean +/- SEM (standard error of the mean) of 3 independent experiments (3 donors of PHH) each performed in biological triplicate. $P \leq 0.05^*$, $P \leq 0.01^{**}$, and $P \leq 0.001^{***}$ were considered statistically significant.



Supporting Figure 1. Efficacy of LNP siPLK1 and toxicity profile in HepG2 cells. (A) HepG2 cells were treated with increasing doses of LNP siPLK1, ranging from 1.3ng/mL to 133ng/mL. RNA were extracted, reverse transcribed and PLK1 mRNA quantified using specific qPCR. Normalized to housekeeping gene and relative to Mock-Treated condition. (B) Cell viability was assessed with CellTiter Blue reagent and plotted along with knockdown of PLK1 RNA under various LNP siPLK1 concentrations.

LNP siPLK1 strongly and specifically inhibits the secretion of HBV virions and antigens by infected primary human hepatocytes

To assay LNP siPLK1 antiviral activity, freshly isolated PHH from three different donors were infected with HBV, and at day-4 post-infection (p.i.), when the average HBsAg production reached around 1000 IU/mL, cells were treated three times every three days with increasing LNP siPLK1 doses or control molecules (**Fig. 2A**). Secretion of viral DNA (i.e., infectious virion) and RNA (i.e., RNA-containing particles; [24]) were significantly inhibited by LNP siPLK1 (**Fig. 2B**). The inhibition was comparable to that observed with LNP siHBV in this model. The lack of dose-dependency of the phenotype suggests that even a weak loss of PLK1 (**Fig. 1B**) is associated with a strong inhibition of virus progeny. Interestingly, we also observed a 70% inhibition of viral antigens HBeAg and HBsAg secretion (**Fig. 2C**), without affecting ApoB secretion, highlighting a specificity of LNP siPLK1 action on those secreted viral proteins. As expected, tenofovir (TFV) did not affect HBV antigens secretion and the inhibition of HBV antigens secretion was stronger with LNP siHBV, as expected due to the direct targeting of HBV RNAs. Such an inhibitory phenotype on viral antigens was not obtained with SM-PLK1i, as previously reported [6]; the decrease of PLK1 protein is more deleterious for the virus than kinase activity inhibition, thus suggesting an enzymatic-independent function of PLK1 towards HBV.

These results were confirmed in the HBV-infected dHepaRG cells with the same experimental setting (**Fig. Sup. 2**). Moreover, the inhibitory phenotype on virion and HBsAg secretion was also observed by *ex vivo* treatment with LNP siPLK1 of freshly isolated hepatocytes from HBV-infected liver-humanized FRG mice [25], which replicate HBV at high level (i.e., around 1.10^9 copies/mL at day-26 post-infection), mirroring the highly viremic « immune tolerant » phase in human. In this model, 300ng/mL of LNP siPLK1 led to a

decrease of 60% in HBV DNA and 65% in HBsAg, whereas treatment with LNP siHBV was very efficient (**Fig. Sup. 3**).

LNP siPLK1 induces a reduction of intracellular viral RNA accumulation without significant modification of cccDNA amount

To further describe the anti-HBV potential of LNP siPLK1 and get insights on the observed phenotype on secreted HBV antigens, intracellular viral parameters were also analyzed. We observed a dose dependent decrease of intracellular HBV DNAs and RNAs in HBV-infected PHH or dHepaRG cells treated with LNP siPLK1 (**Fig. 2D, Fig S2D**). The highest dose of LNP siPLK1 resulted in a decrease of 55% and 60% of HBV DNA and RNA respectively (**Fig. 2D, Fig S2D**). LNP siHBV treatment resulted in a stronger effect, with 80% and 90% loss, whereas only viral DNA amount decreased with TFV (around 60%) as expected. The lower inhibition of intracellular DNA amount for all molecules tested, as compared to what observed with secreted DNA is likely due to the detection of remaining input HBV DNA from HBV inoculum as discussed below. Interestingly and contrary to what we described with SM-PLK1i [6], we observed a decrease in the levels of HBV RNAs upon LNP siPLK1 treatment of HBV-infected hepatocytes without observing any significant changes in cccDNA amount (analyzed by qPCR or Southern Blot) (**Fig. 3**). These data suggest that the decrease of viral RNA observed with LNP siPLK1 is a consequence of a transcriptional and/or post-transcriptional event.

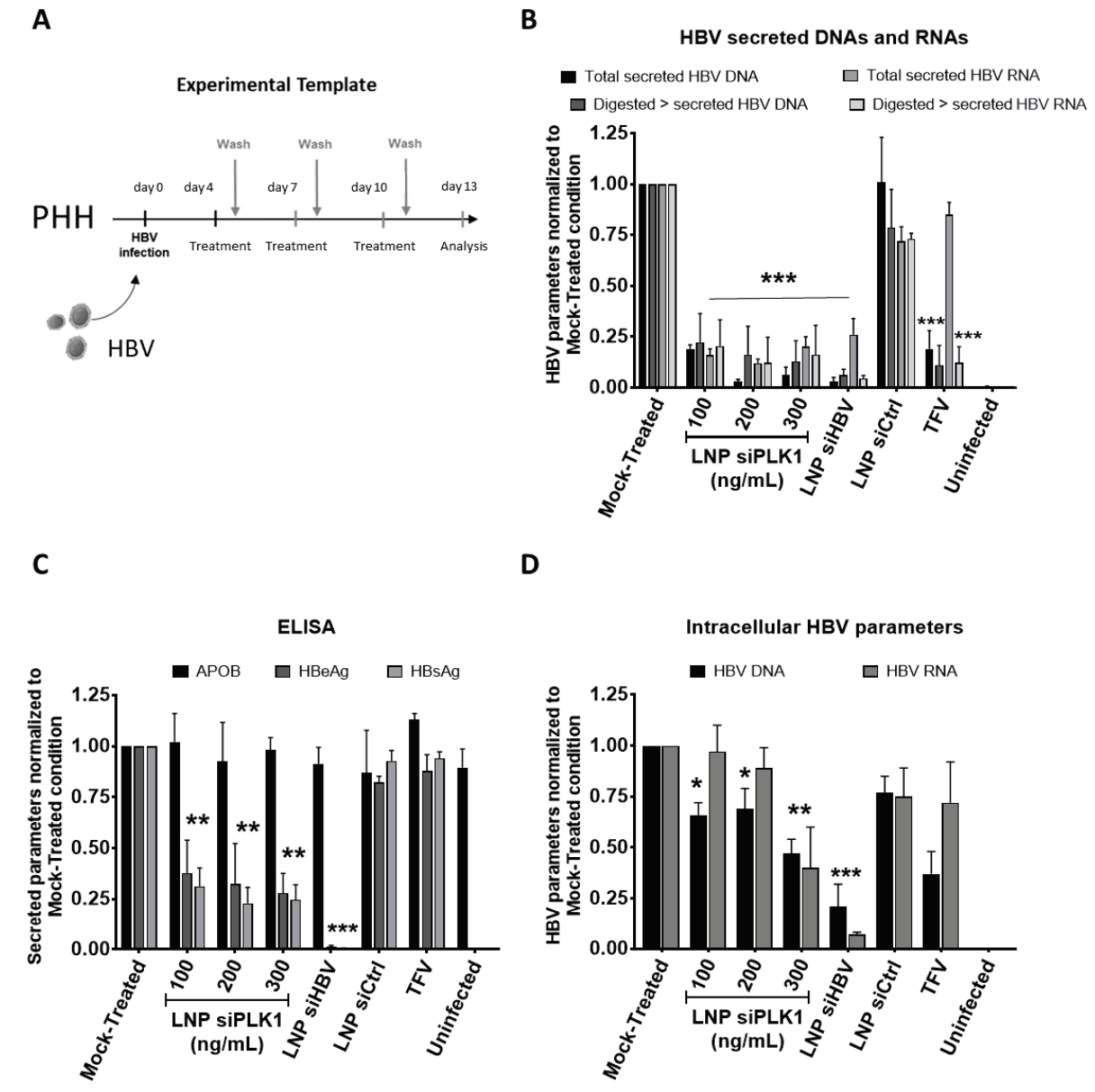
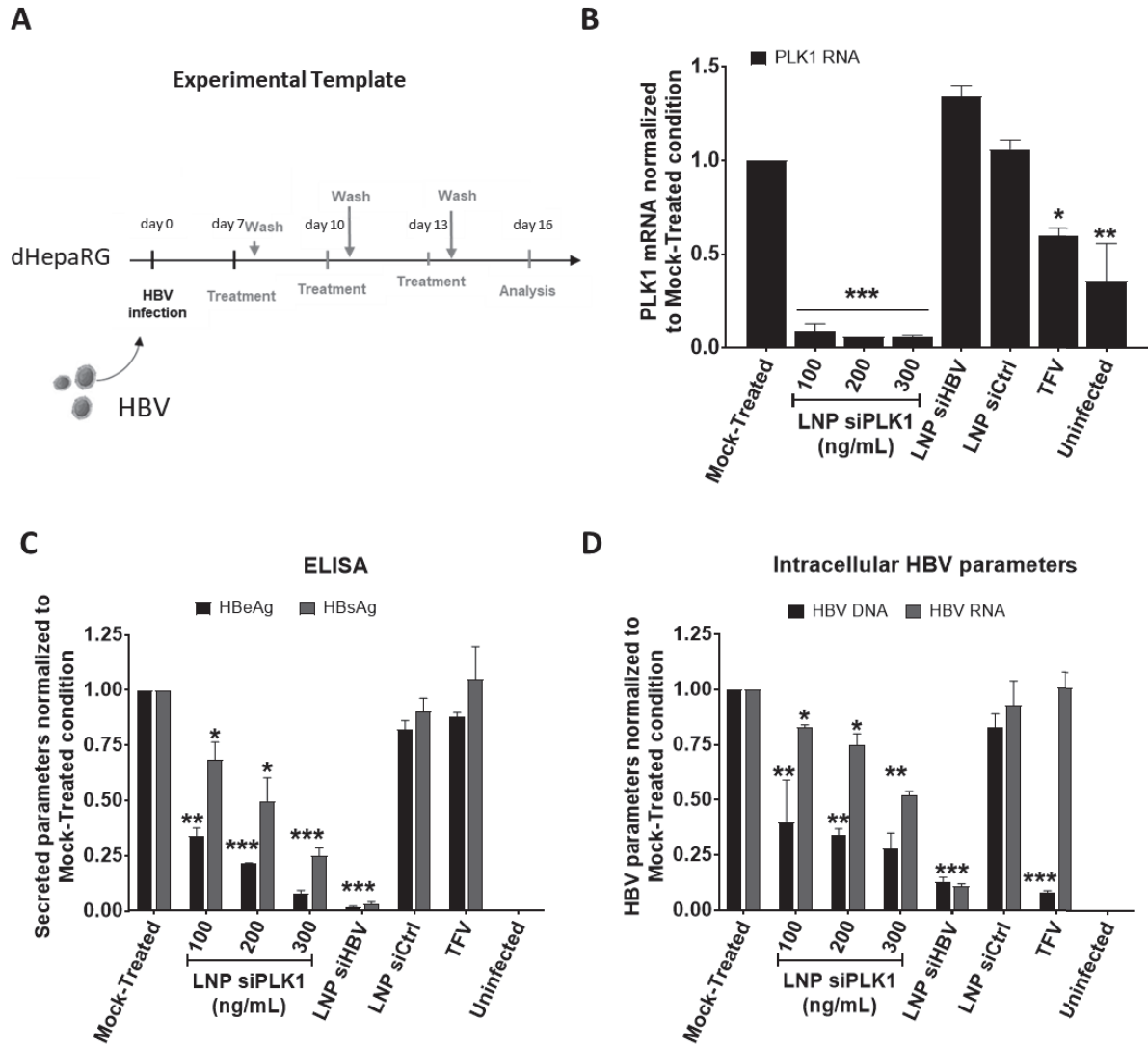
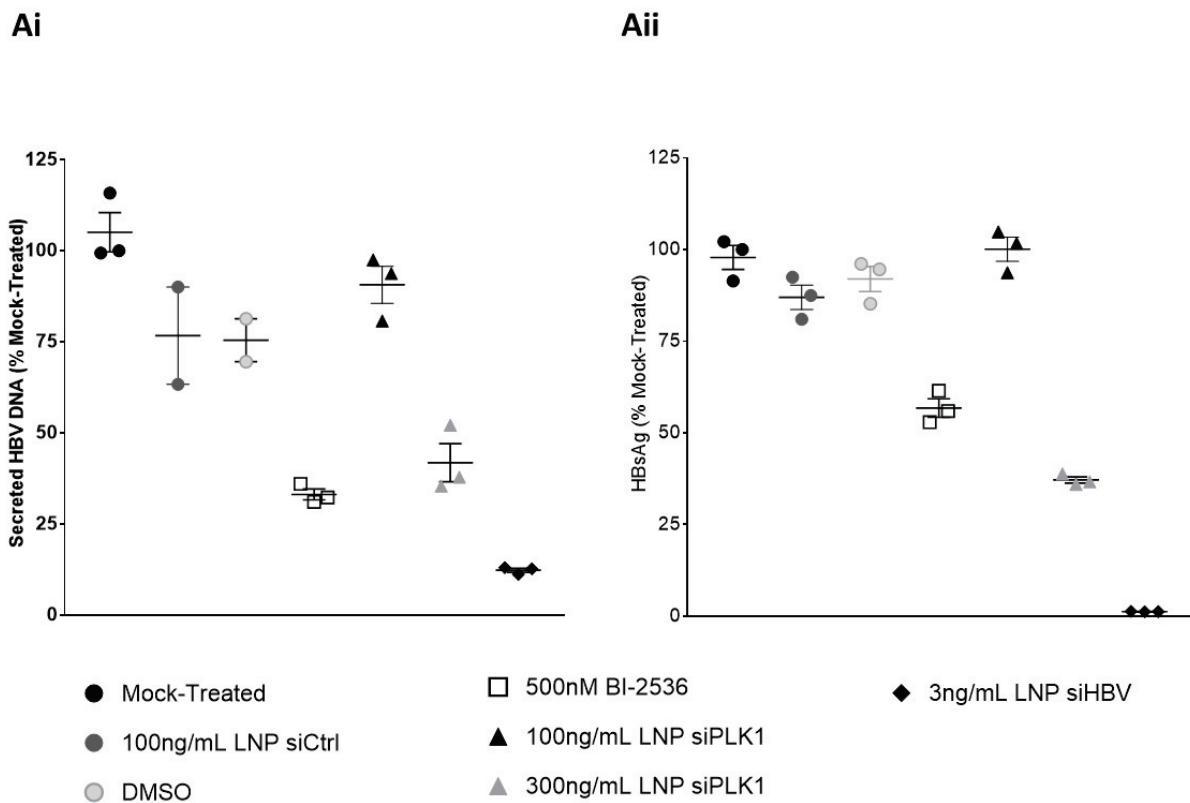


Figure 2. Antiviral activity of LNP siPLK1 in non-transformed and non-dividing HBV-infected PHH. PHH were infected with HBV genotype D at a multiplicity of infection of 200vge/cell and treated with the indicated molecules as shown (A) on the schematic representation of the experiment. LNP siHBV and LNP siCtrl were used at 200ng/mL and TFV at 10 μ M. At the end of the experiment, (B) RNAs and DNAs from the cell supernatants were extracted, digested or not with DNaseI or RNaseH and analyzed by HBV-specific RT-qPCR or qPCR. (C) Levels of secreted ApoB, HBeAg and HBsAg were assessed by ELISA. (D) Intracellular total RNAs and DNAs were extracted and analyzed by HBV-specific RT-qPCR or qPCR. Results presented in graphs are the mean \pm SEM of 3 independent experiments (3 donors of PHH) each performed in biological triplicate and are presented as ratio compared to the Mock-Treated condition. $P \leq 0.05^*$, $P \leq 0.01^{**}$, and $P \leq 0.001^{***}$ were considered statistically significant.



Supporting Figure 2. Antiviral activity of LNP siPLK1 in non-transformed and non-dividing dHepaRG cells. The data were generated the same way as in Figure 2.



Supporting Figure 3. Antiviral activity of LNP siPLK1. PHH from liver-humanized mice. Mice were perfused for 34 days to obtain PHH and were infected with 1.10^9 HBV genotype D. Liver was digested by *in situ* perfusion and PHH seeded into 24-well plates. LNP treatments were conducted every 4 days starting at Day 0 and BI-2536 treatment was started on Day 0 and replenished every tow days. Prior to treatments, the culture supernatants were collected and analysed for secreted HBV DNA and HBsAg levels. Secreted HBV DNA at D16 (Ai) and HBsAg at D16 (Aii).

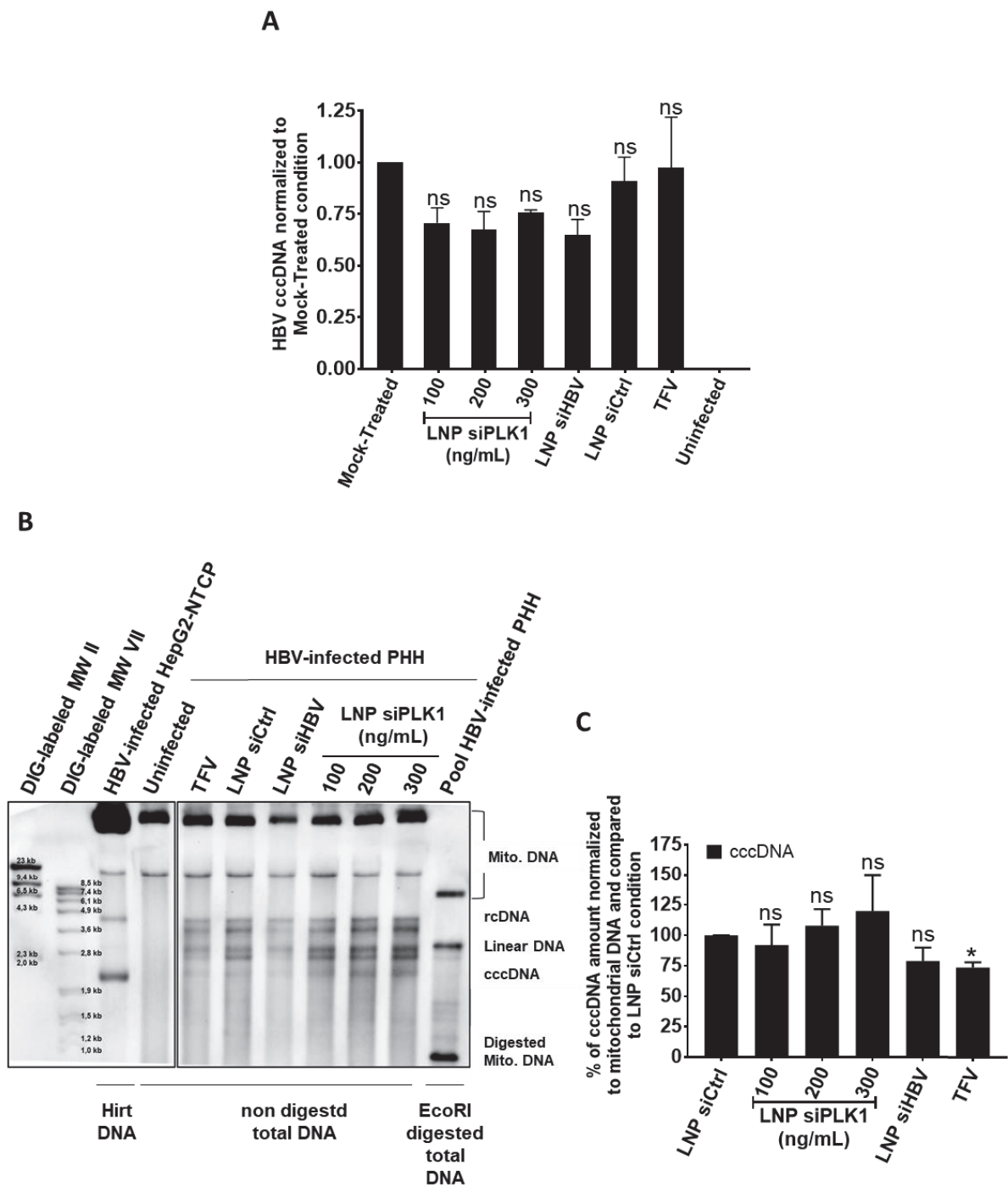


Figure 3. LNP siPLK1 does not affect cccDNA amount. (A) Intracellular cccDNA specific Taqman qPCR [19] from PHH samples from Fig. 2, relative to Mock-Treated. (B) Representative Southern blot of pooled $n=3$ total DNA from Fig. 2. Two micrograms of DNA was loaded by condition and processed as described [22]. EcoRI digestion serves as control as it allows cccDNA linearization of HBV genotype D (shift from coiled 2.2kb band to full length 3.2kb linear DNA). (C) Quantification of Southern blot presented in panel B using image Lab software. Mitochondrial DNA was used as loading control and served for normalization. $P \leq 0.05^*$, $P \leq 0.01^{**}$, and $P \leq 0.001^{***}$ were considered statistically significant.

LNP siPLK1 does not inhibit better HBV replication in pre-infection treatment setting

To determine if PLK1 affects HBV infection establishment, we treated PHH twice with LNP siPLK1 or control molecules, prior to HBV infection, as indicated in **Fig. 4A**. A third treatment was done at the time of HBV inoculation in order to maintain PLK1 protein level low from HBV entry to cccDNA formation. Analyses were done at 5d.p.i. PLK1 mRNA knockdown was still >75% with 100 and 300ng/mL of LNP siPLK1 (**Fig. 4B**). As seen before LNP siHBV allowed a PLK1 mRNA decrease, in accordance to the level found in uninfected cells. Interestingly a similar phenotype was observed with PreS1 peptide, which very efficiently prevented HBV infection in this setting (as shown in other panels of the **Fig. 4**). Regarding antiviral phenotypes obtained with LNP siPLK1, they were very similar to that obtained in post-infection treatment setting (compare results of **Fig. 2 and 4**). Importantly, cccDNA establishment was not blocked by either LNP siPLK1 or LNP siHBV, as compared to that observed with PreS1 peptide, which acts a genuine entry inhibitor. The observed trend of a decrease in cccDNA levels observed with LNP siPLK1 and LNP siHBV was not significant compared to controls (LNP si Ctrl and TFV), and could therefore not be taken into account in the other inhibitory phenotypes observed. It is worth mentioning here that PreS1 peptide did not lead to an absence of detection of intracellular HBV DNA in this setting, as compared to other parameters (cccDNA, intracellular RNA, HBeAg, HBsAg), thus confirming that residual HBV DNA from inoculation perturbs analyses as discussed earlier. This is the reason why results obtained from supernatants analyses are more trustful, and without ambiguity demonstrate the overall strong effect of LNP siPLK1 on HBV replication.

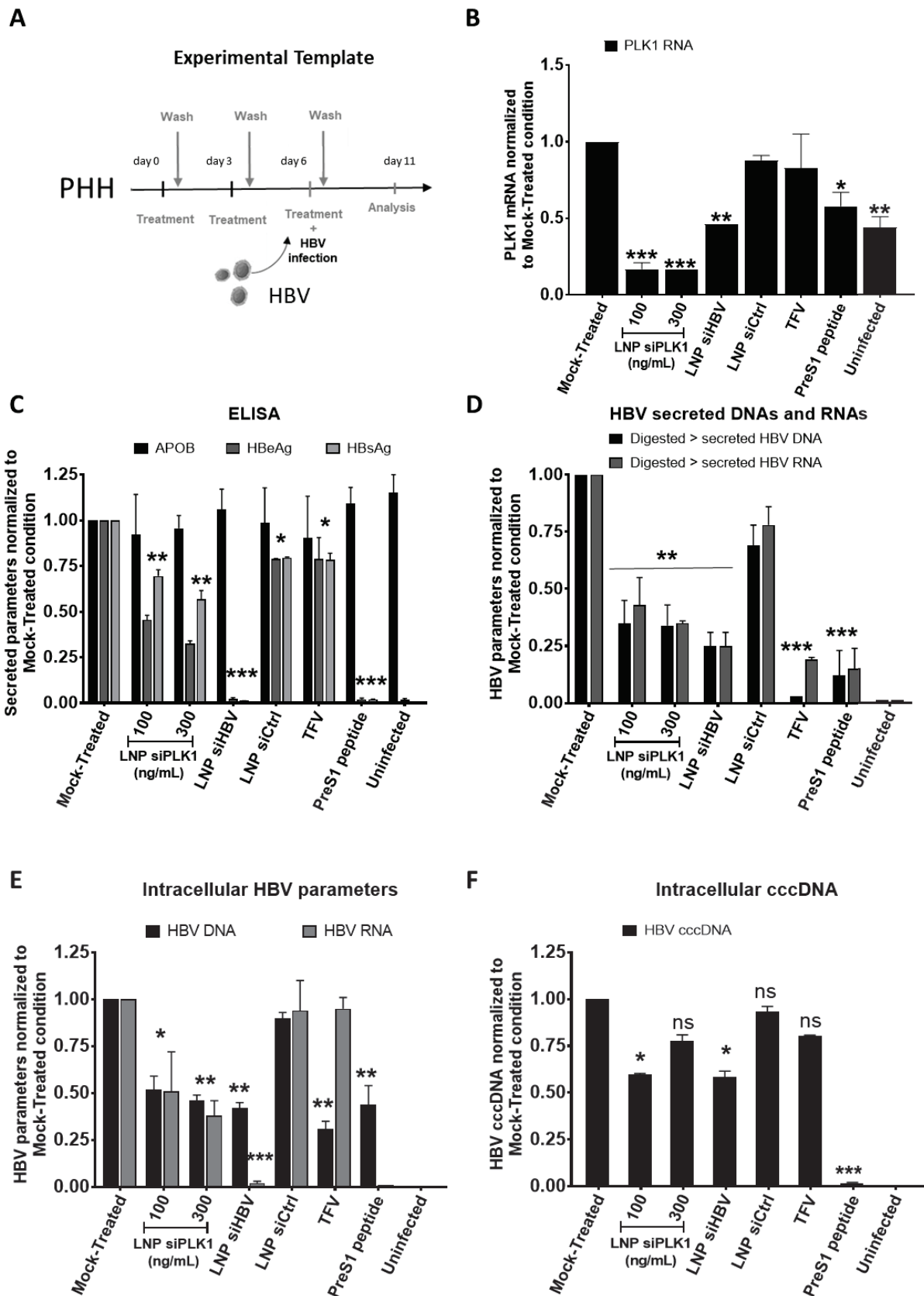


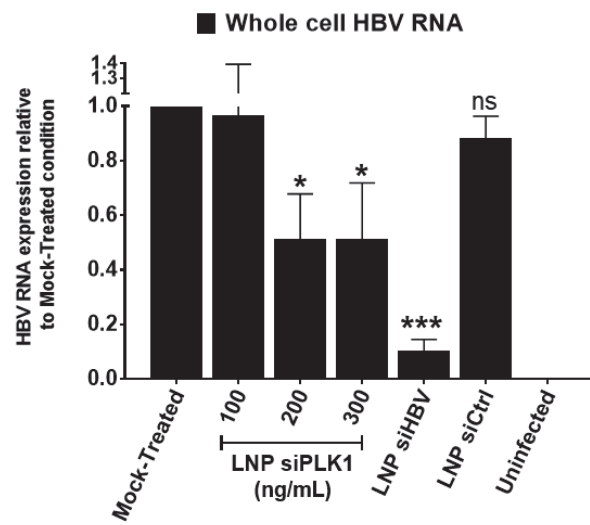
Figure 4. Pre-infection treatment with LNP siPLK1 in primary human hepatocytes. Experiments conducted on three independent PHH batches. LNP siHBV and LNP siCtrl were used at 200ng/mL and TFV at 10μM. PreS1 peptide at 100nM served as genuine entry inhibitor. Results presented in graphs are the mean of 3 independent experiments with SEM. (A) Experimental design of the study. (B) RNA were extracted, reverse-transcribed and

subjected to specific PLK1 qPCR. Normalization was done on PrP and quantification is relative to PLK1 expression in Mock-Treated condition. (C) Quantification of secreted ApoB, HBeAg and HBsAg by ELISA using supernatants of HBV infected PHH, relative to Mock-Treated. (D) RNA and DNA from the supernatant were extracted, followed by a digestion step, were analyzed by HBV-specific RT-qPCR or qPCR, normalized to housekeeping genes, and compared to Mock-Treated cells. (E) Intracellular total RNA and DNA extracted from HBV infected cells were analyzed by HBV-specific RT-qPCR or qPCR, normalized to housekeeping genes, and compared to Mock-Treated. (D) Intracellular cccDNA specific Taqman qPCR [19]. $P \leq 0.05^*$, $P \leq 0.01^{**}$, and $P \leq 0.001^{***}$ were considered statistically significant.

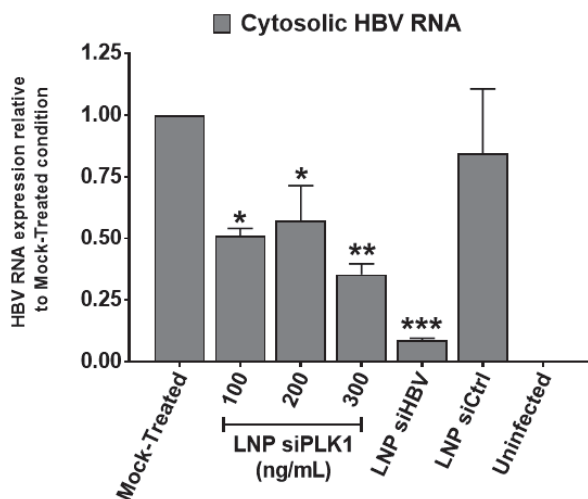
PLK1 knockdown results in a partial nuclear vRNA sequestration in HBV-infected PHH

In HBV-infected PHH, activated pPLK1-S₁₃₇ was mainly localized in the nucleus (**Fig. 1D** and [6]). As we observed a decrease in HBV RNAs in absence of change in the level of cccDNA under LNP siPLK1 treatment, we wondered if PLK1 could be involved in the modulation of HBV RNA levels. To address this, we infected PHHs, treated them as above and harvested cells three days after the last treatment (**Fig. 2A**). We performed a cytosolic/nuclear fractionation in order to quantify HBV RNAs in both compartments. RNA purity of our extracts was checked using nuclear-specific U6 snRNA and cytosolic-specific Tyr-tRNA. LNP siHBV resulted in a decrease of HBV RNAs levels in both compartments, whereas LNP siPLK1 led to a dual phenotype, depending on subcellular compartments. Indeed, in the cytosol, a 50% decrease of HBV RNAs levels was observed, similar to what was observed with whole cell extracts (**Fig. 5A and B**). By contrast, in the nuclear compartment, a dose-dependent accumulation of HBV RNAs was observed under LNP siPLK1 treatment (**Fig. 5C**). As the amount of cytosolic HBV RNAs was overall 10 times higher than that observed in the nucleus, this accumulation of HBV RNAs in the nucleus induced by LNP siPLK1 did not modify the overall observed phenotype (i.e., reduction of total RNA accumulation), but certainly accounted for the decrease in viral protein synthesis.

A



Bi



Bii

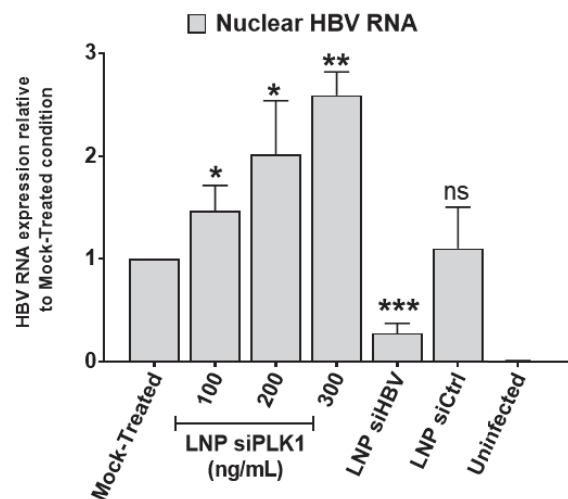
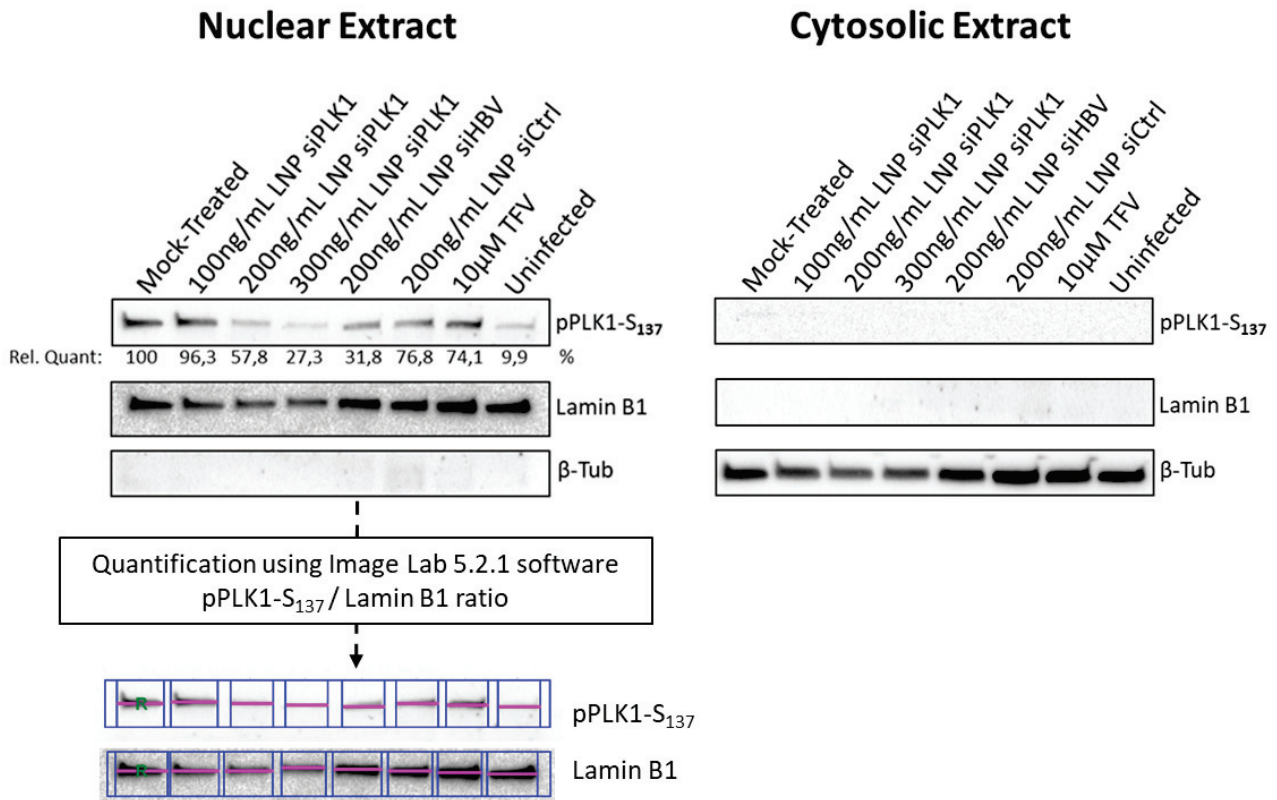


Figure 5. Localization of HBV RNAs under LNP siPLK1 treatment. (A) Total RNA were extracted, reverse transcribed and submitted to HBV specific qPCR. Normalized on housekeeping gene and relative to Mock-Treated condition. (B) Cytosolic and nuclear compartments were recovered using a homemade protocol adapted from *Weil et al.*, [21]. RNA were extracted using trizol and reverse transcribed. Home-designed primers were used to amplify the nuclear specific U6 snRNA and the Human Tyr-tRNA for the cytoplasm compartment and served as purity control as well as housekeeping genes. $P \leq 0.05^*$, $P \leq 0.01^{**}$, and $P \leq 0.001^{***}$ were considered statistically significant.



Supporting Figure 4. pPLK1 is preferentially found in the nucleus of infected PHH. Western Blot of nuclear extracts shown in Figure 1D with above the quantification method used with Image Lab software. The pPLK1-S₁₃₇ / Lamin B1 ratio allows correction of uneven overall protein quantity between each condition. Quantifications are presented relative to Mock-Treated condition, as indicated by the green R. The right panel shows western blot of corresponding cytosolic extracts, 30µg prot./lane. Lamin B1 and β-Tub served as purity control of cyto/nucleus fractionation.

DISCUSSION

Hepatocellular carcinoma ranks 2nd in term of cancer deaths worldwide and is responsible of an estimated 600 000 deaths/year. HCC development is carried out by the interaction of genetic predisposition, environmental factors as well as oncogenic viruses; the latter are involved in 80% of HCC and HBV for itself accounts for 50%. As 257 million people are chronically infected with HBV and only a minority of them are effectively treated, the HBV-mediated HCC burden is far from being eradicated. If a better diagnostic of infected patients and more universally use of antiviral therapies are needed, it is also crucial to identify novel molecules that would both target viral replication and HCC occurrence.

Our previous study demonstrated that PLK1 is activated upon HBV infection and acts as a proviral factor. Consequently, we showed that its inhibition by a SM-PLK1i leads to an inhibition of HBV replication. In addition, the group of Dr O. Andrisani have shown in several studies that PLK1, that is induced by HBx, is a driver of murine hepatocytes transformation *in vitro* and that its inhibition with SM-PLK1i can prevent it [11, 26]. Moreover PLK1 being a validated target in oncology, research efforts to identify potent SM-PLK1i have been important as reviewed recently [27]. The possibility to use siRNA to specifically target kinases involved in cancer has finally opened novel perspectives of use. In this line, the clinical trial (NCT02191878) performed with LNP siPLK1 in subjects with advanced HCC was instrumental and led us to consider LNP siPLK1 as a good candidate for early inhibition of HBV replication.

Here we confirmed that PLK1 is crucial for HBV replication in non-dividing hepatocytes by using of these PLK1-targeting-siRNA encapsulated in lipid nanoparticles (Arbutus Bioparma proprietary composition). In particular, we demonstrated that LNP siPLK1 inhibits more efficiently HBV replication than naked siRNA or SM-PLK1i (reported in [6]) in the absence of

cell toxicity. Indeed 100 to 300ng/mL of LNP siPLK1 (corresponding to 7.5 to 22.5nM siPLK1) led to a >75% decrease in HBV viremia *in vitro*. Moreover, in contrast to BI-2536, the SM-PLK1i that was previously profiled in depth *in vitro* and in liver-humanized FRG mice [6], we found that LNP siPLK1 was also capable to potently inhibit the secretion of HBV antigens in the supernatant of treated cells. This indirectly indicates that a kinase-independent function of PLK1 could account for production and secretion of HBV antigens. To this extent, it was precedently shown by Vitour and colleagues that the polo-box domain of PLK1 could bind to the mitochondria-bound innate immunity adapter MAVS and inhibit its ability to activate the IRF3 and NF-kappaB pathways, without any requirement of the kinase activity [28]. Such an unexpected phenotype on HBV antigens production is particularly relevant, as the next step in the improvement of current HBV therapy in clinic is precisely to be capable to reduced antigenemia in patients to potentially restore immune responses and control infection [3]. SiRNA targeting HBV itself are currently clinically evaluated in this respect (e.g., Phase-2 for ARB-001467; NCT02631096). The co-administration of siRNAs targeting mainly HBV and PLK1, could be an original way to further improve such a therapeutic approach.

The LNP siPLK1-induced inhibition of HBeAg and HBsAg in supernatant correlated with a reduced accumulation of intracellular HBV RNAs in LNP siPLK1 treated cells, in the absence of any change in cccDNA amount. This observation points out toward a role of PLK1 in transcriptional and/or post-transcriptional regulation. In this study, we limited our analysis to the impact of LNP siPLK1 on the nuclear versus cytoplasmic fractionation, and found that HBV RNAs tended to accumulate in the nucleus under LNP siPLK1 treatment, while the total amount of RNA was decreased. Although the precise underlying mechanistic of this transcriptional and/or post-transcriptional regulation (i.e., cccDNA activity, vRNA trafficking and/or fate) is yet to be defined, it is tempting to speculate that the previously described interplay between PLK1 and Hbc could also be at work. Indeed, HBV core protein is known to

interact with cccDNA [29] and potentially in the regulation of transcription [30]. Moreover, it has been suggested that the HBc CTD could be determinant for HBc-cccDNA interaction [30] and in our precedent study, we have identified 3 PLK1-dependent phosphorylation sites in HBc CTD. Finally, HBc is also known to be an RNA-binding protein physically interacting with vRNAs. Taken together, this provided a nice hypothetical landscape for further mechanistic studies to document LNP siPLK1 mode of action (MoA).

Of course, it would also be important to assay the anti-HBV efficacy and MoA of LNP siPLK1 in a liver humanized mouse model to confirm its potential therapeutic interest on the virology side. Regarding the possibility to test the relevance of using LNP siPLK1 as a double bullet compound, which would be able to both contribute to HBV replication inhibition in “add on therapy” with SoC and prevention of HBV-driven carcinogenesis, we would need a preclinical model able to recapitulate the entire pathogenesis process. A mouse model based on immune-competent mice transduced with AAV-HBV vectors could be of interest, as it features a persistent viral replication [31], in the context of an HBV immune tolerance [32], and was also reported to support HCC development [33]. Alternatively other transgenic mouse models featuring the over expression of HBV proteins, HCC development and a demonstrated implication of PLK1 in carcinogenesis could be used to this end. Recently, it was shown that genomic integrity of hepatocytes was disrupted by viral LHBS and that PLK1 acted as a major modulator in LHBS-mediated G2/M checkpoint override. In this model, SM-PLK1i resulted in a G2/M checkpoint restoration and suppression of tumorigenesis [34].

To summarize, in this study we have reported a potent antiviral activity of LNP siPLK1 in HBV-infected PHH, which includes a strong loss of intracellular vRNA accumulation, leading to reduction of HBV antigens secretion. This stands as proof of concept that PLK1 plays other proviral roles in HBV life-cycle that extend beyond its already described involvement in capsid

assembly [6] and further validate its potential interest for the anti-HBV arsenal. Moreover as it is now rather well accepted that PLK1 is an important HCC driver, its inhibition could result in a combined effect on the virus and viro-induced carcinogenesis and this warrants further preclinical investigation.

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Chapter 3: Discussion and Outlooks

An estimated 15% of cancers worldwide are attributed to viruses. Among them, HBV is a well-known hepatocellular carcinoma-inducer and contributes to 800,000 deaths per year with 40% related to cancers (Lozano et al., 2012). Despite the existence of a prophylactic vaccine and relatively efficient nucleotide analogs, there are still 250 million people being chronically infected by HBV, with a high risk of progression to end-stage liver diseases. Regarding the management of non-resectable HCC, there are only two available targeted options, allowing a survival gain of few months only, highlighting the urgent need of efficient drugs development. Checkpoint inhibitors, which are very efficient for other cancer, are yet to be tested in the case of HCC.

As viral infections and oncogenesis often deregulate the same cellular pathways, aiming to enhance cell division and escape from immune system, the community has gained interest in key actors of these pathways. In this respect, PLK1, regarding its global involvement in the cell-cycle regulation, was extensively studied and proposed as a promising target for drugs development in the oncology field. Focusing on HBV-related HCC, several groups have shown an overexpression of PLK1, linked with bad prognosis for patients with cancer. Moreover, PLK1 was shown to be activated by HBx, a potent HBV transactivator, but the mechanism of PLK1 activation early on after HBV infection need still to be determined (Studach et al., 2009b). However, “the chicken and egg” paradigm is still relevant as we do not know yet if PLK1 acts as a key driver of the carcinogenesis or if the overexpression of PLK1 is a result of the high proliferative rate observed in tumours (Cholewa et al., 2013). At last, no one focused on the possible involvement of PLK1 in HBV life cycle early-on after infection in untransformed and quiescent hepatocytes.

Eventually, we have shown the efficacy of targeting PLK1 *in vitro* and in preclinical mouse model to inhibit HBV infection but the exact mode of action of PLK1 is still elusive and seems to be plurimodal, with an involvement of this kinase at several key steps of the HBV life cycle. In this discussion, all these points are addressed in order to delineate a big picture serving as a clue to answer the question:

Is there a place for PLK1 inhibitors to reinforce the anti-HBV arsenal, and can we hope for a bimodal mode of action by acting also on the prevention of neoplastic events occurrence?

How PLK1 is activated upon HBV infection in quiescent and differentiated cells?

The group of Dr Andrisani has shown that PLK1 was activated in 4pX-1 cells expressing the HBx protein after release from double thymidine block. However, this phenomenon occurs after 40 cell generations and seems to be indirectly induced by the polyploidy and is of importance regarding oncogenesis process but may have limited impact on early HBV infection and replication (Studach et al., 2009b). In 2010, using the same model, they demonstrated that HBx mediates activation of PLK1 in the G2 phase of untransformed hepatocytes through the activation of the p38 MAPK pathway (Studach et al., 2010). However, these cells, albeit being untransformed are still undergoing cell division and HBx could be just another cue responsible for PLK1 activation and not playing the driving trigger in this process.

The HepAD38 cell line, which was derived from HepG2 stably transformed with an HBV transgene, is capable to replicate HBV in a tet-off inducible manner. In this model, PLK1 activation, witnessed by the induction of pPLK1_{T210} in immunofluorescence experiments, was found early-on after HBV replication (Zhang et al., 2015). However, although this model recapitulates some steps of HBV life cycle (except the entry steps), cells are not quiescents and were synchronized in G2/M using nocodazole, a cell-cycle stage where PLK1 is described to be already present. Once again one could not exclude that PLK1 was preactivated, possibly being phosphorylated on its Ser137 (*confer* section “**3.3.2. PLK1 activation: a multi-step and dynamic process**”), and HBV in this context might not represent the main inducer of PLK1 activation.

In our hand, using quiescent and untransformed models (e.g., dHepaRG and PHH), where the basal level of PLK1 was neither detectable by western blot nor by immunofluorescence, we observed this early activation of PLK1 upon HBV infection. Indeed, as early as 24h post-infection, we observed a peak in PLK1 mRNA, consistent with an increased amount of pPLK1_{S137} at D3 and a fully activated pPLK1_{T210} at D7 post-infection (*confer* “**A. First study: Polo-like-kinase 1 is a proviral host factor for hepatitis B virus replication**”). This means that HBV could represent a *bona fide* trigger of PLK1 activation, yet the underlying mechanism remained elusive. Indeed, HBx still represents the best candidate as it is the first HBV protein being expressed upon infection and may help transactivating PLK1 expression directly. Alternatively, HBx could act by indirectly, by regulating transcriptions factors found to be associated with the PLK1 promoter, such as STAT5 and/or E4BP4, which were described to interplays with HBx (Lee et al., 2006; Qiao et al., 2013). In our hand, the use of a HBx-deficient virus led to a short delay in the PLK1 activation but was not statistically significant (data not shown). However experimental issues due to viral production did not allow to firmly conclude on the involvement of HBx in PLK1 activation early-on after HBV infection.

This induction being observed quickly after the infection, another hypothesis regarding PLK1 activation resides in the incoming viral material from the HBV inoculum. Indeed as PLK1 and HBc are able to interact (*confer* “**First study: Polo-like-kinase 1 is a proviral host factor for hepatitis B virus**”

replication”), one could suggest that the core protein may sequester PLK1, rendering its activation by Aurora A/Bora impossible due to steric hindrance. Finally it would be worth to address the status of protein known to inhibit PLK1 such as Map205, stabilizing the autoinhibited state of PLK1, or BubR1, a protein shown to physically interact with PLK1 and inhibiting its phosphorylation during interphasic quiescent cells, under HBV infection (Izumi et al., 2009). Moreover, HBx was shown to bind BubR1, thereby deregulating the mitotic checkpoint and enhancing the accumulation of chromosomal instability in HBV-associated hepatocarcinogenesis (Kim et al., 2008). Thus, it is reasonable to speculate that HBx could bind to BubR1 early-on after HBV infection, resulting in a release of BubR1-dependent inhibition of PLK1.

All these described mechanisms could either act in a standalone manner or more possibly act together in order to activate PLK1 in a short time after HBV infection of quiescent hepatocytes. Furthermore, some of them could last long enough to make a bridge between activation of PLK1, which is mandatory for HBV replication, and high level of PLK1 found in HCC.

However, as shown in the section **“Second study: Antiviral activity of PLK1-targeting siRNA delivered by lipid nanoparticles in HBV-infected hepatocytes”**, a knockdown of PLK1 before HBV infection does not lead to a defect in HBV infection establishment, suggesting a minor role of PLK1 in steps from HBV entry to cccDNA formation. Thus the early activation of PLK1 upon infection could be induced by HBV in order to support subsequent steps of the viral life cycle (from cccDNA transcription to rcDNA-containing capsids formation).

Finally, the overexpression of PLK1 at the early steps of HBV infection could be the result of a transient NF- κ B activation via the TLR4/MyD88/I κ B α axis. Indeed PLK1 was associated with the downregulation of TLR2/TLR4-induced inflammation which is known to be activated by HBsAg (Jing et al., 2018). The initial inoculum we used contains up to 10000 fold more subviral particles than infectious virions and thereby could explain such activation. As HBV is described to be a stealth virus or more possibly is able to shut down very quickly the innate immune response, the activation of PLK1 through this axis could in part explain this phenomenon by attenuating the NF κ B pathway via direct inhibition of the IKK complex (Higashimoto et al., 2008). In a second time, after cccDNA formation and active replication, the virus could deflect PLK1 activity to this advantage as we identified this kinase to be involved in pgRNA encapsidation and reverse-transcription (*confer* **“First study: Polo-like-kinase 1 is a proviral host factor for hepatitis B virus replication”**).

To summarize, two pictures that may explain the PLK1 activation upon HBV infection are proposed here. On the one hand, HBV through direct mechanisms could lead to PLK1 activation in order to support the upcoming replication. On the other hand, PLK1 activation could come from the transient activation of the innate immune response by HBV infection, and PLK1, by downregulating

the IKK complex, could be part of the explanation on how HBV succeed in inhibiting the innate immune response (**Figure 37**).

In accordance with kinase cascades which are often observed (e.g., MAPK, PI3K/Akt/mTOR) to transduced a signal; one could address the question of the PLK1 activation kinetic/dynamic. Are PLK1 activators such as Aurora A found overexpressed/deregulated under HBV infection? Recently the nonsynonymous polymorphism of the AURKA gene Ile31Phe (encoding the kinase Aurora A) was associated with increased risk of occurrence of HBV-related hepatocellular carcinoma in Chinese population (Bao et al., 2017). Although the authors have not deciphered the underlying mechanisms, it would be interesting to focus on the role of this polymorphism regarding early HBV infection and study its potential impact on PLK1. This could strengthen the link between the overexpression of PLK1 induced quickly after HBV infection and elevated PLK1 levels found in HBV-related HCC.

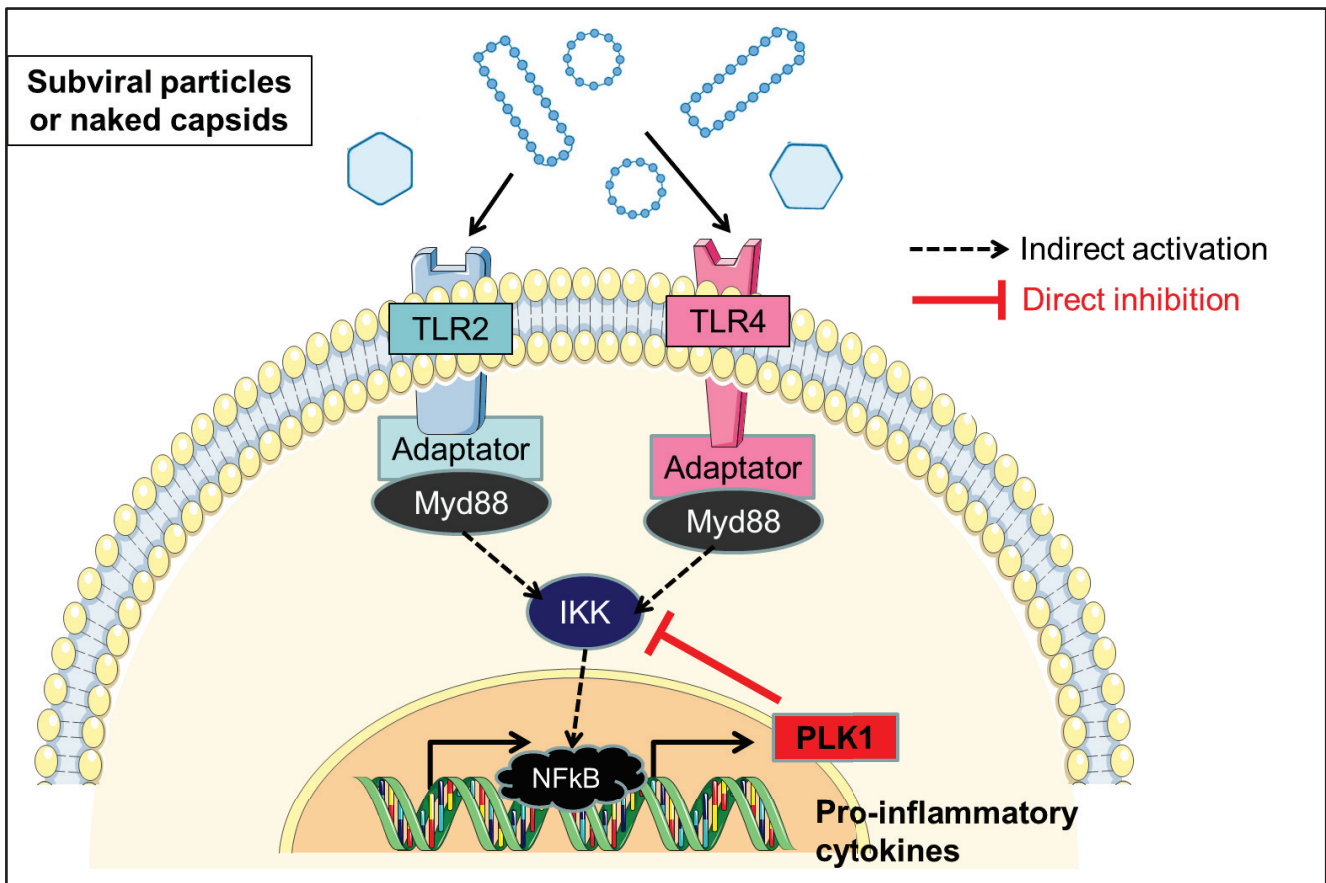


Figure 37: Hypotetic model of PLK1 activation based on innate immune sensing of HBV. Subviral particles and/or naked capsids are known to be recognized by TLR2/TLR4, thereby activating the NFkB pathway. HBs has been shown to interact directly with the β -2-glycoprotein I (β 2GPI) via the TLR4/Myd88 route and through the activation of NFkB by phosphorylation of Ser32/36 by IKKa (Jing et al., 2018). NFkB is a well described transcription factor of PLK1, leading to increase its transcription. PLK1 was identified as a gammaBD kinase, which negatively regulates IKK activation (Higashimoto et al., 2008) and could explain how HBV shut down innate immune response quickly after infection.

PLK1 is involved in several steps of HBV life cycle, strengthening the interest of targeting this kinase for antiviral therapies

As discussed before, although PLK1 is found overexpressed and activated early-on after HBV infection, its inhibition before infection does not impede the establishment of HBV cccDNA, implying a minor involvement of PLK1 regarding entry step, rcDNA translocation and cccDNA formation (*confer* “**Second study: Antiviral activity of PLK1-targeting siRNA delivered by lipid nanoparticles in HBV-infected hepatocytes**”). From our studies, we identified PLK1 as a proviral factor for HBV and found that the neosynthesis of HBV DNA was the only parameter impacted drastically by small molecules inhibitors of PLK1 (e.g., BI-2536). The critical step for the rcDNA generation being the pgRNA encapsidation and subsequent reverse transcription, we focused our efforts on the Hbc-PLK1 interaction. As explained, a dynamic process of phosphorylation of the HBV capsid is mandatory for a proper pgRNA encapsidation and reverse transcription. Moreover, several groups have already identified kinases able to phosphorylate the capsid, such as CDK2 which phosphorylates SP sites located at the C-terminus of Hbc, including S155, S162, and S170 (Ludgate et al., 2012) or SRPK1/2 (Daub et al., 2002). PLK1 is known to mostly interact with already phospho-primed peptides and was found to co-immunoprecipitates with Hbc in HepaD38 cells. All these observations and bibliographic data led us to hypothesis that PLK1 may play a role in HBV life cycle by regulating the encapsidation/reverse transcription of the pgRNA via a phosphorylation process of the HBV core protein. We confirmed our hypothesis and have mapped PLK1 phosphorylation sites on Hbc C-ter domain to be S168, S176, and S178 (reported in the first study) but requiring prior phosphorylation at positions S155, S162 and S170, sites shown to be phosphorylated by CDK2 (Ludgate et al., 2012)(Figure 38).

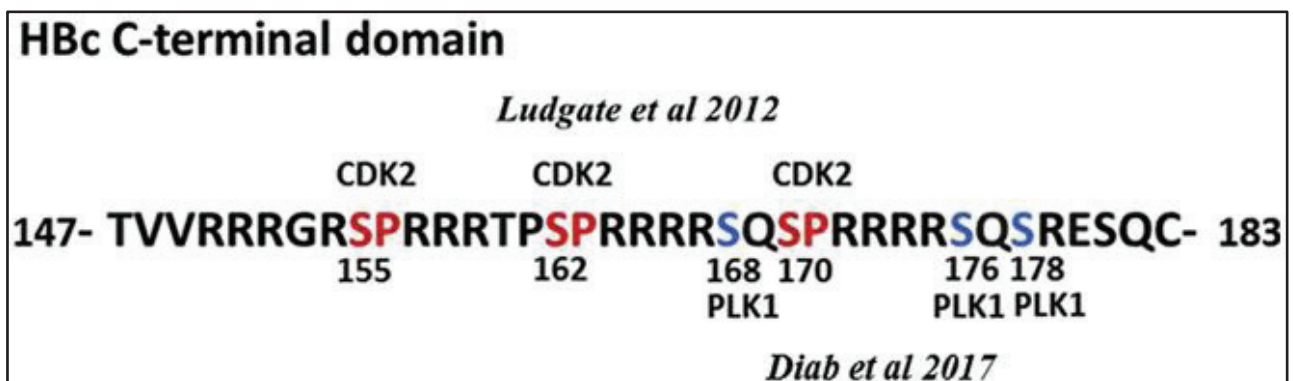


Figure 38: HBc CTD primary sequence, highlighting important sites of phosphorylation. In red are the three SP sites described by Ludgate and colleagues to be phosphorylated by CDK2. They represent a first wave of phosphorylation that is required for PLK1 to interact and further phosphorylate the CTD of Hbc (in blue). Extracted from the review I have written on the “The diverse functions of the hepatitis B core/capsid protein (Hbc) in the viral life cycle: Implications for the development of Hbc-targeting antivirals”. Please *confer* to Appendix review (Diab et al., 2018) for precisions.

This antiviral activity using the PLK1 inhibitor BI-2536 is very potent in decreasing HBV DNA with EC_{50} ranging from 5 to 50 nM, depending on the cell type used; such an antiviral efficacy is rarely obtained with host targeting agents and corresponds to the efficacy of direct acting agents including nucleoside analogs or assembly inhibitors. Accordingly, an *in vivo* antiviral activity was also obtained with BI-2536 in HBV-infected liver humanized FRG mice, and this antiviral activity was comparable to that obtained with entecavir or IFN- α in the same model.

However, as kinase inhibitors often demonstrate limits in term of specificity, we decided to knockdown PLK1 using siRNA. Thanks to Arbutus Biopharma, we had access to an innovative delivery strategy that allow a specific targeting of the hepatocytes; **the siRNA-containing lipid nanoparticles (LNP) (Figure 39)**. Using this technology and siRNA targeting PLK1 which were already clinically trialed for anti-HCC activity ([NCT02191878](#)), we knocked-down PLK1 in a context of HBV infection.

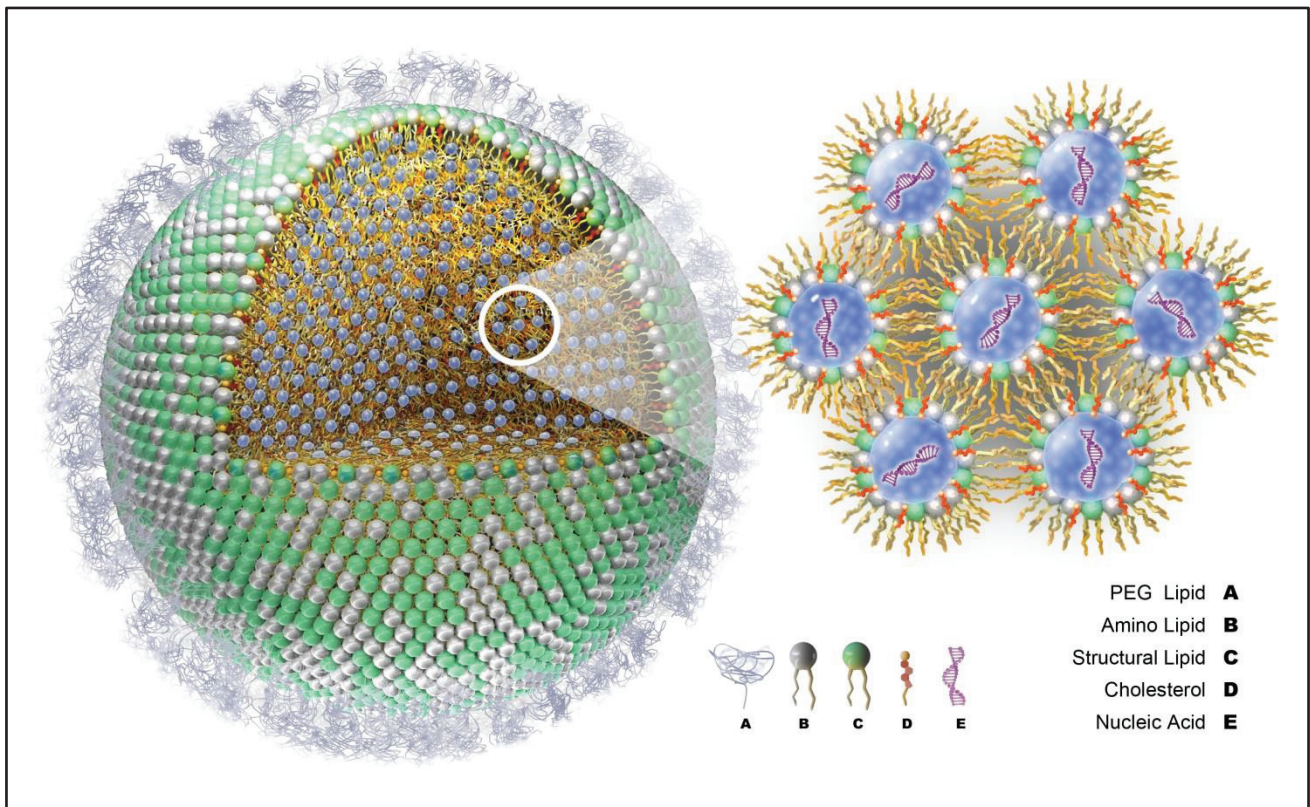


Figure 39: Schematic representation of the lipid nanoparticles technology. The large spherical structure can be coated with PEG and different molecules (**A**), depending on the cell type to target, and contains thousands of nanoparticles. Inside each nanoparticle, a nucleic acid can be incorporated, such as siRNA (**E**). This technology allows efficient delivery of the compound with limited side effects. Extracted from Arbutus Biopharma portfolio.

Surprisingly, knocking-down of PLK1 at a protein level, instead of solely inhibiting its kinase activity, uncovered new phenotypes regarding the anti-HBV activity. As mentioned earlier, the use of the small molecule PLK1 inhibitor BI-2536 resulted in an inhibition of HBV DNA neosynthesis .

The use of LNP siPLK1, which abolish totally the expression of PLK1 protein, resulted in a loss in HBV DNA, but also in HBV RNA and secreted HBe/HBsAg (*confer* “**Second study: Antiviral activity of PLK1-targeting siRNA delivered by lipid nanoparticles in HBV-infected hepatocytes**”). This implies that PLK1 may play roles in HBV life cycle in a kinase-independent manner and regulates several steps of the HBV cycle. To this extent, it was precedently shown by Vitour and colleagues that the polo-box domain of PLK1 could bind to the mitochondria-bound innate immunity adapter MAVS and inhibit its ability to activate the IRF3 and NFκB pathways, without any requirement of the kinase activity (Vitour et al., 2009). Furthermore, the lack of a functional kinase domain in PLK5 highlights the question of whether other PLKs have kinase-independent roles. Notably, PLK2 leads to modulation of distal dendrite synapses during chronic excitation in a kinase- and PBD-independent manner (Evers et al., 2010). Besides the “classical” functions of PLKs in the regulation of the cell-cycle originally described in *Drosophila* and yeasts, it is now obvious that these are important regulators of non-proliferative events, such as cell differentiation, and these functions may be independent of the kinase activity.

The mechanisms behind this antiviral activity is not characterized yet, however we saw an accumulation of HBV RNAs in the nucleus of cells treated with LNP siPLK1 treatment, while the total amount of vRNA was decreased, indicating an involvement of PLK1 in cccDNA activity, vRNA trafficking and/or fate. Undergoing experiments attempt to answer this question, mostly by ChIP/RIP analysis to see if activation/repression marks on the cccDNA are altered under PLK1 inhibition. The second point of interest regarding this axis will be to determine if PLK1 is found associated with vRNA, playing a role in their stability/trafficking. It is tempting to speculate that the previously described interplay between PLK1 and HBc could also be at work. Indeed, HBV core protein is known to interact with the HBV cccDNA (Bock et al., 2001) and potentially plays a role in the regulation of its transcription. Moreover, it has been suggested that the HBc CTD could be determinant for HBc-cccDNA interaction (Chong et al., 2017) and in first precedent study, we have identified 3 PLK1-dependent phosphorylation sites in HBc CTD. Finally, HBc is also known to be an RNA-binding protein physically interacting with vRNAs. Taken together, this provided a nice hypothetical landscape for further mechanistic studies to document LNP siPLK1 mode of action.

Such an unexpected phenotype on HBV antigens production is particularly relevant, as the next step in the improvement of current HBV therapy in clinic is precisely to be able to reduced antigenemia in patients to potentially restore immune responses and control infection.

Other groups in the HBV field have found that PLK1 plays proviral roles. Recently, the team of Jake Liang from the NIH has performed a genome-wide screen on HepG2-NTCP, targeting 21,500 genes using three unique siRNA sequences per gene in order to identify host factors related to HBV. In addition, they did a counter-screen with a cell viability assay measuring ATP as an endpoint to assess the effect of knockdown on cytotoxicity. Interestingly they found that siPLK1 led to a loss of secreted HBV antigens (HBV meeting 2018, oral presentation N°167; [A genome-wide siRNA screen for host factors involve in hepatitis B virus infection](#)), strengthening our results. Furthermore, they identified HNF4 α in the screen to have an impact on HBV infection. This master regulator of hepatocyte differentiation has been described to bind to the cccDNA, thus, activating its transcription in infected HepG2-NTCP cells. The IL6 inhibitory effect on HBV transcription could be in part mediated by a loss HNF4 α binding to the cccDNA and a redistribution of STAT3 binding from the cccDNA to IL6 cellular target genes (Palumbo et al., 2015). HNF4 α has also been linked to PLK1 expression. Indeed, under normal conditions, HNF4 α tends to inhibit hepatocytes proliferation while PLK1 is known as a potent cell-cycle promoter. In the liver of mice deleted for HNF4 α , PLK1 is found upregulated in a 13 fold manner at the genomic level (Walesky et al., 2013). However, one may suggest that in the case of an HBV infection, the relocatization of HNF4 α to the cccDNA could allow an inhibition release of the PLK1 transcription, leading to increased expression/activity of PLK1 that is mandatory for a proper HBV replication. Regarding this hypothesis, it would be interesting to perform ChIP experiments to determine whether HNF4 α could bind to PLK1 promoter/gene and to see if HBV infection in primary human hepatocytes leads to a relocalization of HNF4 α from PLK1 gene to the cccDNA (Figure 40).

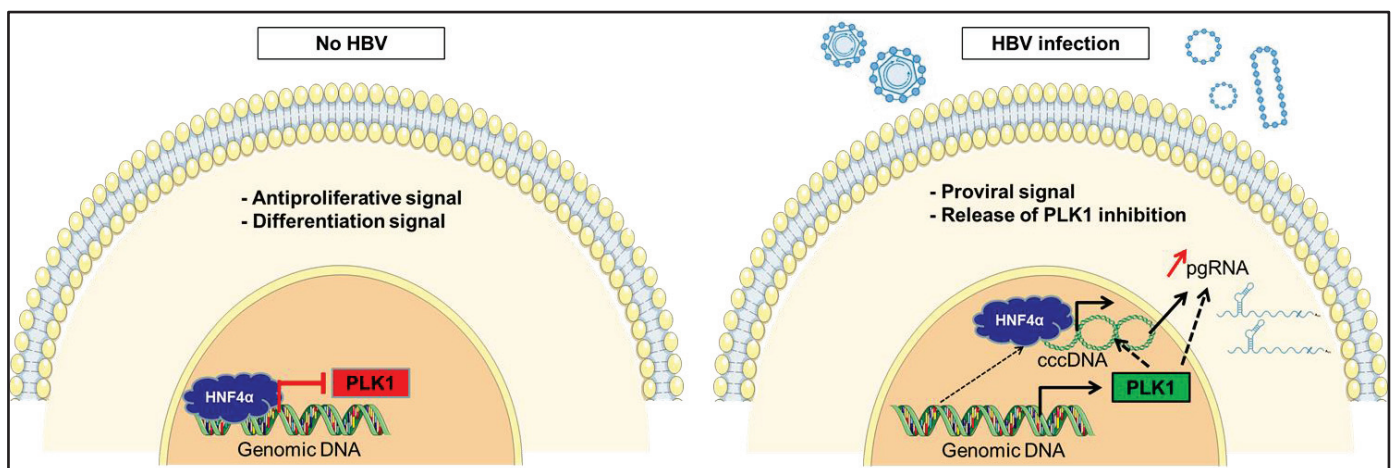


Figure 40: Hypotetic model of PLK1-inhibition release under HBV infection. In non-infected hepatocytes (**left**), HNF4 α acts as a repressor of PLK1 transcription, thereby promoting the quiescence of the hepatocytes and the differentiation (Walesky et al., 2013). Under HBV infection (**right**), HNF4 α is found to bind the cccDNA where it promotes the viral transcription (Palumbo et al., 2015). The dashed black line represents the relocalization of HNF4 α from genomic DNA to the cccDNA, thus, releasing the inhibition of PLK1. In turn, PLK1 act as a proviral factor either by increasing the cccDNA activity or by promoting stability/trafficking/maturation of the viral RNAs.

Does the PLK1 proviral effect towards HBV is pan-genotypic?

The majority of the experiments were done using HBV genotype D concentrated from HepAD38 cells supernatants. However, recently we have developed new tools allowing the production of the eight HBV genotypes (A-H) either in HepG2 or in HepaRG backbone. As explained in the introduction section, the different HBV genotypes lead to divergences in terms of natural history and progression of the disease. This highlights the fact that molecular mechanisms are affected in a different way, depending on the HBV genotype. Actually, the community put efforts on testing potent antiviral molecules in several HBV genotypes. From our side, PLK1 was found overexpressed in all HepG2 cells producing A-H genotypes compared to naive HepG2 and the antiviral effect of LNP siPLK1 was confirmed in genotype C (data not shown). These preliminary results seem to indicate that PLK1 is involved in the replication of all HBV genotypes and that its inhibition could be of benefit in a pan-genotypic manner. Indeed, the CTD of HBc being extremely well conserved among all the genotypes, one would expect that PLK1 inhibition should at least exhibit the same antiviral phenotype regarding the encapsidation/reverse-transcription of the pgRNA.

PLK1 as a first-choice target for anti-HBV and anti-HCC therapies?

Besides its involvement in the HBV life cycle, PLK1 is a key player in HBV carcinogenesis. PLK1 is expressed ubiquitously in eukaryotic cells and is highly expressed in most of human cancers, including HCC (Mok et al., 2012). PLK1 can be activated by HBV X protein to allow propagation of DNA damage by both attenuating the DNA damage checkpoint Chk1 activity and destabilizing DNA repair protein Mre11 (Studach et al., 2010). Furthermore, PLK1, together with the long non-coding RNA HOTAIR, induces proteasomal degradation of two transcription repression factors, SUZ12 and ZNF198, to alter the chromatin landscape, leading to epigenetic reprogramming associated with oncogenic transformation (Zhang et al., 2015). Finally, L-HBsAg was found to be involved in HCC development through induction of cytokinesis failure, leading to aneuploidy in hepatocytes. This phenotype appears to be mediated via DNA damage and G2/M checkpoint override due to PLK1 overexpression.

However, we do not know yet if the initial overexpression of PLK1 upon HBV infection is related to the PLK1 elevated levels found in HBV-related carcinogenesis (Figure 41). To answer this question it would be of value to follow the PLK1 expression in a long term kinetic of HBV infection. The main issue is the limits of experimental models. Regarding the *in vitro* part, PHH cannot support more than three weeks of plating without dedifferentiation and loss of viability. The HepaRG model can last hypothetically for months/years in differentiation state, yet, we saw a decrease in HBV replication after approximately a month of infection. Regarding *in vivo* model, FRG liver-humanized mice tend to develop spontaneously liver tumours of murine origin and thus are not the optimal

model to perform a long-term follow up of HBV infection. The solution may arise from the HepaRG-producing all the HBV genotypes in a stable manner. Although being pauciclonal, these cells lines could theoretically produce virion and all HBV intermediates in a long-lasting way. It would be interesting to monitor the PLK1 expression level in these cells in order to:

- Know if HBV genotypes induce PLK1 expression in a different quantitative manner.
- See if PLK1 overexpression (plasmid transduction of PLK1 constitutively active form) in these cells leads to cellular transformation.

If the two latter points are proven exact, can we imagine different time-courses regarding cellular transformation as we know that genotypes C2 or F1b are more aggressive and lead rapidly to HCC.

The developpement of new innovative tools (e.g., 3D spheroids culture, humanized-miced) and efforts bring to understand the interface between PLK1 involvement in HBV replication and PLK1 functions in carcinogenesis will eventually converge to the development of new drugs, which will act in a bimodal way, acting on both HBV replication and prevention of the occurrence of neoplastic events. In this respect, inhibiting PLK1 may emerge as a pionner strategy. Furthermore, due to its large involvement in carcinogenesis, several molecules targeting specifically this kinase have been developed and clinically trialed. Thus, a repositioning of drugs (e.g., Volasertib, Rigosertib) could be foreseen to reinforce the anti-HBV/HCC arsenal. At last, direct acting agents such as HBV RNA destabilizers, CpAMs, siHBV, NAs should be combined with host targeting agents like kinase inhibitors to combat in an efficient way HBV and to maintain the hepatocytes landscape in a near normal state to prevent carcinogenesis.

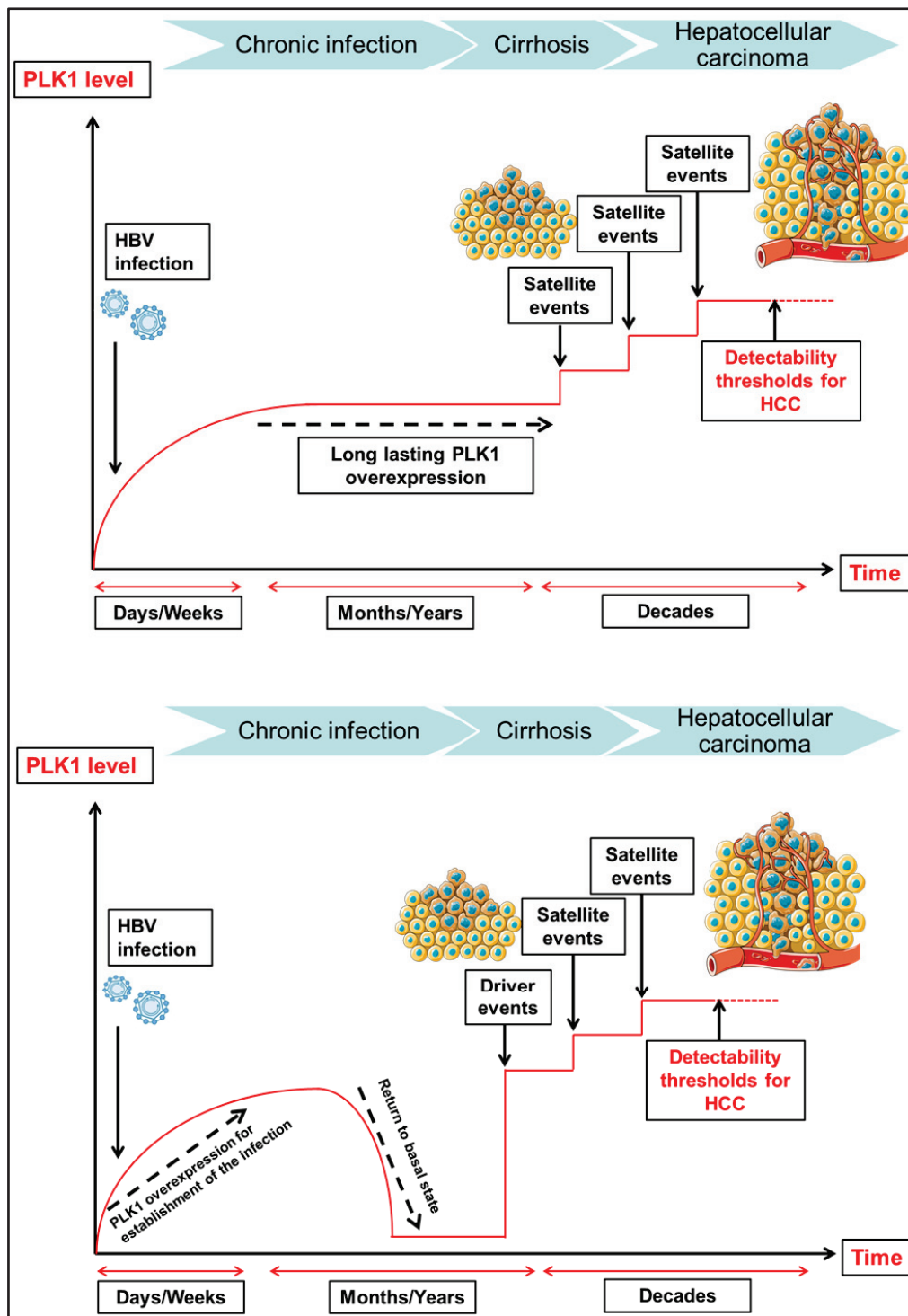


Figure 41: Two models presenting how PLK1 could be involved in HBV-related carcinogenesis. Top: Upon HBV infection, PLK1 is overexpressed and stays at an elevated level in a long-lasting way. This alters the hepatocytes and microenvironment landscape by promoting proliferation, DNA damages, chromosomal instabilities ... and eventually along with the occurrence of satellite events, lead to cellular transformation. In this model PLK1 acts as a key driver of carcinogenesis. **Bottom:** Initially the situation remains unchanged, PLK1 is overexpressed due to HBV infection. However, once the infection is stably established, the PLK1 level returns to a basal state until a driver carcinogenic event occurs such as p53 mutation, which leads in an indirect manner to PLK1 overexpression. In this situation PLK1 overexpression plays a role of satellite event towards the transformation process and is just the result of the high-proliferative rate of uncontrolled cells. In both cases it is of importance to note that with our actual technology, we can pretend in the best cases to detect tumour approximately 10 years after the emergence of the first transformed cell.

Take-home message

This thesis project aimed at exploring the impact of cellular partners on HBV and has led to the identification of PLK1 as a new proviral factor. Using several approaches such as ATP-competitive inhibitors of PLK1 or innovative lipid nanoparticles system to deliver siRNA, we brought forward the proof-of-concept that PLK1 could be an attractive druggable target in HBV infection. We uncovered partially the mechanisms behind this antiviral effect and found that PLK1, through its kinase activity, is able to phosphorylate the HBV core protein, thus affecting the pgRNA encapsidation and subsequent reverse-transcription. Furthermore, PLK1 seems to be involved in other steps of the HBV life cycle, in a kinase-independent manner albeit the exact mechanism needs still to be determined. Finally, as PLK1 is found overexpressed in HBV-related HCC, we hypothesized that its inhibition could act in a bimodal way, both acting on HBV replication and preventing the occurrence of neoplastic events.

Place of my project within the research group

At last, my thesis project on PLK1 involvement in the HBV life cycle led the group to explore new opportunities regarding host-virus interactions, with a special focus on cellular kinases. Indeed, interactomes of Hbc were done and allowed to identify kinases involved in RNA metabolism (e.g., maturation, splicing, trafficking) which could be of importance for HBV replication. Notably, a patent was established regarding the inhibition of a specific kinase which could serve as a new drug target to reinforce the anti-HBV arsenal. The next step would be to do a kinome between uninfected and infected PHH to decipher the general pattern of kinases being deregulated under HBV infection. Thus, my work allowed the team initiating a new axis of research around host-virus interactions with a special focus on kinases that may be involved in vRNA metabolism as well as strengthening several links between subgroups.

My contribution outside this project

During my PhD, I was not only focused on my personal project but always get involved in neighboring projects and some examples are presented in Appendix.

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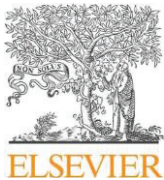
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PART III: APPENDIX



The diverse functions of the hepatitis B core/capsid protein (HBc) in the viral life cycle: Implications for the development of HBc-targeting antivirals

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ABSTRACT

Virally encoded proteins have evolved to perform multiple functions, and the core protein (HBc) of the hepatitis B virus (HBV) is a perfect example. While HBc is the structural component of the viral nucleocapsid, additional novel functions for the nucleus-localized HBc have recently been described. These results extend for HBc, beyond its structural role, a regulatory function in the viral life cycle and potentially a role in pathogenesis. In this article, we review the diverse roles of HBc in HBV replication and pathogenesis, emphasizing how the unique structure of this protein is key to its various functions. We focus in particular on recent advances in understanding the significance of HBc phosphorylations, its interaction with host proteins and the role of HBc in regulating the transcription of host genes. We also briefly allude to the emerging niche for new direct-acting antivirals targeting HBc, known as Core (protein) Allosteric Modulators (CAMs).

1. Introduction

Hepatitis B virus (HBV) is a major hepatotropic human pathogen, with at least 2 billion people having been exposed to date to the virus according to World Health Organization (Revill and Yuan, 2013). HBV exposure leads to chronic infection in 85–95% of infected neonates/ children and 5–15% of infected adults (Hadziyannis, 2011). Chronic infections are associated with a higher risk of developing liver cancer (Revill and Yuan, 2013), with 50% of hepatocellular carcinoma (HCC) attributed to HBV. Viral persistence is due to the ability of the virus to escape the host immune system, and due to establishment of an episome in the nucleus of infected cells, known as covalently closed circular double-stranded DNA (cccDNA). This cccDNA resembles the host genome, as it assumes a chromosome-like structure, and serves as main template for viral transcription. However, cccDNA is devoid of telomeres and replication origins, implying that it can be lost when cells divide; consequently, its reservoir is maintained by recycling of newly synthesized, relaxed-circular DNA (rcDNA) containing nucleocapsids. For an in-depth discussion of cccDNA biology see recent reviews (Allweiss and Dandri, 2017; Lucifora and Protzer, 2016). CccDNA is a 3.2 kb molecule that expresses at least 6 overlapping RNAs, from 4 open reading frames (ORF), leading to production of 7–8 proteins, including viral polymerase (P), regulatory X protein (HBx), envelop proteins (S, M, and L), HBcAg, and core/capsid (HBc). In this review, we focus our discussion on the function of HBc.

HBc, a small versatile protein of 21 kDa, has been

classically viewed as a structural protein that self-assembles to form the viral nucleocapsid. In turn, the nucleocapsid initially contains the pregenomic RNA (pgRNA), subsequently converted into rcDNA by reverse transcription. The versatile nature of HBc is primarily a consequence of post-translational modifications occurring at its C-terminal domain (CTD), and thus, this protein directs several viral processes. Accumulated evidence supports that HBc functions extend well beyond this structural role. HBc plays a role in nearly every stage of the HBV life cycle, including: subcellular trafficking and release of HBV genome, RNA metabolism, capsid assembly and transport, and reverse transcription (Fig. 1) (Basagoudanavar et al., 2007; Nassal, 1992; Perlman et al., 2005; Rabe et al., 2003; Zlotnick et al., 2015). Moreover, recent findings implicate an active role for HBc in epigenetic regulation of the viral and host genomes. Collectively, this multiplicity of functions elevates HBc as an excellent target for mechanism-based antiviral therapeutics (Durantel and Zoulim, 2016; Zlotnick et al., 2015). This review will highlight how the unique structure of HBc, its capacity to multimerize, as well as its post-translational modifications, can explain its multiple roles in the HBV life cycle.

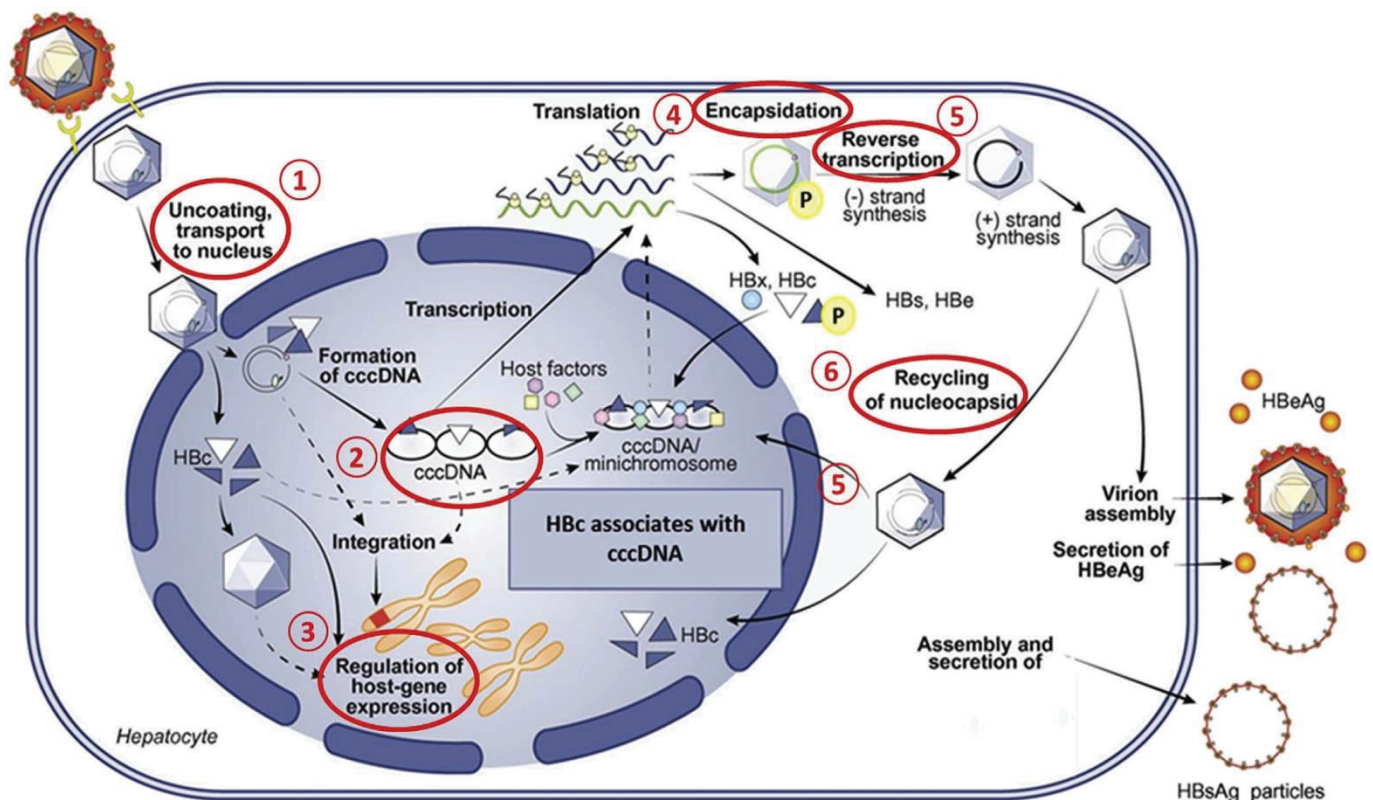


Fig. 1. Stages of the HBV life cycle regulated by HBc. 1) HBc regulates transport and nuclear release of the viral genome (Blondot et al., 2016). 2) HBc associates with cccDNA (Guo et al., 2011). 3) HBc modulates host gene expression (Xie et al., 2017). 4) HBc is required for pgRNA encapsidation (Nassal, 1992). 5) HBc is required for reverse transcription (Lewellyn and Loeb, 2011), and 6) persistence of cccDNA involves recycling of nucleocapsids into the nucleus (Nassal, 2015).

1. HBc: from translation to capsid structure and self-assembly

The core protein is encoded by the HBV pgRNA which also encodes the viral polymerase, and serves as template for rcDNA synthesis via reverse-transcription. The viral polymerase acts as a molecular switch to regulate these two otherwise competitive processes allowing the virus to replicate efficiently (Ryu et al., 2010, 2008). The 21 kDa HBc protein is the building block or capsomer of the HBV genome-containing icosahedral capsid. The first crystal structure, resolved by Wynne and colleagues, revolutionized our understanding of HBc biology (Wynne et al., 1999). HBc is a helical protein with five alpha-helices and lacking β -sheets. Helices 2 and 3, making a long alpha-helical hairpin, are responsible for the HBc folding pattern. Interestingly, most nucleocapsid proteins that form icosahedral shells usually fold into barrel structures instead of helical ones, e.g., those seen in the HIV-1 capsid (Wynne et al., 1999). A hydrophobic region makes up the base of the protein. HBc assembles first into dimers via alpha-helical hairpins, which form a four-helix bundle, assuming an inverted T shape. A disulfide bridge between the two Cys-61 residues, stabilizes the association, although this cystine formation is not necessary for dimer assembly (Nassal, 1992; Zheng et al., 1992; Zlotnick et al., 1996). Residues Tyr-132, Arg-127, Pro-129, and Ile-139 appear to be involved in dimer formation (Wynne et al., 1999). Following dimer formation, trimers of dimers assemble into hexamers, eventually forming icosahedral capsid particles (Birnbaum and Nassal, 1990). Multimerization of 120 HBc dimers, i.e., 240 capsomers, leads to production of a 22 nm, icosahedral capsid particle, without need of other viral proteins (Birnbaum and Nassal, 1990). The dimers form the icosahedral capsid with a triangulation number $T = 4$. Of note, a small fraction of capsids consist of only 90 dimers with a triangulation number $T = 3$ (Crowther

et al., 1994). However, it is unknown whether virions comprised of smaller capsids are infectious or merely represent dead-end products caused by aberrant assembly.

Focusing on the HBc primary sequence, the full-length protein consists of 183 amino acids with distinct N- and C-terminal domains connected by a hinge region (Nassal, 1992). The N-terminal domain of HBc, spanning amino acid residues 1–140, contains the capsid assembly domain, as truncated HBc₁₋₁₄₀ is sufficient for self-assembly (Birnbaum and Nassal, 1990). On the other hand, the CTD of HBc, spanning amino acids 141–183, is dispensable for *in vitro* viral assembly. Notably, capsids lacking the last 34 amino acids maintain assembly capacity *in vitro*, but do not support genome replication in cells (Nassal, 1992), suggesting a role for the CTD in this process. Of the 34 amino acid residues comprising the CTD, 14 are arginines, and at least seven other residues are either serines or threonines, putative phospho-acceptor sites.

Studies to understand the role of HBc CTD in HBV replication have led to the proposal that electrostatic interactions between the highly basic CTD in assembled capsids and the viral RNA/DNA regulate reverse transcription of HBV DNA (Newman et al., 2009). Charge-driven destabilization of capsids, by either rcDNA maturation and/or changes in post-translational modifications of CTD, for example by phosphorylation, could explain capsid disassembly at the nuclear pores (Basagoudanavar et al., 2007; Perlman et al., 2005; Rabe et al., 2009). In addition, results of a study by Lewellyn and Loeb favor a hypothesis that CTD could serve as a nucleic acid chaperone domain, i.e., CTD could mediate changes in nucleic acid structure hence enabling pregenomic RNA (pgRNA) reverse transcription into rcDNA (Lewellyn and Loeb, 2011). These two models are not necessarily mutually exclusive, since the function of HBc as chaperone may be dependent on the overall charge of its CTD.

A recent study employing cryo-EM scanning has determined the high-resolution 3D structure of assembled HBV capsids (Yu et al.,

2013). It was proposed that HBc CTD shuttles between the interior and exterior of capsids, as evidenced by the presence of CTDs either on the exterior of some capsids or RNA-bound in the interior of other capsids (Yu et al., 2013). This model is attractive as it provides a mechanism by which HBc can be accessible for interaction with host proteins involved in intracellular trafficking, capsid maturation, or capsid disassembly. In this model, the positively charged CTD interacts transiently with the negatively charged Asp2 and Glu43 that line the local three-fold channel, presumably allowing movement of CTD in and out of capsids (Yu et al., 2013). In congruence with the cryo-EM study, biochemical binding assays demonstrate a nucleic acid sensitive and transient exposure of the CTD tail (Chen et al., 2011), further confirming the presence of CTD on the exterior of capsids. Specifically, RNA-filled capsids failed to bind serine-arginine protein kinase (SRPK), a host chaperone that binds full length HBc capsids, but not HBc₁₋₁₄₉ capsids (Chen et al., 2011), confirming the dynamic exposure of CTDs is mediated by CTD-nucleic acid interactions. These results also agree with biochemical studies showing the CTD of assembled capsids is not only sensitive to proteases, but also accessible to host proteins (Chen et al., 2011; Gallina et al., 1989). The exact mechanism of this dynamic CTD shuttling remains unknown, although a role of phosphorylation has been suggested by site directed mutagenesis of HBc (Selzer et al., 2015). However, further detailed analyses of the effect of CTD phosphorylations and dephosphorylations in CTD shuttling are required.

2. HBc, a nucleic acid chaperone

Nucleic acid chaperones are proteins that catalyze structural rearrangement of genetic material into a functional conformation (Semrad, 2011). The binding of nucleic acid chaperones to their targets is characteristically weak and transient, lacks sequence specificity, and does not require ATP hydrolysis (Semrad, 2011). An array of viral proteins displays nucleic acid chaperone activity. For example, nucleocapsid proteins of HIV (Levin et al., 2005), dengue virus (Pong et al., 2011), and hepatitis delta virus (Huang and Wu, 1998) are described as nucleic acid chaperones. The CTD of HBc contains an arginine-rich domain resembling histone tails with more than 50% of the residues being arginine (Lewellyn and Loeb, 2011). The CTD binds to nucleic acids *in vivo* due to its positive charge (Nassal, 1992). In addition to its characteristic positive charge, CTD is also structurally disordered (Yu et al., 2013), another key feature of nucleic acid chaperones (Chu et al., 2014). The localization of CTD to the interior of capsids (Zlotnick et al., 1997) and its ability to bind nucleic acids *in vivo* support the model that CTD works as a nucleic acid chaperone. This chaperone activity was recently confirmed *in vitro* using recombinant full length HBc, as well as CTD synthetic peptides (Chu et al., 2014). HBc CTD facilitated annealing and unwinding of DNA and enhanced hammerhead ribozyme cleavage *in vitro*, rendering HBc CTD a *bona fide* nucleic acid chaperone (Chu et al., 2014). However, the high degree of functional complexity associated with the CTD, makes it challenging to demonstrate the nucleic acid chaperone activity of HBc *in vivo*, without disturbing other HBc functions.

3. HBc localization: a dynamic process involving active-transport

Reports of subcellular localization of HBc *in vivo* demonstrated both cytoplasmic as well as nuclear distribution. Histologic analysis of tissues from HBV-infected patients indicated a distribution of HBc/capsids in both cytosolic and nuclear compartments. Sharma et al., as well as Akiba et al., described a predominant nuclear distribution (Akiba et al., 1987; Sharma et al., 2002) while others observed mainly a cytosolic core localization (Michalak and Nowoslawski, 1982; Petit and Pillot, 1985). *In vitro* analysis has shown HBc subcellular localization is in fact cell-cycle dependent (Yeh et al., 1993). The amount of core protein in the nucleus increases in G1 phase, diminishes in S phase, and increases again to detectable levels in non-proliferating cells (Yeh et al., 1993).

HBc shuttles between cytoplasm and nucleus rapidly and continuously (Li et al., 2010). These latter points could explain differences described above. Although HBc is 21 kDa, suggesting passive transport to and from the nucleus, this trapping, as exists for human T-lymphotropicvirus-1 (HTLV-1) (Goff, 2007) and human papillomaviruses (Aydin et al., 2014), would be fairly inefficient due to the quasi-quiescent state of hepatocytes. Given that HBc is a phosphorylation target of well-characterized cell-cycle regulated kinases, such as cyclin-dependent kinase 2 (CDK2) (Ludgate et al., 2012) and polo-like kinase 1 (PLK1) (Diab et al., 2017), it is highly plausible that such phosphorylation may contribute to HBc/capsid localization. Investigating this question in a physiologically relevant infection model will most certainly expand our understanding of the dynamics of HBc subcellular localization.

Furthermore, it remains unresolved whether HBc enters the nuclear pore complex (NPC) as a dimer or as a mature capsid. Thus, signals modulating HBc import and export from the nucleus are probably distinct from those that guide transport of mature capsids. An early *in vitro* model for capsid transport by Kann and colleagues suggested mature capsid disassembles into HBc dimers at the cytosolic side of the NPC, releasing the rcDNA-pol complex (Kann et al., 1997). Subsequently, HBc dimers bind importin- β and are transported through a NPC. At this point, HBc dimers dissociate from transport factors and could remain free in the nucleus, eventually playing a role as transcription factors, as described below, or reassemble as empty-capsid forms. On the other hand, as viral polymerase does not contain an importin- β binding (IBB) domain, formation of importin- α/β is required to import the genome complex (Kann et al., 1997). Once at the nuclear side, the rcDNA-pol complex dissociates from importin α/β . Since the viral genome comprises pathogen-associated molecular patterns (PAMPs), it could trigger sensing by cytosolic pattern recognition receptors (PRRs) (Paludan, 2013). This model of genomic nuclear import, showing a release of viral rcDNA in the cytosol could explain the weak innate immunity activation upon HBV infection (Faure-Dupuy et al., 2017; Lucifora et al., 2014; Vanwolleghe et al., 2015). However, this model is based on Woodchuck Hepatitis Virus where capsids were denatured using urea treatment in order to release the rcDNA-pol complex (Kann et al., 1997). Under such conditions, changes in conformation of the viral polymerase could enable its interaction with importin- α .

A more recent alternative model (see Blondot et al., 2016 for in-depth review), suggests a direct interaction between mature capsids and an essential component of the NPC's basket, the nucleoporin 153 (Nup153). It is known that importin- α interacts directly with Nup153, promoting importin- α/β -mediated nuclear import (Ogawa et al., 2012). Of note, it was shown earlier that mature capsids are transported to nuclear pores by an importin α/β complex (Kann et al., 2007). The new model provides two options for mature capsids going through nuclear pores: first, direct binding to Nup153 at the basket of the NPC (cargo size 36 nM) or via coating by importin- α/β and binding to Nup153 (cargo size 38 nM). According to this model, the HBc CTD is not necessary to anchor the capsid at the nuclear basket since importin α/β is required for capsid transport. While both models intend to explain the mechanism of capsid transport through nuclear pores, neither reveals the trigger of such transport.

Studies of the CTD tail revealed a role in directing HBc subcellular localization (Blondot et al., 2016; Li et al., 2010). Several attempts have been made to map the nuclear localization sequence (NLS) to the arginine repeats of CTD. However, the results are conflicting, probably due to the redundancy of arginine repeats within the CTD (Liao and Ou, 1995; Rabe et al., 2003). Recently, an exhaustive mutagenesis analysis of the CTD localized the NLS sequence to the 1st and 3rd arginine repeats, while the 2nd and 4th repeats contained the cytoplasmic retention signals (CRS) (Fig. 2) (Li et al., 2010). The presence of more than one NLS in a protein is known to maximize import efficiency (Mears et al., 1995). Other viral proteins, such as ICP27, of herpes simplex virus type 1 (HSV-1) (Mears et al., 1995) and NS1 of Influenza (Melen

Li et al., 2010

NLS	CRS	NLS	CRS		
RRRGRS	PRRRT	PSP	PRRRRS	QSP	PRRRRS
QSR	RES	Q	Q		
SV					
HBV	HBc				
RRGRGRER	ARGGS	RER	ARGRGR	RGRGR	GEKPRSPSSQS
EBV	EBNA1				
EQ	ARRNR	RRRWR	ER	QR	
HSV-1	ICP27				
R	GGRR	RRRGRGR			
HIV-1	REV				

Fig. 2. Arginine-rich motifs of different viral proteins exhibit an abundance of arginine residues within short 10–20 residues. The arginine-rich domain of HBc has distinct nuclear localization (NLS) and cytoplasmic retention signals (CRS) (Li et al., 2010).

et al., 2012), also contain multiple NLS sites. However, one must also consider the accessibility of these sequences in view of the dynamic structural changes of assembled HBc in viral capsids.

The dynamic positioning of HBc CTD in assembled capsids makes it quite challenging to define clearly the different signals regulating HBc transport. Nuclear export of HBc resembles ICP27 of HSV-1 and EBNA1 of Epstein Barr virus (EBV), in that they all bind to the RNA export factor TAP, and all contain the characteristic arginine rich domains (Fig. 2) (Yang et al., 2014). Since subcellular localization of ICP27 and EBNA1 are regulated by arginine methylation (Souki et al., 2009), it is reasonable to envisage a similar mechanism driving HBc localization. However, it is still unknown if the arginine repeats of CTD are methylated, an hypothesis worthy of future investigation. Of note, arginine methyltransferase 5 (PRMT5) was reported as a negative effector of HBV replication and transcription via epigenetic repression of cccDNA transcription and interference with pregenomic RNA encapsidation (Zhang et al., 2017). Interestingly, PRMT5 was shown to bind HBc, and HBc-deficient virus (HBV-ΔHBc) had less PRMT5 bound cccDNA when compared to the wildtype HBV (Zhang et al., 2017).

The contribution of HBc CTD to capsid localization is only beginning to be resolved. The ambiguity is based on the long-standing notion that the CTD localizes to the interior of capsids based on cryo-EM studies (Zlotnick et al., 1997), and has a well-documented role in pgRNA packaging (Nassal, 1992). However, as previously discussed, more recent studies suggest a dynamic model in which HBc CTD shuttles between the interior and exterior of the capsid (Chen et al., 2011; Li et al., 2010; Selzer et al., 2015).

Transport of cargo through the nuclear envelope is often facilitated by regulatory phosphorylation/dephosphorylation of specific residues (Nardozi et al., 2010). Earlier studies reported phosphorylated HBc was only observed in the cytoplasm, based on subcellular fractionation of *in vivo* ³²P-radiolabeled cell lysates (Yeh et al., 1993). However, the molecular mechanism regulating subcellular localization of monomeric/dimeric vs. assembled HBc in capsids remains ambiguous. The potential contribution of HBc phosphorylation to the nuclear import of capsids comes from known import mechanisms for other viral proteins, including EBNA-1 (Kitamura et al., 2006) and SV40 T-antigen (Hubner et al., 1997). Specifically, phosphorylation of EBNA-1 increases its affinity for the import adaptor importin-α5 which mediates the nuclear transport via interaction with importin-β (Kitamura et al., 2006). SV-40 large T antigen has the best characterized NLS, and phosphorylation upstream of the classic NLS enhances nuclear import (Hubner et al., 1997). Thus, the phosphorylation status of HBc CTD could facilitate interaction with nuclear pore complex components. Indeed, in

pull-down experiments using HBc CTD-GST fusion proteins, serine to alanine substitution at the three serine/proline (S/P) sites (Fig. 3) showed increased binding to importin-α (Liu et al., 2015), suggesting phosphorylation impedes trafficking of HBc to the nucleus. While the *in vivo* relevance of these observations remains to be determined, other groups have reported that HBc phosphorylated *in vitro* by PKC in assembled capsids, preferentially bound importin-β (Kann et al., 1999). However, the phosphorylation sites of HBc required for interaction with importin-β have not been mapped (Kann et al., 1999). Furthermore, it is unresolved whether protein kinase C (PKC) is the *in vivo* kinase that phosphorylates HBc CTD (Daub et al., 2002). Thus, further studies are necessary to address this issue, employing well-characterized and functionally documented mutants of HBc.

Alternatively, as shown by Schmitz et al., capsid maturation could be a driving force of HBV nuclear import (Schmitz et al., 2010). At the nuclear pore, HBc capsids dissociate into dimers followed by entry into the nucleus (Rabe et al., 2009). Mature capsids directly interact with Nup153, an essential component of the nuclear basket that facilitates nuclear import via importin-β (Schmitz et al., 2010). Interaction of mature capsids with Nup153 resulted in capsid disintegration followed by release of viral genomes into the nucleus (Schmitz et al., 2010). Interestingly, capsids with immature genomes, i.e., RNA-filled capsids, accumulate at the nuclear pore. Recently, it was reported that serine to glutamate substitutions at the 3 S/P sites of HBc CTD (Fig. 3) reduced CTD exposure to the exterior of capsids suggesting a role for these phosphorylations in directing CTD exposure—inside or outside the capsid (Selzer et al., 2015). However, the mechanism responsible for distinguishing mature capsids vs. RNA-filled capsids remains unclear (Hu and Liu, 2017). Phosphorylation of HBc, shuttling of the HBc CTD to the interior vs. exterior of capsids, and maturation of the genome, may regulate exposure of different localization signals to host transport machinery. Such a multilayer and dynamic regulation network can best explain the difficulty in deciphering the mechanism of HBc subcellular localization during the HBV life cycle. To understand these important dynamic regulatory networks, it is essential to gain better understanding of HBc structure, HBc interacting partners, and HBc post-translational modifications.

4. HBc phosphorylation

Since the HBV genome does not encode a protein kinase, endogenous/cellular kinases must mediate post-translational HBc modifications (Lanford and Notvall, 1990). PKC (Kann and Gerlich, 1994; Wittkop et al., 2010), serine arginine protein kinase 1 (SRPK1) (Daub

HBc C-terminal domain

Ludgate et al 2012

CDK2	CDK2	CDK2	
TV	RRRRGR	SPRRRT	SPRRRR
155	162	168 170	176 178
		PLK1	PLK1 PLK1

Diab et al 2017

Fig. 3. HBc C-terminal domain (CTD) is a phosphorylation substrate for CDK2 (SP sites at positions 155, 162 and 170) and PLK1 (phosphorylation sites at positions 168, 176 and 178), as shown by Ludgate et al. (2012) and Diab et al. (2017), respectively.

Table 1
Kinases that phosphorylate HBc.

Kinase	Effect on viral replication	Immuno-detected in capsids	Phosphorylation sites	References
PKC	Required for envelopment	Yes	ND	Kann and Gerlich, 1994; Wittkop et al., 2010
GAPD-PK	ND	No	ND	Duclos-Vallee et al., 1998
SRPK1/2	Decrease replication	ND	S155,S162,S170	Daub et al., 2002
CDK2	No effect	Yes	S155,S162,S170	Ludgate et al., 2012
PLK1	Enhance replication	ND	S168,S176,S178	Diab et al., 2017

et al., 2002) and CDK2 (Ludgate et al., 2012) have been suggested as endogenous kinases that associate with or are packaged within viral capsids. The robust phosphorylation of HBc has been demonstrated *in vitro* by phospho-proteomic approaches (Chen et al., 2011), and *in vivo* by labeling with ^{32}P -orthophosphate (Jung et al., 2014). The HBc CTD contains multiple serine/threonine phospho-acceptor sites which are remarkably well-conserved among related viruses (Jung et al., 2014). Phospho-proteomic analyses of HBc have identified at least seven conserved serine and threonine sites that are phosphorylated *in vivo* (Chen et al., 2011). Notably, proline-directed serine (S/P) sites at positions 155, 162 and 170, are highly conserved and several S/P kinases have been reported to phosphorylate these sites (Fig. 3) (Daub et al., 2002; Ludgate et al., 2012). Other putative kinases, including: SRPKs, Glyceraldehyde-3-phosphate dehydrogenase protein kinase (GAPD-protein kinase), PKC, and an unknown kinase of 46 kDa, have also been reported (Daub et al., 2002; Duclos-Vallee et al., 1998; Kann et al., 1999; Ludgate et al., 2012) (Table 1). Yet none of these studies have mapped phosphorylation to specific sites, with the exception of SRPK1 and SRPK2 at the 3 S/P sites (Daub et al., 2002). However, the exact contribution of the SRPK-mediated phosphorylation to viral replication is enigmatic, because, although overexpression of SRPK1 or SRPK2 impaired HBV replication, this effect was independent of kinase activity (Zheng et al., 2005). These results support that SRPK-mediated phosphorylation of HBc observed *in vitro* is due to promiscuous substrate specificity of these kinases (Ludgate et al., 2012), and that SRPKs exert other functions affecting viral replication. For example, SRPKs could act as non-canonical chaperones in capsid assembly (Chen et al., 2011). Regarding PKC-mediated HBc phosphorylation, inactivation of PKC led to defective capsid envelopment, but had no effect on genome maturation (Wittkop et al., 2010). There is, however, lack of consensus regarding PKC being the candidate host kinase since specific inhibition of PKC had no effect on CTD phosphorylation (Daub et al., 2002; Ludgate et al., 2012). CDK2 was shown to phosphorylate the core protein of HBV both *in vitro* and *in vivo* (Ludgate et al., 2012). Intriguingly, CDK2 inhibitors had no observable effect on viral replication (Ludgate et al., 2012). The absence of such effect could be due to lack of specificity of the chemical inhibitors (Sakurikar and Eastman, 2016) or, more likely, due to redundancy among host kinases in phosphorylating HBc. Knockdown cell lines for CDK2 or other kinases via expression of shRNAs could elucidate their contribution to HBc phosphorylation and viral replication.

Although the kinases that phosphorylate HBc are still to be determined, phosphorylations of HBc do regulate its function at many levels (Gazina et al., 2000; Jung et al., 2014; Kann et al., 1999; Ludgate et al., 2011). Specifically, phosphorylation and de-phosphorylation of the HBc protein is required for capsid maturation as demonstrated by mass spectrometric analyses and site-directed mutagenesis studies of HBc (Basagoudanavar et al., 2007; Perlman et al., 2005). Phosphorylation of different serine residues of HBc CTD regulates reverse transcription, pgRNA encapsidation, DNA synthesis, subcellular localization, and virion secretion (Basagoudanavar et al., 2007; Gazina et al., 2000; Lan et al., 1999; Perlman et al., 2005). HBc phosphorylation is also required for the chaperone activity of CTD (Chu et al., 2014). It was also reported that the HBc phosphorylation status influences capsid stability (Ludgate et al., 2016), since capsids with glutamate phospho-

mimetic substitutions at the 3 S/P sites (S/P to E/P) were more stable than wild type capsids. It is likely that during various stages in the life cycle of HBV or as a function of the metabolic and proliferative status of the infected hepatocyte, distinct kinases target HBc phosphorylation, perhaps in an overlapping manner. The identification of those kinases, and the mechanisms by which they regulate HBV replication, is of great significance and will provide valuable targets for mechanism-based therapeutics.

To this end, our recent study has identified the mitotic PLK1 as another cellular kinase that phosphorylates HBc *in vitro* (Diab et al., 2017). PLK1 substrates are usually primed by phosphorylation at S/P directed sites mediated by CDKs, including CDK1 or 2 (Elia et al., 2003). Recombinant HBc, as well as immuno-purified HBc from transfected mammalian cells serves as robust PLK1 substrate *in vitro* (Diab et al., 2017). We have mapped the phospho-acceptor sites to S168, S176, and S178 (Fig. 3) (Diab et al., 2017), all of which have been identified as *in vivo* phospho-acceptor sites by an unknown kinase (Jung et al., 2014). Remarkably, inhibition of PLK1 by specific chemical inhibitors or siRNA-mediated knockdown of PLK1 inhibited viral replication, suggesting HBc phosphorylation by PLK1 is functionally important in virus biosynthesis (Diab et al., 2017). Significantly, we made the interesting observation, in accordance with the known substrate preference of PLK1, that alanine substitutions at the 3 S/P sites (S/P to A/P) resulted in loss of PLK1 phosphorylation *in vitro*. By contrast, PLK1 phosphorylation was robustly recovered in phospho-mimetic mutants, containing serine to aspartic acid substitutions at all three S/P sites (Diab et al., 2017). This suggests that CDK2 functions as the priming kinase for HBc, mediating the phosphorylation of the S/P sites which in turn enables the subsequent phosphorylation by PLK1 (Elia et al., 2003). Based on our results that PLK1 is a positive effector of HBV replication, a mechanistic understanding of the role of PLK1 phosphorylation of HBc in HBV replication has promising therapeutic potential. So far, PLK1 is the sole host-kinase whose chemical inhibition results in impaired HBV replication *in vivo* (i.e., in liver humanized mouse model) at concentrations comparable to other known anti-HBV agents (Diab et al., 2017). Finally, based on this dependence of PLK1 phosphorylation of HBc on its prior phosphorylation by CDK2, we reason combination treatment with inhibitors of CDK2 and PLK1 kinases will be effective in suppressing these modifications and thus effectively inhibit virus biosynthesis. This hypothesis is particularly attractive since safety of PLK1 (Gjertsen and Schoffski, 2015) and CDK2 (Asghar et al., 2015) inhibitors has been established in clinical trials for different types of human cancer.

5. HBc and interaction with host factors

HBc modulates nearly every stage of the HBV life cycle. With such extensive and intricate functions, HBc has evolved to modulate several host-protein functions. For example, HBc dimers interact with HSP90, and HSP90 facilitates formation of HBV capsids both *in vitro* and *in vivo* (Shim et al., 2011). On the other hand, the host HSP40 protein also binds, but de-stabilizes HBc by accelerating its degradation (Sohn et al., 2006). The binding of HBc to other cellular factors, such as NIRF, an E3 ubiquitin ligase, is implicated in HBc degradation (Qian et al., 2012). Thus, dynamic interactions of HBc with host chaperones regulate both

HBc stability and capsid assembly (Qian et al., 2012; Shim et al., 2011; Sohn et al., 2006).

Viruses hijack host RNA processing machinery (Salveti and Greco, 2014) and HBV is no exception. HBc utilizes the nuclear export factor 1 (NXF1/p15) and transcription export (TREX) machinery for nuclear export as a ribonucleoprotein complex along with the viral pgRNA (Yang et al., 2014); however, TREX-mediated export of HBc and pgRNA are independent of each other (Yang et al., 2014). Other viruses that hijack the NXF1/p15 pathway for mRNA processing and export include EBV (Juillard et al., 2009) and HSV-1 (Johnson et al., 2009). Interestingly, depletion of different TREX components has no apparent effect on accumulation of viral DNA, suggesting redundancy among host factors utilized by the virus.

Another class of host proteins commonly targeted by viruses is serine, arginine rich-proteins (Howard and Sanford, 2015) which, along with the kinases (SRPKs) that phosphorylate them, play central roles in alternative RNA splicing (Howard and Sanford, 2015). Intriguingly, HBc CTD resembles serine, arginine-rich proteins and it is not surprising that SRPKs associate with and potentially phosphorylate HBc (Chen et al., 2011; Daub et al., 2002; Zheng et al., 2005). Interestingly, mass spectrometry analyses of HBc interacting proteins has identified SRPK1 as well as several known SRPK1 interacting partners, including: SRSF1, SRSF9, DHX9, CDKN2A, DDX21, and DDX50 (Diab et al. unpublished data). The functional significance of these HBc interactions in the viral life cycle is unknown and worthy of investigation. We speculate that HBV usurps the host RNA-processing machinery for production and processing of its own viral RNA. It is also likely that such interactions may allow HBc to interfere with host gene expression. This has been evident in the case of E1E4 protein of the human papilloma type 1 virus (HPV1), which not only binds to SRPK1, but also inhibits phosphorylation of its host serine arginine substrates (Prescott et al., 2014). A yeast two-hybrid screen of a human liver cDNA library identified the human protein GIPC1 as another HBc interaction partner (Razanskas and Sasnauskas, 2010), but the significance of such interaction remains to be determined. GIPC1 contains a PDZ domain, a protein-protein interaction domain (reviewed in Hung and Sheng, 2002). PDZ-containing host proteins are commonly targeted by viral proteins; examples include the Tax protein of the T-cell leukemia virus type 1 (Rousset et al., 1998) and the E6 protein of the human papilloma virus type 18 (Favre-Bonvin et al., 2005).

It is reasonable to speculate that HBc exerts its various roles by interacting with specific host factors at different stages of the viral life cycle in a phosphorylation dependent manner. A better dissection of host factors that interact with HBc could facilitate development of new antiviral strategies or help elucidate core assembly modulators (CAMs).

6. HBc as a regulator of transcription

In chronically infected patients, HBc is detected in both cytoplasm and nucleus of hepatocytes (Chu and Liaw, 1987). *In vitro* models of HBV infection also show nuclear as well as cytoplasmic localization of HBc. Nuclear localization of HBc, in biopsies from HBV infected patients, has been linked to high viremia and deficient immune response (Chu and Liaw, 1987; Nguyen et al., 2009). These results prompted investigations of the nuclear function of HBc, in relation to virus biosynthesis and influence upon host gene expression.

In the nucleus of infected cells, the viral cccDNA assumes chromatin-like structure in association with histone and non-histone proteins, along with viral HBx and HBc proteins (Bock et al., 2001; Lucifora and Protzer, 2016). Association of HBc with the cccDNA mini-chromosome was reported to alter nucleosome spacing (Bock et al., 2001). Furthermore, it was reported that HBc preferentially associated with CpG island 2 of the HBV genome that overlaps enhancers (Enh) I and II involved in viral transcription; HBc binding to CpG island 2 positively correlated with the rcDNA/cccDNA ratio and serum levels of HBV DNA in infected patients (Guo et al., 2011). HBc may also act as

transcriptional activator of the HBV pre-Core promoter by enhancing binding of NF- κ B upstream of viral Enh II (Kwon and Rho, 2002). However, how HBc modulates the function of the cccDNA mini-chromosome remains to be determined. Since HBc is also essential for viral DNA replication, a definitive genetic approach involving mutagenesis or depletion of HBc is not feasible as a means to delineate its role in cccDNA mini-chromosome functions. Therefore, novel approaches are required to target nuclear HBc, influencing its capacity to associate with the cccDNA mini-chromosome, without affecting HBc cytoplasmic functions.

Regarding effects of HBc on host gene expression, HBc enhanced cAMP-Response-Element (CRE)-mediated transcription via the CRE/ CREB/CBP pathway (Xiang et al., 2015) in a manner similar, but weaker to previously reported HBx-mediated CRE activation (Andrisani, 1999; Williams and Andrisani, 1995). HBc may also function as a transcriptional repressor of host genes. For example, binding of HBc to E2F1 reduced the DNA-binding ability of E2F1 at the p53 promoter, thereby inhibiting p53 transcription (Kwon and Rho, 2003). In hepatoma cell lines expressing the core protein, HBc blocked the human death receptor 5 (DR5) by repressing its promoter, and inhibition of DR5 desensitized hepatocytes to TRAIL-induced apoptosis (Du et al., 2009). These results suggest a role for HBc in development of chronic infection by preventing hepatocyte death via blocking DR5 expression. The gene regulatory function of HBc may have evolved as a means whereby the virus may evade host immunity. HBc has been shown to downregulate IFN-induced host antiviral responses in hepatoma cell lines, by interacting with the promoter of MxA, an IFN-inducible gene widely implicated in antiviral response (Fernandez et al., 2003). However, these conclusions must be further validated by determining *in vivo* association of HBc with the MxA promoter, in a physiological cellular context of HBV infection. Interestingly, MxA has been shown to inhibit HBV replication by direct interaction with HBc, preventing capsid formation (Li et al., 2012), and providing yet another example of antagonism between viral and host proteins. Ectopic expression of HBc in HepG2 cells and primary hepatocyte cultures sensitized cells to TNF- α induced apoptosis, by disrupting the interaction between mitogen-activated protein kinase kinase 7 (MKK7) and receptor of activated protein kinase C 1 (RACK1) (Jia et al., 2015). This suggests a direct role of HBc in driving liver pathogenesis in chronically infected patients. These results require validation in a rigorous experimental model.

In continuing this effort to understand the effect of HBc on host gene expression, recent studies have generated the genome-wide profile of HBc in HBV-infected hepatocytes, employing chromatin immunoprecipitation microarray studies (ChIP-on-chip). These studies revealed that promoters of nearly 3100 host genes exhibited increased binding to HBc (Guo et al., 2012). However, further studies are necessary to understand the functional significance of HBc binding to the host genome in affecting HBV-mediated disease pathogenesis. Toward this goal, Lucifora et al. recently demonstrated interaction of nuclear deaminases APOBEC3A and APOBEC3B with HBc-bound cccDNA in host nucleus, leading to cccDNA deamination and degradation (Lucifora et al., 2014). Whether HBc recruits these nuclear deaminases to host genes is unknown and of great importance, given the role of APOBEC proteins as cancer driver genes in humans (Harris, 2015; Morganello et al., 2016; Nik-Zainal et al., 2012). Multi-omics analyses of HBc transfected cells linked HBc over expression up-regulated glycolysis and amino acid metabolism, providing further insights into the role of HBc in the molecular pathogenesis of HBV-mediated tumorigenesis (Xie et al., 2017).

7. HBc as a target for clinical intervention against chronic HBV infection

Due to the multiple functions of HBc in the HBV life cycle, this protein has become a preferential target for developing novel, direct-acting agents against HBV replication. Molecules targeting capsid

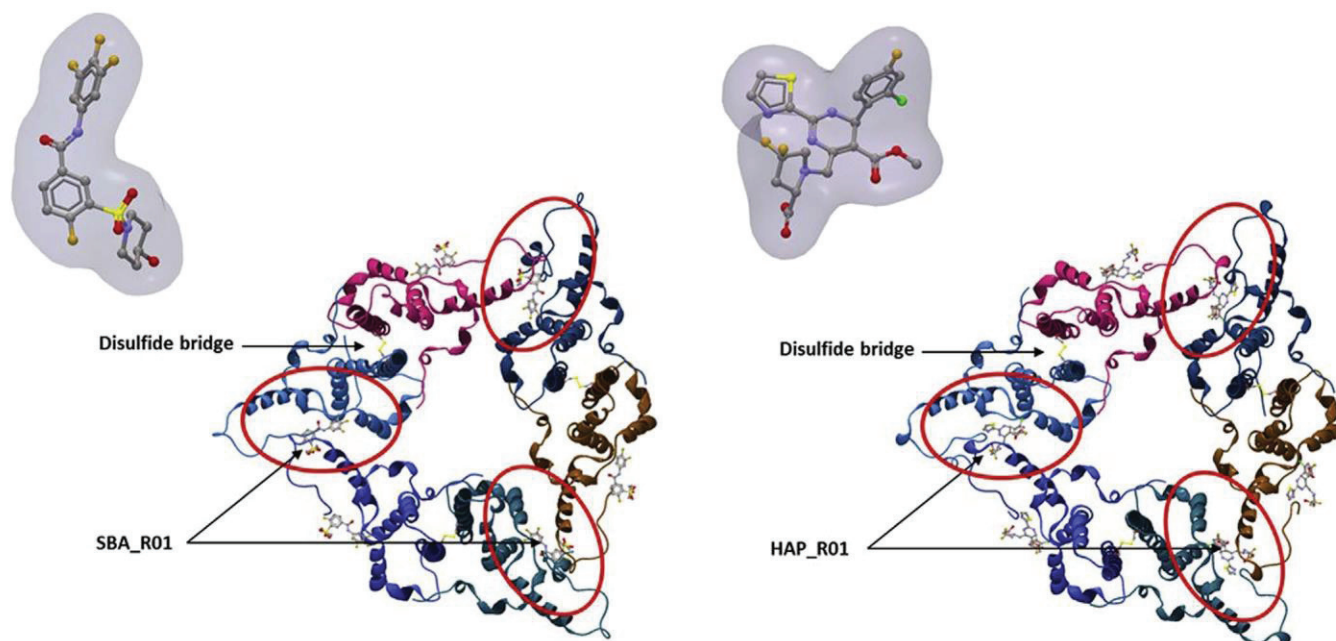


Fig. 4. Top view of an HBc hexamer in complex with CAM sulfamoylbenzamide SBA_R01, PDB: 5T2P (Left) and CAM heteroaryldihydropyrimidine HAP_R01 PDB: 5WRE (Zhou et al., 2017) (Right).

assembly have been known for some time with the discovery BAY41-4109, a heteroaryldihydropyrimidine derivative (Deres et al., 2003), and AT130, a phenylpropanamide derivative (Delaney et al., 2002). This class of inhibitors were subsequently abbreviated core assembly/allosteric modulators (CAMs), due to their mode of action, which is mainly based on allosteric modulation (Zlotnick et al., 2015). Interestingly, these molecules were shown to be active against nucleoside analogue resistant strains, which render them very attractive when viral resistance was a genuine clinical challenge (Billioud et al., 2011; Delaney et al., 2002; Wang et al., 2012). Moreover, CAMs are active against most HBV genotypes (Berke et al., 2017). Surprisingly, CAM therapy has not reached the clinic –probably due to safety or pharmacological issues. Since this early time, active research on structural and biological features of the HBc protein has led to the discovery of many other CAMs. Currently there are 3 types of CAMs according to their different molecular architectures, including heteroaryldihydropyrimidine, phenylpropanamide, and sulfamoylbenzamides derivatives (Fig. 4) (Zlotnick et al., 2015). These CAMs are thought to inhibit nucleocapsid-associated reverse transcription of pgRNA into rcDNA by preventing encapsidation of pgRNA, by accelerating formation of HBV nucleic-acid-free, yet normal-in-shape capsids, or by promoting formation of abnormal capsids. This defines two classes of CAMs based on these *in vitro/in tubo* structural criteria. In addition to their role in nucleocapsid formation and resulting conversion of pgRNA into rcDNA, CAMs have other modes of action. CAMs have been shown to prevent, in a post-entry manner, formation of cccDNA (Berke et al., 2017). This could be due to retention of rcDNA at the nuclear basket; in this respect, CAMs could bind to nucleocapsid and stabilize the structure, thus preventing its disassembly. CAMs also prevent the release from hepatocytes of RNA-containing and genome-free viral particles (Lam et al., 2015; AASLD abstract). Very recently it has been reported that specific CAMs prevent release of the HBe antigen (Lahlali et al., HBV meeting 2017). HBeAg, a secreted antigen sharing primary structure with HBc, has potential immune-modulatory functions. An active and fast inhibition of HBeAg secretion by CAMs could promote HBeAg seroconversion, an immunological process that occurs during natural history of HBV infection, and considered an important clinical end-point during therapy.

However, bearing in mind some of the other functions described in

this review (i.e. regulation of host gene expression, modulation of host and viral RNA metabolism, etc.), one might anticipate CAMs could play a role in HBV replication inhibition and HBV-induced pathogenesis. To date several CAMs are undergoing clinical trials: NVR 3–778 (NCT02112799; Novira Therapeutics), ABI-H0731 (NCT02908191; Assembly Biosciences), JNJ-56136379 (NCT02662712; Janssen), and GLS-4 (trial with China-CFDA; Morphothiadine Mesilate, HEC Pharm), and many others are the focus of pre-clinical evaluations.

8. Unanswered questions and future directions

As discussed in this review, HBc is involved in various aspects of HBV life cycle: formation of the viral capsid, pgRNA encapsidation followed by its reverse transcription, and as a component of the viral minichromosome, contributing to its chromatin structure. The unique structure of HBc CTD and its modifications, mainly phosphorylation, are essential for viral biosynthesis. Accordingly, HBc appears to be an ideal target for mechanism-based therapeutics. Yet many questions remain unanswered when it comes to HBc biology. A definitive answer to the identity of the kinases responsible of HBc phosphorylation *in vivo* is yet to be determined. Additionally, systematic investigation of HBc-host interactions, in the context of physiologic infection as well as details of its molecular properties, will expand our knowledge of how HBc is regulated by post-translational modifications, as well as how HBc regulates host functions. Moreover, given the unique structure of the CTD, the seemingly promiscuous affinity of HBc for RNA and nucleic acids in general, (Birnbaum and Nassal, 1990; Nassal, 1992), suggests potential interactions between HBc and host RNA metabolism. This corroborates our recent findings of long-noncoding (lnc) RNAs NEAT1 and NEAT2 as HBc interacting partners in cellular models of HBV replication and infection (Diab et al., HBV meeting 2017). Ribonucleoprotein immunoprecipitation (RIP) of HBc confirmed HBc associates with NEAT1 and NEAT2, known scaffolds of ribonucleoprotein complexes that constitute nuclear speckles (Hutchinson et al., 2007). Employing affinity purification of HBc, combined with mass spectrometry-based proteomics, we identified nuclear speckle proteins heterogeneous nuclear ribonucleoprotein M (HNRNPM), splicing factor proline/glutamine-rich (SFPQ), and non-POU domain containing protein (NONO) as HBc interacting partners. Thus, we propose that HBc, as

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Novel Potent Capsid Assembly Modulators Regulate Multiple Steps of the Hepatitis B Virus Life Cycle

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ABSTRACT The assembly of hepatitis B virus (HBV) core protein (HBc) into capsids represents a critical step of viral replication. HBc has multiple functions during the HBV life cycle, which makes it an attractive target for antiviral therapies. Capsid assembly modulators (CAMs) induce the formation of empty capsid or aberrant capsid devoid of pregenomic RNA (pgRNA) and finally block relaxed circular DNA neosynthesis and virion progeny. In this study, the novel CAMs JNJ-827 and JNJ-890 were found to be potent inhibitors of HBV replication with respective half-maximal effective concentrations of 4.7 and 66 nM, respectively, in HepG2.117 cells. Antiviral profiling in differentiated HepaRG (dHepaRG) cells and primary human hepatocytes revealed that these compounds efficiently inhibited HBV replication, as well as *de novo* establishment of covalently closed circular DNA (cccDNA). In addition to these two known effects of CAMs, we observed for the first time that a CAM, here JNJ-827, when added postinfection for a short-term period, significantly reduced hepatitis B e antigen (HBeAg) secretion without affecting the levels of cccDNA amount, transcription, and hepatitis B surface antigen (HBsAg) secretion. This inhibitory activity resulted from a direct effect of JNJ-827 on HBeAg biogenesis. In a long-term treatment condition using persistently infected dHepaRG cells, JNJ-827 and JNJ-890 reduced HBsAg concomitantly with a decrease in viral total RNA and pgRNA levels. Altogether, these data demonstrate that some CAMs could interfere with multiple functions of HBc in the viral life cycle.

KEYWORDS hepatitis B virus, core/capsid/HBc protein, nucleocapsid assembly, capsid assembly modulators, mechanism of action, HBV, antiviral, capsid inhibitors

Chronic hepatitis B (CHB) infections caused by the hepatitis B virus (HBV), a member of the *Hepadnaviridae* family (1), remain a major public health problem worldwide. According to the World Health Organization, over 250 million people are chronic carriers of the virus (Fact Sheet 204). These patients have a higher risk of developing severe liver diseases, such as decompensated cirrhosis and hepatocellular carcinoma (HCC); the latter being the third leading cause of mortality worldwide (2, 3).

HBV is a noncytopathic DNA virus that specifically enters hepatocytes through the sodium taurocholate cotransporting polypeptide (NTCP) receptor to replicate and produce virion progeny (4). After delivery to the cytosol, the viral nucleocapsid is translocated to the nuclear pores for disassembly and release of the relaxed circular DNA (rcDNA) (5). Within the nucleus, the rcDNA is converted to a covalently closed circular DNA (cccDNA), which is “chromatinized” to form a long-lived life viral minichromosome that serves as the main template for all viral RNA transcript (pre-C, pregenomic [pg], pre-S, S, and X) synthesis. From these transcripts, seven proteins are translated:

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HBeAg (hepatitis B e antigen; secreted dimer protein); HBV Pol (viral polymerase); HBc/core (capsid protein); the large (which contains PreS1, PreS2, and S domains), medium (which contains PreS2 and S domains), and small surface envelope glycoproteins; and HBx (transcriptional transactivator) (1, 6). The pgRNA is encapsidated in a nucleocapsid and converted into rcDNA by the reverse transcriptase activity of the HBV polymerase, which represents the main replication step. The polymerase-bound pgRNA (Pol-pgRNA) serves as a specific intermediate for the association with several dimers of HBc (nucleation step) from which the fully matured nucleocapsid will arise containing rcDNA (7–9). Mature nucleocapsids containing rcDNA are then either used to produce progeny virions, following envelopment in HBV-envelope-protein-containing cellular membranes, or are redirected to the nucleus to amplify or maintain the cccDNA pool (this process is called “recycling”) (1, 10). The secretion of HBV RNA-containing virion-like particles was also recently reported both *in vitro* and in patient serum. These types of particles, which were shown to be produced in even higher quantity during nucleos(t)ide analogue (NA) treatment, have been very recently described as replication deficient (11). Importantly, the levels of HBV RNA virion-like particles could fluctuate during the natural history of HBV infections and be used as a biomarker of response to CHB treatments (12, 13).

Currently approved CHB treatments are limited to pegylated interferon alpha (Peg-IFN- α) and NAs. A 48-week Peg-IFN- α treatment leads to complete viral suppression (i.e., undetectable HBV DNA in blood) in approximately 25% of patients but is associated with significant side effects (14, 15). NA administration (e.g., tenofovir or entecavir) is well tolerated and induces a strong viral suppression in the majority of patients, but NAs usually have to be taken lifelong to prevent relapses (14, 15). Despite viral suppression, a functional cure (i.e., the loss of serum HBsAg [hepatitis B surface antigen] with or without seroconversion) is achieved in only 10% of treated patients after a 5-year follow up (14, 15). Therefore, the identification of new targets and development of new antiviral strategies are needed.

The core/HBc protein of HBV has recently reemerged as a promising antiviral target due to its multiple functions in the HBV life cycle (16). Indeed, besides its roles in capsid assembly and viral replication in the cytoplasm, nuclear HBc has been reported to modulate cccDNA transcription or posttranscriptional events, as well as host gene expression (17–23). It is also worth noting that HBc contains the major epitopes recognized by T cells during an HBV-targeted host immune response, thus further emphasizing the importance of this viral protein (1). Molecules targeting HBc, now generically named capsid assembly modulators (CAMs) or core protein allosteric modulators, are being developed as novel direct-acting antivirals. CAMs either induce the formation of morphologically intact empty capsids, referred to as class I mechanism of action (MoA) compounds (e.g., phenylpropanamide derivatives such as AT130 [24, 25] and sulfamoylbenzamides derivatives [26]) or the formation of aberrant empty structures, referred to as class II MoA compounds (e.g., heteroarypyrimidines [HAPs] such as Bayer41-4109 [here abbreviated BAY41] [27, 28]). Altogether, they mainly act by preventing and/or blocking the encapsidation of the Pol-pgRNA complex and thus its reverse transcription into rcDNA (29, 30). HAPs were the first CAMs demonstrating potent antiviral activity *in vitro* (31) and *in vivo* in both HBV transgenic mice (32) and HBV-infected chimeric mice (33). Importantly, CAMs efficiently inhibit replication of HBV mutants resistant to NAs (30, 34) and are active against multiple HBV genotypes (35). Moreover, CAMs inhibit the production of extracellular RNA-containing particles (36) and prevent *de novo* formation of cccDNA by acting at a postentry step (37). The inhibition of the establishment of HBV infection could be due to an accelerated breakdown of capsid in cytoplasm before rcDNA could be delivered to nucleus and converted into cccDNA or to a stabilization of the capsid structure, which would prevent uncoating at the nuclear pore (38, 39). In addition, it was suggested that CAMs could interfere with the nuclear functions of HBc, including the regulation of cccDNA transcription (40) and host gene expression (19). To date, several CAMs are in preclinical evaluations or have entered clinical trials, including GLS-4 (trial with China-CFDA;

morphothiadine mesilate, HEC Pharm), NVR 3-778 (NCT02112799; Novira Therapeutics, now part of Janssen), JNJ-56136379 (NCT02662712; Janssen), ABI-H0731 (NCT02908191; Assembly Biosciences), and RO7049389 (NCT02952924; Hoffmann-La Roche).

The aim of this study was to determine whether CAM antiviral effects can extend beyond their two well-described MoA compounds *in vitro*, which are the inhibition of HBV replication and *de novo* cccDNA formation. To answer this question, two novel CAMs, JNJ-827 and JNJ-890, were profiled *in vitro*. Altogether, our data demonstrate that some CAMs could interfere with multiple functions of HBc during the viral life cycle.

RESULTS

***In tubo* and *in cellulo* effects of JNJ-827 and JNJ-890 on capsid assembly.** CAMs were reported to induce either the formation of morphologically intact HBV capsids (class I MoA compounds) (41) or the formation of pleiomorphic/aberrant structures (class II MoA compounds) (28). In this study, two novel CAMs, JNJ-61030827 and JNJ-54138890 (Fig. 1A), abbreviated here as JNJ-827 and JNJ-890, were tested for their anti-HBV activity and cytotoxicity. Before testing them for their antiviral effect in an infectious model, electron microscopy (EM) and size exclusion chromatography (SEC) analyses were performed to categorize JNJ-827 and JNJ-890 as having a class I or II MoA compounds. The compounds were incubated together with the recombinant HBV core assembly domain (amino acids 1 to 149) for 24 h in the presence of 150 mM NaCl before visualization by EM. Images were compared to those obtained in our previous study (37) with the phenylpropenamide AT130 (25) and the heteroaryldihydropyrimidine Bayer-41-4109 (here abbreviated as BAY41) (31) compounds with class I and class II MoA compounds, respectively. EM images showed the formation of morphologically intact capsids in the case of JNJ-827 or aberrant structures after treatment with JNJ-890 (Fig. 1B). SEC analysis confirmed these observations (Fig. 1C). The control sample without compound (dimethyl sulfoxide [DMSO]) showed, as expected, two absorbance peaks, one corresponding to capsid and the other to HBc dimers. Control class I MoA compound AT130 increased the formation of capsid as expected, whereas control class II MoA compound BAY41 altered the retention time, indicating the formation of aberrant structures. With JNJ-827, a unique peak was observed at the elution volume corresponding to morphologically intact capsids, whereas a peak was observed at the elution volume corresponding to aberrant structures for the sample treated with JNJ-890. Two inactive compounds (inactive Cp#1 and Cp#2) did not change the SEC profile. Altogether, these studies indicate that JNJ-827 is a class I CAM and that JNJ-890 is a class II CAM.

To study the effect of both CAMs on capsid assembly *in cellulo*, differentiated HepaRG (dHepaRG) cells were infected with HBV and then treated from day 7 postinfection for a total of 7 days, during which the compound was refreshed three times. Intracellular capsid formation was visualized by a native agarose gel migration assay. A cell lysate from HepG2.2.15 cells was used as a positive control. As expected, capsid formation was not affected with tenofovir (TDF), whereas there was no band migrating at that same height with BAY41 and JNJ-890 (Fig. 1D). Capsid formation was increased by 2-fold upon AT130 treatment, whereas JNJ-827, which was classified as class I, induced a 2-fold decrease in capsid formation (Fig. 1E).

In vivo studies investigating the subcellular distribution of HBc have reported both cytoplasmic and nuclear localization (42, 43). Here, we wanted to know whether CAM treatment could affect HBc subnuclear localization. To address this question, the cellular distribution of HBc was investigated by immunostaining using an antibody, which solely recognizes HBc as part of a capsid and not its monomeric or dimeric form (44). Assembled HBc was mainly found in the nucleus of nontreated HBV-infected dHepaRG (see Fig. S1 in the supplemental material). In untreated (NT) and in TDF- and AT130-treated cells, HBc staining was diffuse in the nucleus, whereas the BAY41 and JNJ-890 treatments induced HBc clustering (see Fig. S1 in the supplemental material). Interestingly, these HBc clusters colocalized with large nuclear bodies containing the

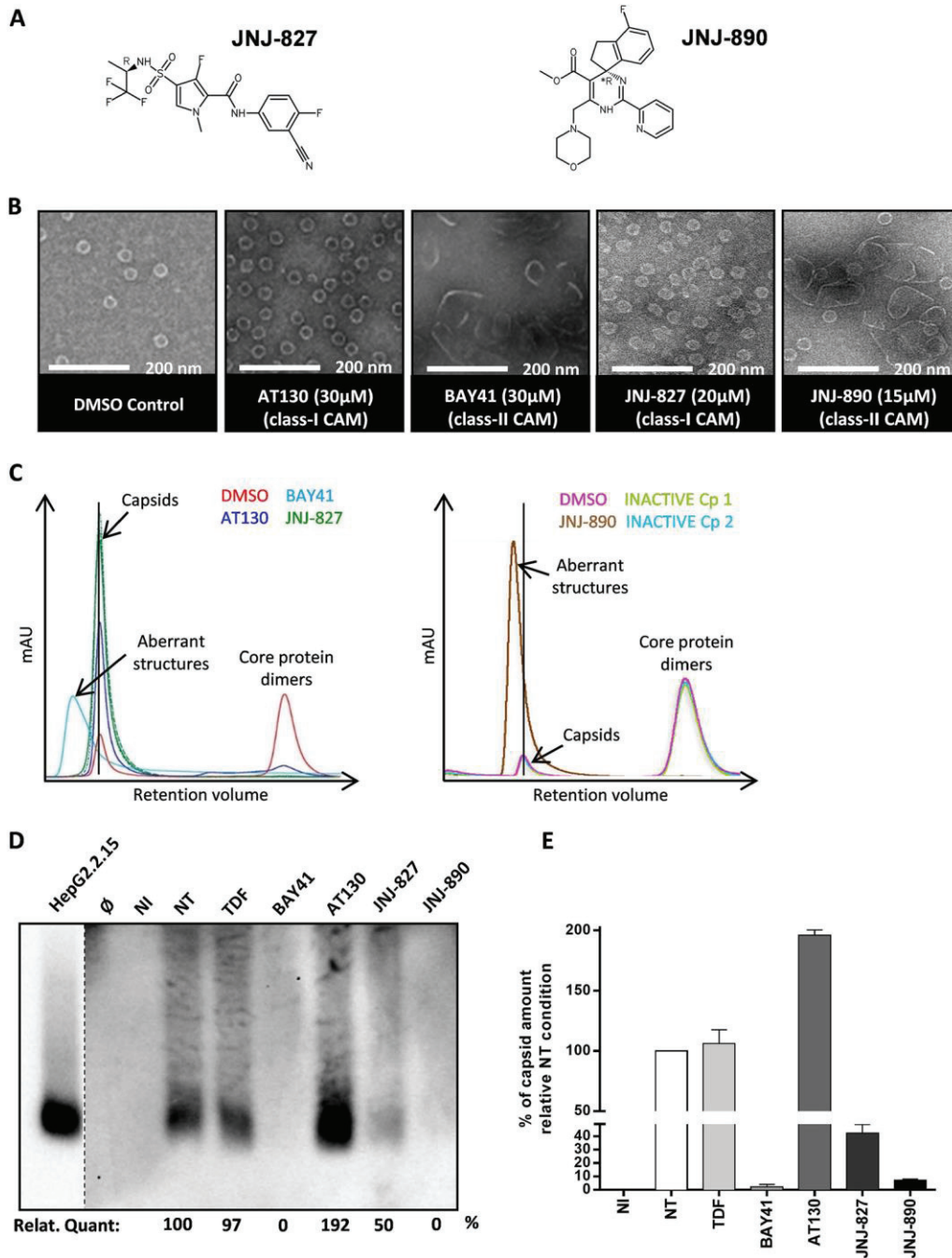


FIG 1 Effect of JNJ-827 and JNJ-890 on capsid assembly *in tubo* and *in cellulo*. (A) Structures of JNJ-827 and JNJ-890. (B) EM images of *in tubo* capsid formation obtained using the indicated amounts of molecules as described in Materials and Methods. (C) Graphs of SEC analyses performed with the same amounts of compounds as in panel B. Compound 1 (Cp 1) and Cp 2 correspond to inactive controls of JNJ-827 and JNJ-890. (D) Differentiated HepaRG were infected with HBV (500 virus genome equivalent [vge]/ml) and then treated from day 7 postinfection for 7 days every 2 or 3 days (three treatments) with 10 µM concentrations of the indicated compounds. Noninfected (NI) and infected not treated (NT) controls were also added. Protein lysates were loaded and run in native conditions on an agarose gel, the proteins were transferred onto an enhanced chemiluminescence membrane, and Hbc was detected by chemiluminescent immunoblotting with an anti-Hbc antibody. The blot is representative of three experiments. (E) Quantification (means plus the standard errors of the mean [SEM]; *n* = 3) of the capsid signal by chemiluminescence determined using a ChemiDoc XRS+ System (Bio-Rad).

promyelocytic leukemia (PML) protein and the proteasomal 20S subunit, two proteins associated with sites of protein modification/degradation (45). An intermediate phenotype was obtained with JNJ-827. Altogether, the data showed that CAM treatment can affect Hbc nuclear localization and capsid formation in infected hepatocytes.

TABLE 1 Anti-HBV activity and cytotoxicity in HepG2.117 and HepG2.2.15 cells

Compound	Type	Mean (range) EC ₅₀ (nM)	Mean (range) CC ₅₀ (nM)		Selectivity index	
		HepG2.117 or HepG2.2.15 cells	HepG2.117/2.15	HepG2	HepG2.117/2.15	HepG2
AT130	Intra	1,020 (120–4,620), <i>n</i> = 24	ND	>50,000, <i>n</i> = 11	ND	>49
	Extra	127 (<98–207), <i>n</i> = 4			ND	>393
JNJ-827	Intra	4.7 (2.1–11), <i>n</i> = 55	>25,000 (>5,000–>25,000), <i>n</i> = 3	>25,000, <i>n</i> = 11	>5,319	>5,319
	Extra	5.7 (<3.9–9.8), <i>n</i> = 11			>4,386	>4,386
BAY41	Intra	67 (21–216), <i>n</i> = 467	33,900 (21,400–>50,000), <i>n</i> = 3	35,400 (10,600–>120,000), <i>n</i> = 56	506	528
	Extra	101 (25–216), <i>n</i> = 99			336	350
JNJ-890	Intra	66 (63–70), <i>n</i> = 2	ND	>100,000, <i>n</i> = 4	ND	>1,515
	Extra	<39, <i>n</i> = 2			ND	>256

^aThe anti-HBV activity of AT130, JNJ-827, BAY41, and JNJ-890 in stable HBV-replicating cells was tested in a dose-response assay. Intracellular (HepG2.117 cells) and extracellular (HepG2.2.15 cells) HBV DNA was extracted, and DNA levels were assessed using qPCR. In each experiment, the EC₅₀s were determined based on the mean inhibition from two wells per compound concentration. The cytotoxicity for HepG2 cells was assessed using a resazurin readout. EC₅₀, 50% effective concentration; CC₅₀, 50% cytotoxic concentration; Intra, intracellular; Extra, extracellular; *n*, number of experiments; ND, not done.

Anti-HBV activity and cytotoxicity of JNJ-827 and JNJ-890. The half-maximal effective concentration (EC₅₀) was determined in HepG2.117 cells (intracellular EC₅₀ [iEC₅₀]) and in HepG2.2.15 cells (extracellular EC₅₀ [eEC₅₀]) after quantification of total intracellular or extracellular HBV DNA by quantitative PCR (qPCR). The mean iEC₅₀ values for JNJ-827, JNJ-890, BAY41, and AT130 were 4.7, 66, 67, and 1,020 nM, respectively, whereas the eEC₅₀ values were 5.7, <391, 101, and 127 nM, respectively (Table 1). Moreover, we observed in infected dHepaRG cells a dose-dependent reduction in the amount of intracellular HBV DNA, without cytotoxicity and cell dedifferentiation, as measured by the cellular secretion of the apolipoprotein B (ApoB) (see Fig. S2 in the supplemental material). JNJ-827, JNJ-890, and AT130 did not show cytotoxicity in HepG2 cells at any of the concentrations tested (>25, >100, and >50 μM, respectively), whereas the 50% cytotoxic concentration (CC₅₀) of BAY41 was 33.9 μM (Table 1). The selectivity index (SI) values (i.e., the CC₅₀/iEC₅₀ value) for JNJ-827 and JNJ-890 in HepG2 cells were >5,319 and >1,515, respectively (Table 1). For the remainder of the mechanistic studies, CAMs were used at a single, noncytotoxic concentration of 10 μM. **Ex vivo effect of JNJ-827 and JNJ-890 in infected PHHs.** Primary human hepatocytes (PHHs) represent the golden-standard in terms of evaluating drug metabolism and hepatotoxicity. Moreover, the susceptibility of the cell culture model to HBV infection makes it ideal for studying viral replication *ex vivo* (46). Three different batches of freshly isolated PHHs were infected with HBV and treated three times with JNJ-827, JNJ-890, control CAMs, and TDF from day 5 postinfection for a total of 7 days. JNJ-827 and JNJ-890 reduced total intracellular HBV DNA by 80 and 60%, respectively, whereas only weak decreases were observed with BAY41 and AT130, and 90% intracellular HBV DNA reduction was obtained with TDF (Fig. 2A). Interestingly, JNJ-827 and JNJ-890 induced also a weak but significant reduction in intracellular total HBV RNA levels, whereas JNJ-890 was the sole compound able to significantly reduce PreC/pg-RNA by 2-fold (Fig. 2A). All treatments had no effect on the established pool of cccDNA in this short treatment duration setting.

At the extracellular level, TDF, as already reported for lamivudine (36), induced a >90% reduction of secreted HBV DNA, along with an accumulation of secreted HBV RNA (Fig. 2B). Among all the CAMs tested, JNJ-827 was the only compound able to strongly decrease both secreted HBV RNA and DNA levels. Moreover, JNJ-827 was the sole CAM observed to affect HBeAg secretion, whereas none of the molecules affected HBsAg secretion (Fig. 2B). The EC₅₀ of JNJ-827 regarding HBeAg secretion inhibition was around 5 μM (see Fig. S3 in the supplemental material). None of the molecules tested were toxic at the concentrations tested, as indicated by the unchanged levels of HBsAg and ApoB in supernatants (Fig. 2B). Note that results obtained with the three individual batches of PHHs, for cccDNA as well as for secreted viral and ApoB parameters, are also presented as absolute quantifications in Fig. S4 in the supplemental material.

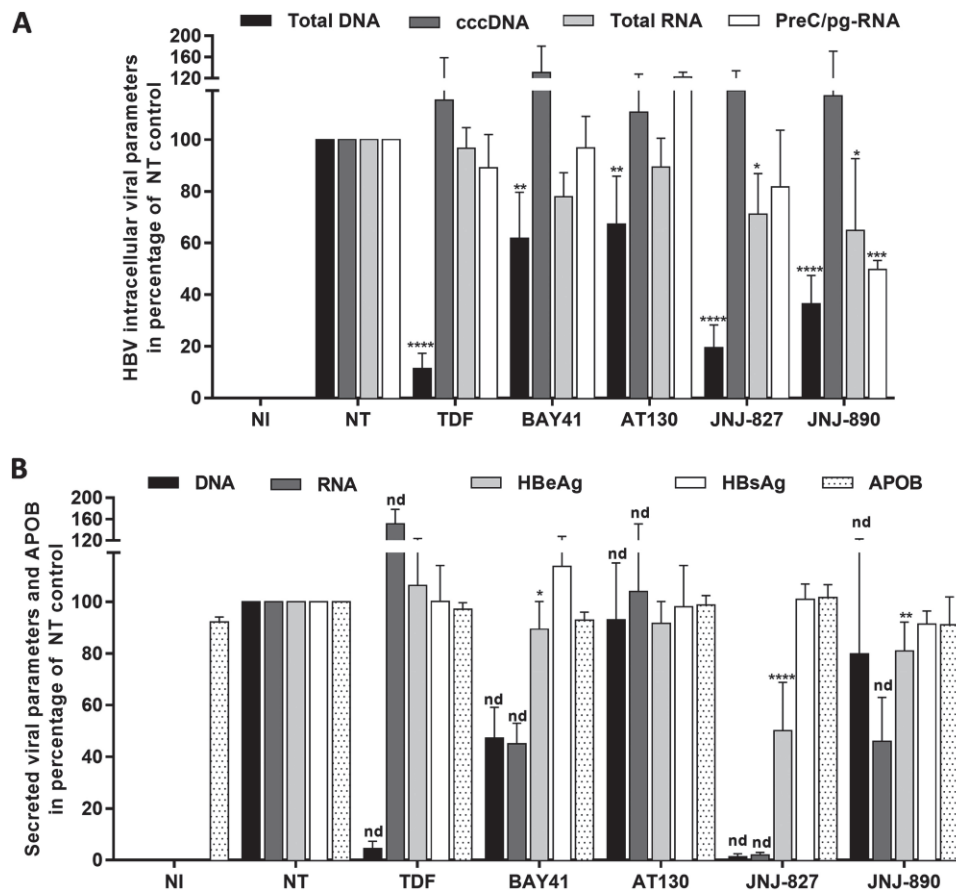


FIG 2 Antiviral properties of JNJ-827 and JNJ-890 in PHHs. PHHs were mock infected (NI condition) or infected with HBV (200 vge/ml), cultivated during 5 days postinfection, and then not treated (NT condition) or treated three times for 7 days (treatment every 2 or 3 days) with 10 μ M concentrations of the indicated compound. (A) Intracellular HBV total DNA, cccDNA, total RNA, and PreC/pgRNA levels were quantified by qPCR, qPCR FRET (fluorescence resonance energy transfer), and quantitative reverse transcription-PCR (RT-qPCR), respectively. (B) The HBeAg, HBsAg, and ApoB levels in cell culture supernatant were monitored by ELISA. Extracellular HBV DNA and RNA levels were quantified by qPCR or RT-qPCR after nucleic acid extraction from supernatant. The results are means plus the SEM ($n = 3$) performed with three different PHH donors. Statistics were determined using a two-tailed Mann-Whitney test, with the following calculated probabilities: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$; and nd, not determined.

Altogether, these results identified JNJ-827 as having the most pronounced effects among the CAMs tested. Moreover, JNJ-827 and JNJ-890 featured interesting novel properties that were further investigated in the rest of the study.

Effect of JNJ-827 and JNJ-890 on the formation of cccDNA in a *de novo* infection setting.

It was recently described that CAMs could also prevent cccDNA formation, thus defining a secondary MoA compound of these molecules based on a postentry mechanism (37). To evaluate JNJ-827 and JNJ-890 with respect to this MoA compound and compare with control CAMs or the PreS1 peptide (i.e., an entry inhibitor), which is known to inhibit HBV entry by interfering with hNTCP binding (47), compounds were added 1 day before and during viral inoculation of PHHs or dHepaRG cells. This is in contrast to what was done in previous experiments (Fig. 2) that aimed at evaluating the postinfection action of these molecules. TDF was added 24 h postinfection and used here as a technical control for cccDNA quantification, since NAs do not alter the cccDNA level. cccDNA quantification was performed at day 7 postinfection. None of the drugs were toxic in this setting at the concentration tested, as shown by an absence of variation in ApoB secretion (Fig. 3B and D). As expected, the PreS1 peptide prevented viral entry thus preventing cccDNA establishment and subsequent synthesis of viral intermediates (Fig. 3A). Interestingly, JNJ-827 treatment also reduced cccDNA formation by >90%, resulting in a concomitant

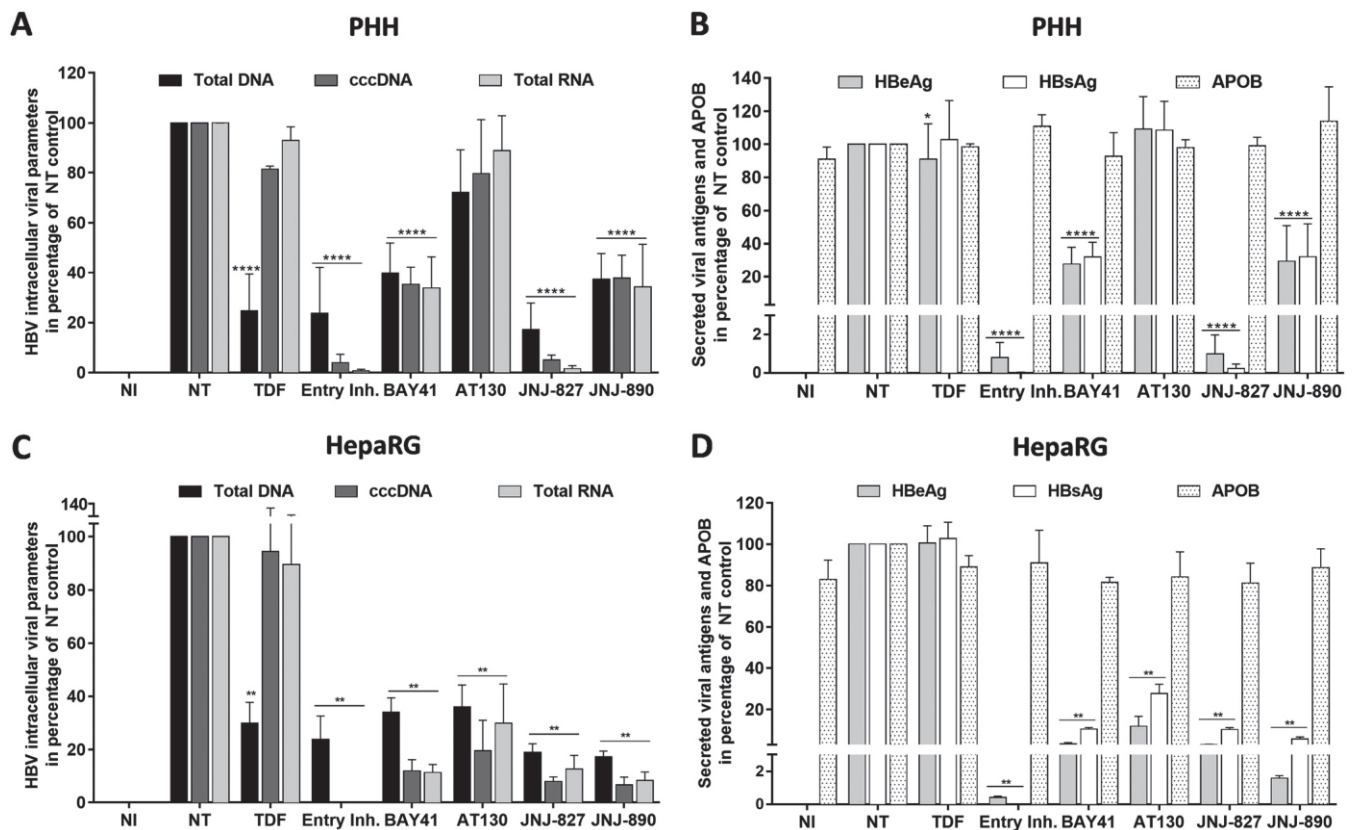


FIG 3 Effect of JNJ-827 and JNJ-890 on HBV infection establishment in dHepaRG cells and PHHs. PHH (A and B) or dHepaRG (C and D) cells were pretreated for 24 h with 10 μ M concentrations of the indicated CAMs or 100 nM preS1 peptide entry inhibitor and then inoculated with HBV (200 vge/ml) in the presence of drug for 24 h. One day after infection, the medium was changed, and the cells were cultured for 1 week. For the TDF control, cells were treated twice after infection. A noninfected (NI) control was included. (A and C) Intracellular HBV total DNA, cccDNA, and total RNA levels were quantified by qPCR, qPCR FRET, and RT-qPCR, respectively. (B and D) HBeAg, HBsAg, and ApoB levels in cell culture supernatant were monitored by ELISA. The results are means plus the SEM ($n = 3$) performed with three different PHH donors or three different differentiations of HepaRG. Statistics were determined using a two-tailed Mann-Whitney test, with the following calculated probabilities: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; and ****, $P < 0.0001$.

decrease of intracellular HBV DNA and RNA levels (Fig. 3A), as well as HBeAg and HBsAg secretion (Fig. 3B). JNJ-890 and BAY41 were found to be far less potent than JNJ-827, whereas AT130 was similarly inefficient as TDF at preventing cccDNA establishment. The EC_{50} s of JNJ-827 for cccDNA establishment in PHH were between 19 and 61 nM and around 100 nM for HBV total DNA, HBeAg, and HBsAg (see Fig. S5 in the supplemental material). Phenotypically, JNJ-827 and the PreS1 peptide behaved the same, indicating that a bona fide entry or a postentry inhibition could be equal in terms of antiviral effect. In the dHepaRG model, all CAMs were found to be efficient to inhibit cccDNA establishment (Fig. 3C), replication intermediates (Fig. 3C), and antigen production (Fig. 3D), suggesting that the compounds might be differentially metabolized in both cell types. Note that results obtained with the three individual batches of PHHs and three independent HepaRG differentiations, regarding cccDNA as well as secreted HBe/ HBsAg and ApoB parameters, are also presented in absolute quantification in Fig. S6 and S7 in the supplemental material.

Long-term treatment with JNJ-827 and JNJ-890 affects viral RNA accumulation. Short-term postinfection treatment with JNJ-827 and JNJ-890 in PHHs suggested a modest effect on viral RNA accumulation (Fig. 2A). It was previously speculated that CAM treatment could impact cccDNA transcription and/or posttranscriptional events (40). To further analyze this potential MoA of JNJ-827 and JNJ-890 compared to other CAMs, we made use of dHepaRG that can be maintained for weeks or months in culture in contrast to PHHs, which quickly dedifferentiate when cultured *ex vivo*. dHepaRG cells were infected with HBV and then treated from day 7 postinfection either for a total of 7 days (total of 3 administrations) or 1 month (total of 15 administrations). Under short-term conditions using dHepaRG, all CAMs induced a strong reduction of intracellular HBV DNA accumulation (Fig. 4A), which is in line with the observed, though less

pronounced, effects in PHHs, most likely due to different metabolization properties of each cell type (compare Fig. 4A with Fig. 2A). This inhibition of nucleocapsid assembly was translated into a strong inhibition of secreted HBV DNA and RNA, yet without any effect on HBsAg and ApoB, secretions (Fig. 4B). Regarding the effect on intracellular RNA accumulation, only JNJ-890 led to a weak reduction in dHepaRG (Fig. 4A). It is also worth noting that a significant reduction in HBeAg secretion was observed with both JNJ-827 and JNJ-890, in the absence of any correlative inhibition of HBsAg (Fig. 4B). Such an inhibition was also observed in the PHH model for JNJ-827 (Fig. 2B), suggesting a peculiar feature of JNJ-827 and JNJ-890 with respect to HBeAg biogenesis and secretion.

In the long-term setting, JNJ-827 and JNJ-890 were able to reduce both intracellular total HBV RNA and PreC/pgRNA levels (Fig. 4C), as well as antigens and HBV DNA/RNA secretions (Fig. 4D). Intracellular total HBV RNA and PreC/pgRNA levels were also impacted by BAY41 and AT130, but to a lesser extent. All CAMs induced a strong inhibition of secreted HBV DNA and RNA, whereas TDF gave rise to an expected profile, i.e., decreasing DNA and increasing RNA secretion. JNJ-890 induced a weak but significant reduction of ApoB secretion (Fig. 4D). Altogether, these data suggest that JNJ-827 and JNJ-890 could reduce HBV RNA synthesis/stability in a long-term treatment context and that this reduction can be associated with a decrease in HBsAg production. Unfortunately, in dHepaRG the amount of cccDNA is very low (<0.05 copy/cell), making a reliable quantification of this parameter not possible. Note that results obtained with the three independent HepaRG differentiations in short- and long-term treatment conditions, regarding secreted HBe/HBsAg and ApoB parameters, are also presented in absolute quantification in Fig. S8 and S9 in the supplemental material.

Effect of JNJ-827 and JNJ-890 on HBeAg biogenesis and/or secretion. In both PHH and dHepaRG cells and a short-term treatment setting, we observed a reduction in HBeAg secretion with JNJ-827 without intracellular total HBV RNA decrease, leading us to speculate that this CAM has putative effects on HBeAg biogenesis/secretion. To specifically address this question, a HepaRG cell line expressing HBeAg alone in a tetracycline-dependent manner was used (HepaRG-TR-HBe). First, we confirmed that HBeAg secretion increased with the tetracycline amount (see Fig. S10A in the supplemental material). We choose to use 1 $\mu\text{g/ml}$ of tetracycline, for which the HBeAg secretion level (about 150 NCU/ml) was comparable to what we obtained after an infection of dHepaRG cells. By native agarose gel migration assay using also a HepaRG-TR-HBc cell line as a positive control, we confirmed that the HepaRG-TR-HBe cells do not express HBc and as such do not produce capsid (see Fig. S10B in the supplemental material). HepaRG-TR-HBe cells were induced to secrete HBeAg for 1 day and then treated for 5 days (total of two treatments). We observed a reduction in HBeAg secretion of >90% with JNJ-827 (Fig. 5A). The same observation was made with other CAMs tested, but to a lower extent. As expected, TDF had no effect on HBeAg secretion. Note that intracellular quantification of p22/pre-HBeAg by enzyme-linked immunosorbent assay (ELISA) led to the same ranking of compounds, although with a lower level of inhibition (Fig. 5B).

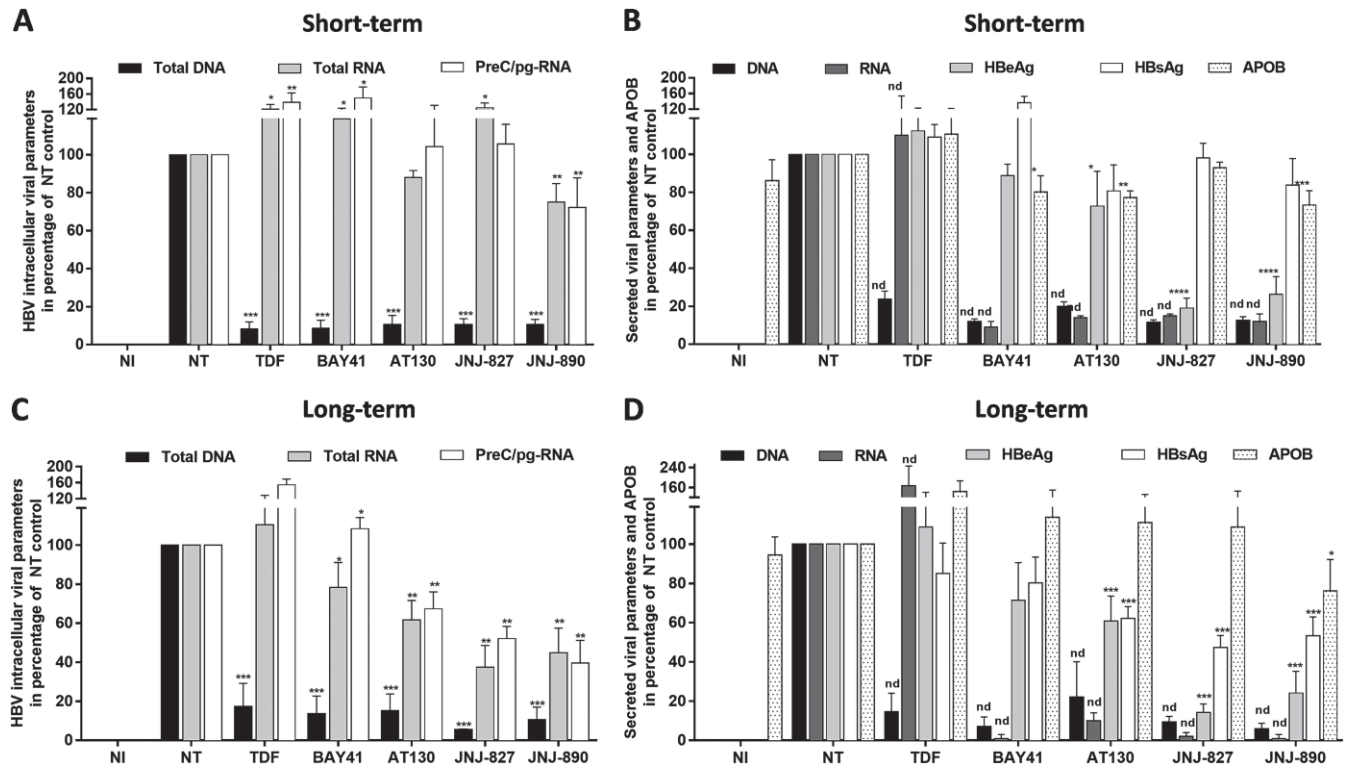


FIG 4 Antiviral properties of JNJ-827 and JNJ-890 in dHepaRG cells: short-term versus long-term treatment. dHepaRG were infected with HBV (200 vge/ml), cultured for 1 week, and then treated for 7 days (treatment each 2 or 3 days; 3 treatments in total) (A and B) or 1 month (treatment each 2 or 3 days; 15 treatments in total) (C and D) with a 10 μ M concentration of the indicated compound. The antiviral effects of the compounds were monitored at endpoint. (A and C) Intracellular HBV total DNA, total RNA, and PreC/pgRNA levels were quantified by qPCR and RT-qPCR. (B and D) HBeAg, HBsAg, and ApoB levels in cell culture supernatant were monitored by ELISA. Extracellular HBV DNA and RNA levels were quantified by qPCR or RT-qPCR after nucleic acid extraction from the supernatant. The results are means plus the SEM ($n = 3$) performed with three different differentiations of HepaRG. Statistics were determined using a two-tailed Mann-Whitney test, with the following calculated probabilities: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$; and nd, not determined.

To further extend the analysis to the primary level of p22/pre-HBe/HBeAg expression, supernatants were also submitted to denaturing Western blotting after trichloroacetic acid precipitation to confirm that the observed effect was genuinely due to an inhibition of HBeAg secretion and not to a problem of detection of the protein by ELISA. At the intracellular level, p22/pre-HBeAg amounts were only affected by BAY41 treatment, whereas other CAMs only reduced extracellular accumulation (Fig. 5Ci, ii, and iii). Altogether, these data suggest that all CAMs tested, except for BAY41, do not tend to prevent the primary expression of p22/pre-HBeAg, while some of them can also potentially interfere with HBeAg biogenesis/secretion. In this respect, JNJ-827 was the most potent in this assay (i.e., use of HepaRG-TR-HBe), and this potency can translate into an inhibition of HBeAg secretion in HBV-infected PHHs (Fig. 2), suggesting that this molecule has a particular feature.

DISCUSSION

Treatment of CHB is limited to the use of NAs and Peg-IFN- α . Unfortunately, these therapeutic options lead to a functional cure (i.e., HBsAg loss with or without HBsAg to HBsAb seroconversion) in only 10% of cases after a 5-year follow-up (14, 15). Therefore, there is a need for new therapeutic options.

The core/HBc protein plays multiple roles during the HBV life cycle (e.g., forms nucleocapsid, interacts with cccDNA and human host genes, and may be involved in the regulation of their transcription) (1, 17, 19, 20). Interfering with the multiple functions of HBc using a direct antiviral agent represents a promising strategy to disrupt HBV replication at several steps in the viral life cycle. Currently, several CAMs are being evaluated in clinical trials, and many others are in preclinical development (additional information is available [<http://www.hepb.org/treatment-and-management/drug-watch/>]).

JNJ-827 and JNJ-890 are two novel CAMs. SEC and EM studies revealed that JNJ-827 possesses a class I MoA compound, whereas JNJ-890 possesses a class II MoA compound. However, and in contrast to the hallmark of a class I MoA compound, JNJ-827 was capable of reducing the amount of intracellular capsid by 2-fold, as visualized on native agarose gel. This discrepancy might be explained by the much higher potency of JNJ-827 compared to the other class I CAM in the panel, AT130 (217-fold less active than JNJ-827 in HepG2.117). For the JNJ-890 and BAY41, the iEC_{50} s were similar.

In the present study, CAMs were mostly used at a high concentration (10 μ M) to highlight novel antiviral effects and MoA. In HBV-infected PHHs we confirmed that JNJ-827 and JNJ-890 possess the two described antiviral effects of CAMs: inhibition of HBV replication in already established infections (primary mechanism) and inhibition of cccDNA *de novo* formation (secondary mechanism) (37, 38). JNJ-827 was the most potent CAM tested in stably HBV-transformed HepG2 cell lines, with an EC_{50} in the single-digit nanomolar range. Like the PreS1 peptide entry inhibitor, JNJ-827 inhibited cccDNA formation with an EC_{50} around 50 nM. BAY41 and JNJ-890 were similarly efficient in this model. The improved efficacy of JNJ-827 at inhibiting HBV infection by a postentry mechanism was likely due to the higher potency of JNJ-827 (iEC_{50} at 4.7 nM), as reflected by the lack of inhibition with AT130 (iEC_{50} at 1,020 nM). It is worth noting that all CAMs, including AT130, were similarly efficient at preventing HBV infection in the dHepaRG model. This can either point to the difference in metabolic properties of these cells compared to PHH or the lower infection rate in dHepaRG cells compared to PHHs at the same multiplicity of infection (as an indication, HBsAg production ranges between 50 and 100 IU/ml in dHepaRG versus 1,000 to 5,000 IU/ml in PHHs when a multiplicity of infection of 100 genome equivalent of HBV/cell is used). The concentration necessary to achieve a 50% reduction of the formation of cccDNA (EC_{50} for cccDNA formation inhibition) was 10 times higher than that necessary to achieve the main inhibitory action (i.e., inhibition of HBV DNA neosynthesis). It remains to be determined whether this inhibition of the establishment of HBV infection is due to an accelerated breakdown of capsid in cytoplasm before rcDNA could be delivered to nucleus and converted into cccDNA (38, 39) or to a prevention of disassembly at the nuclear pore. In the first case, it would be interesting to determine whether rcDNA could serve as a pathogen-associated molecular pattern for innate sensing by the dsDNA sensing machinery.

In postinfection and short-term treatment conditions, among all CAMs tested, JNJ-827 was the most potent at inhibiting intracellular HBV replication (i.e., neosynthesized rcDNA) in PHHs. Very importantly, as reported both for NVR-3-1983 (36) *in vitro* and NVR-3-778 (48) *in vivo*, we found that, in PHHs, JNJ-827 strongly reduced both extracellular HBV DNA and RNA levels, whereas TDF only decreased extracellular HBV DNA levels (and increased the HBV RNA level), and other CAMs tested were inefficient. This is due to the fact that JNJ-827, in contrast to NAs, blocks the encapsidation of pgRNA, and therefore these results in a reduction of encapsidated DNA and RNA in the cell culture supernatant (11–13, 49). The function of secreted HBV-RNA virion-like particles in HBV life cycle is unclear. HBV RNA virion-like particles were recently reported to be mainly replication-deficient (11), but they could have immunological functions. Therefore, the inhibition of their release into the blood of CHB patients could be beneficial.

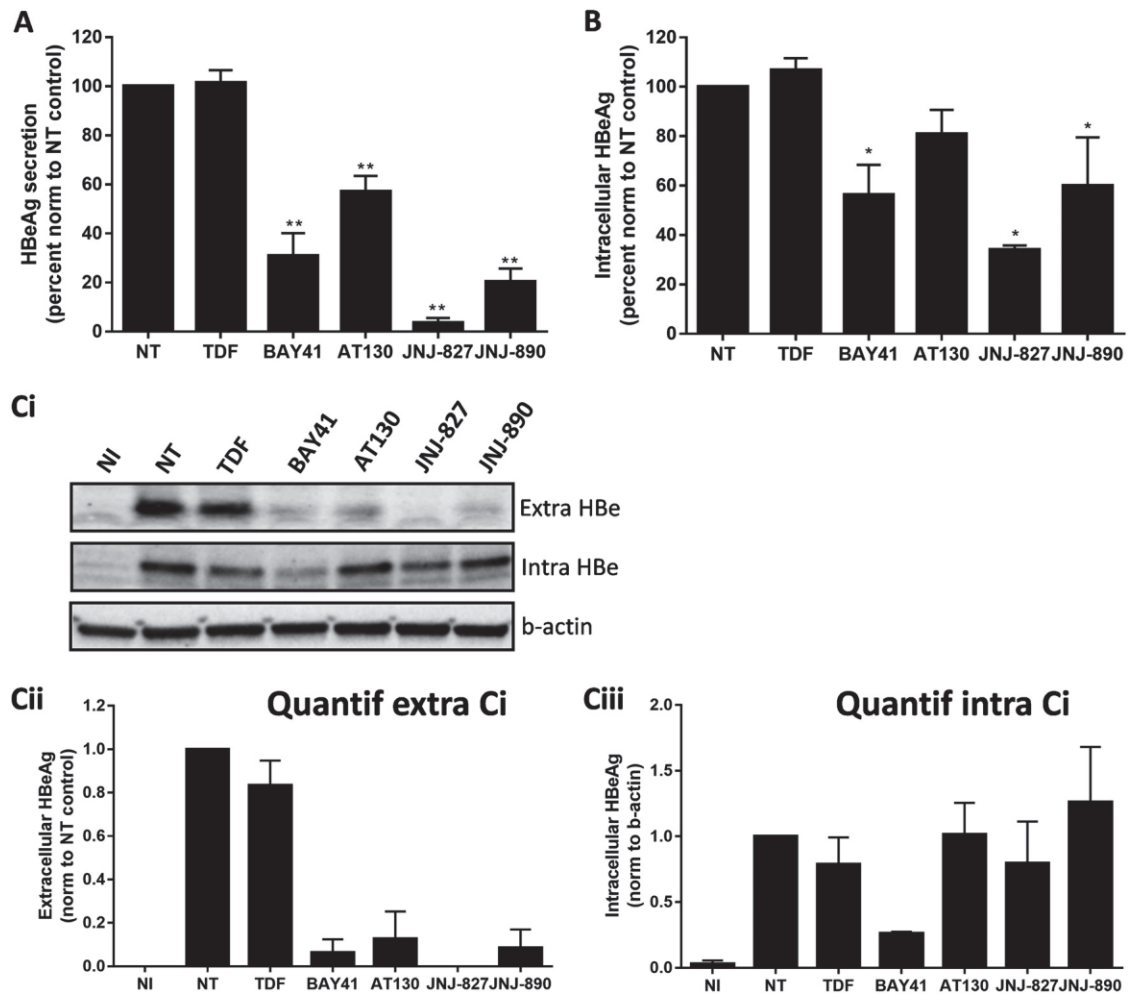


FIG 5 Effect of JNJ-827 and JNJ-890 on HBeAg biogenesis and/or secretion. HepaRG-TR-HBe cells were treated with tetracycline (1 $\mu\text{g/ml}$) for 48 h to allow expression of HBeAg from tetracycline-regulated promoter and then treated for 5 days (two treatments) with 10 μM concentrations of the indicated compounds. (A) The HBeAg secretion level in cell culture supernatant was monitored by ELISA. (B) Intracellular HBeAg amount was also monitored by ELISA. For panels A and B, the results are means plus the SEM ($n = 3$), and the statistics were determined using a two-tailed Mann-Whitney test (**, $P < 0.01$). (Ci) Evaluation of intracellular and extracellular HBeAg levels by immunoblotting (results from a representative experiment [$n = 2$]). (Cii and iii) Quantification of extracellular and intracellular HBeAg signal by chemiluminescence done with a ChemiDoc XRS+ System (Bio-Rad). The results are means plus the SEM ($n = 2$).

Nuclear HBc was suggested to regulate HBV transcription by interacting with cccDNA (17, 22). Interestingly, we found, like a very recently published study (50), that CAM treatment induces the relocalization of nuclear HBc to subnuclear sites staining positive for the 20S proteasomal subunit and PML bodies, which are the sites of protein posttranslational modification, activation, sequestration, or degradation (45). Here, we found that BAY41 treatment led to the formation of large HBc aggregates, whereas AT130 treatment had no effect or only a slight effect on nuclear HBc distribution. These observations correlate with the MoA of the compounds and suggest that CAMs could differentially disrupt nuclear functions of HBc. In this respect, in both dHepaRG and PHH cells, JNJ-890 was the sole CAM capable of decreasing intracellular total HBV RNA and PreC/pgRNA levels after 1 week of treatment. However, after 1 month of treatment of dHepaRG cells, all CAMs tended to decrease total HBV RNA levels, as well as HBeAg and HBsAg secretion, with JNJ-827 and JNJ-890 being again the most potent compounds tested. Even if we cannot exclude an effect on cccDNA amount after a long-term treatment (we failed to reliably detect cccDNA in this experimental context by qPCR-based methods), our observations suggest that HBc could be a positive regulator of cccDNA transcription or/and posttranscriptional events and suggest also that (i) CAMs interfere with the nuclear function of core *in vitro* and that (ii) compound class is not necessarily an indicator of the effect of CAM on HBc nuclear functions; compound potency could also be involved.

HBeAg is a secreted antigen sharing primary structure with HBc. The intracellular precursor of HBe (called p22 or pre-HBe) features 10 additional amino acids at the N-terminal extremity compared to HBc and needs to be processed by both N- and C-terminal maturation to give rise to secreted HBeAg (51, 52). Secreted HBeAg is in fact a dimer of p17 polypeptide. One possible function of secreted HBeAg could be to transiently suppress the immune response against HBV. This could be done by negatively regulating the TLR2 signaling pathway (51, 52) and/or by modulating the immune response against HBc (53, 54). HBeAg seroconversion is an immunological process that occurs during the natural history of HBV infections due to the occurrence of stop or PreC/core promoter mutations in the cccDNA integration events and can be increased upon treatment with Peg-IFN and NAs. It is associated with higher immune activity and is considered an important clinical endpoint during therapy (53, 54). In this study, we observed a strong and fast inhibition of HBeAg biogenesis/secretion by CAMs in the HepaRG-TR-HBe model, more particularly with JNJ-827, which was the only compound to recapitulate this phenotype in PHHs with an EC₅₀ of around 5 μ M. The inhibition is unlikely due to an inhibition of the precursor (p22/pre-HBe) synthesis, since Western blot analyses under denaturing conditions did not reveal any differences, but is likely mediated by a direct interaction of these compounds with the intracellular precursor of HBeAg, leading to an impaired processing and dimerization (i.e., biogenesis of the antigen), as well as to an impaired secretion. Although HBc and HBe monomer share very similar primary structures, the quaternary dimeric form of HBeAg is drastically different from HBc (55). This is a consequence of an intramolecular disulfide bridge between the cysteine 61 (common to HBeAg and HBc) and cysteine 7 (specific to HBeAg) that is specifically added in the lumen of the endoplasmic reticulum, in which HBeAg is translocated during translation (56). HBeAg dimers with the C7–C61 disulfide

bridge do not assemble in capsid-like structures, while the reduced form does in the cytoplasm (56, 57). CAMs target HBc by binding to a hydrophobic pocket at the dimer-dimer interface (58–60). To explain how some CAMs target HBeAg, we can hypothesize that they could force HBeAg dimer-dimer assembly, leading to HBeAg aggregation, as observed for HBc, and could promote a fast degradation.

To summarize, CAMs possess two well-characterized mechanisms of action. They accelerate capsid assembly, which results in an inhibition of HBV replication (primary MoA) and prevent *de novo* formation of cccDNA by acting at a postentry step (secondary MoA). Here, we demonstrated that treatment with some CAMs can extend beyond these two mechanisms. Indeed, some CAMs can directly reduce secreted HBeAg and act also either at transcriptional or posttranscriptional levels, leading to a reduction in both total HBV RNA and PreC/pgRNA accumulation, as well as HBsAg production. These additional effects with some CAMs require further investigation and may lead to the development of second-generation compounds. Due to their multiple and additional effects compared to NAs, one could expect that some CAMs could lead to higher rates of functional cure in CHB patients, and clinical evaluation of this type of

multifunctional CAMs is eagerly awaited.

MATERIALS AND METHODS

Compounds. BAY41, AT130, JNJ-827, and JNJ-890 were provided by Janssen. JNJ-827 and JNJ-890 (see structure in Fig. 1A) were prepared according to methods described in the patent applications WO2014184350 and WO2013102655, respectively. The PreS1 peptide (myristoylated amino acids 2 to 48 from the N-terminal part of PreS1) was synthesized by GenScript, and TDF was obtained from Gilead Sciences (Foster City, CA). All compounds had a high-pressure liquid chromatography-certified purity of >95%.

Antiviral assay using HepG2.2.15 cells. Determinations of the JNJ-827 and JNJ-890 EC₅₀ and EC₉₀ values were performed as previously described (35).

Cytotoxicity assay using HepG2 cells. Determinations of the JNJ-827 and JNJ-890 CC₅₀ and CC₉₀ values were performed as previously described (35).

EM and SEC studies. Determination of JNJ-827 and JNJ-890 effects on capsid formation *in tubo* were performed as previously described (35).

Culture of HepaRG cells and primary human hepatocytes and HBV infection. Human liver progenitor HepaRG cells (61) and an engineered HepaRG-TR-HBe cell line expressing only HBeAg in a tetracycline-dependent manner (obtained by double transduction with lentiviral vectors [from Invitrogen; T-Rex System] containing tetracycline repressor and HBe expression transgenes [a detailed method is available on request]) were cultured as previously described (61). Primary human hepatocytes (PHHs) were isolated from surgical liver resections, after informed consent of patients (kindly provided by M. Rivoire [CLB, Lyon]; agreements DC-2008-99 and DC-2008-101) as previously described (62) and cultured in complete William's medium supplemented with 1.8% of DMSO. Differentiated HepaRG (dHepaRG) cells and PHHs were infected as previously described (63), with HBV genotype D inoculum prepared either from HepG2.2.15 or HepAD38 cells. The multiplicities of infection (expressed as virus genome equivalent/cell) are indicated in the figure legends.

Antiviral treatment of PHHs and dHepaRG cells. JNJ-827, JNJ-890, and TDF (used at 10 μ M), as well as PreS1 peptide (used at 100 nM), were evaluated for their effect on the establishment of HBV infections or on established HBV infections. Under the preinfection condition, CAMs and PreS1 peptide were added 1 day before and during infection. TDF was added twice postinfection. Cells and supernatants were harvested at day 6 postinfection. Under the postinfection condition, PHHs or dHepaRG cells were infected by HBV for, respectively, 5 or 7 days and then treated three times at days 5, 7, and 9 (PHHs) or days 7, 9, and 11 (dHepaRG cells) postinfection. Cells and supernatants were harvested at day 12 or 14 postinfection (i.e., 7 days posttreatment).

Analysis of viral parameters. From cell culture supernatants, secreted HBe and HBs antigens were quantified by ELISA, using a chemiluminescence immunoassay kit (Autobio, China) according to manufacturer's instructions. Extracellular viral DNA and RNA were extracted from cell culture supernatant using MagMAX kit (Thermo Scientific) and MagNAPure (Roche), respectively, according to the manufacturer's protocols and treated either with DNase I or RNase A. Intracellular total HBV DNA or RNA were extracted from infected cells using, respectively, NucleoSpin 96 Tissue or NucleoSpin 96 RNA kits (Macherey-Nagel). RNAs were transcribed into cDNA using the SuperScript III reverse transcriptase (Invitrogen). Real-time PCR for total intracellular HBV DNA and RNA was performed using a LightCycler 480 (Roche) and normalized to PRP, a housekeeping gene, with the following primers: 5'-GCTGACGCAACGCCCACT-3' (HBV-sense), 5'-AGGAGTTCGCGCAGTATGG-3' (HBV-antisense), 5'-TGCTGGGAAGTGCATGAG-3' (PRP-sense), and 5'-CGGTGCA TGTTTCACGATAGTA-3' (PRP-antisense). An HBV standard was used for quantification of extracellular viral DNA and RNA. For cccDNA quantification, total DNA was digested by using Plasmid-Safe DNase (New England Biolabs) for 6 h at 37°C and then subjected to qPCR using a LightCycler 480 Probes master kit (Roche) with a home-made probe-primer mix. cccDNA was normalized to 13-globin quantification. For PreC/pgRNA quantification, qPCR was performed using TaqMan Fast Advanced master mix (Life Technologies) using a home-made probe primer mix. PreC/pgRNA levels were normalized to GUSB using a commercial probe primer mix (Life Technologies, catalog no. 4448491; GUSB: Hs99999908_m1).

ELISA. Apolipoprotein B (ApoB) was quantified in cell culture supernatants using a total human apolipoprotein B ELISA (Alerchek, Inc.) according to the manufacturer's recommendations. The amounts of HBeAg and HBsAg in the supernatant and sera were quantified by commercially available ELISA (Autobio, Ltd., China) according to the manufacturer's instructions. An intracellular HBeAg ELISA was performed after cell lysis in a buffer containing 400 mM potassium acetate, 25 mM KHEPES (pH 7.2), 15 mM magnesium acetate, 1% (vol/vol) NP-40, and 0.5% (wt/vol) deoxycholate supplemented with 1 mM phenylmethylsulfonyl fluoride and 1X protease inhibitor cocktail.

Native agarose gel migration assay and immunoblotting. The intracellular formation/accumulation of HBV nucleocapsid in infected dHepaRG cells was performed from cells lysed in a buffer containing 50 mM Tris-HCl (pH 8), 1 mM EDTA, and 1% (wt/vol) NP-40. Cell lysates were centrifuged for 1 min at 10,000 rpm and 4°C. The supernatants were recovered and incubated at least 2h at 37°C with 10 μ g/ml of a DNase I/RNase A mix in buffer containing 20 mM Mg(CH₃COO)₂. Lysates were loaded into a native 1.2% agarose gel, and electrophoresis was performed in 1X Tris-HCl acetate EDTA (TAE) buffer. Capillary transfer of proteins to ECL membrane was performed in 1X Tris-HCl/NaCl/EDTA (TNE) buffer. Immunoblotting was conducted according to a standard protocol with an antibody against HBeAg (Dako, catalog no. B0586) at 1:1,000.

For the detection of HBeAg in cell supernatants, supernatants were 10X concentrated by trichloroacetic acid (Sigma) precipitation and subjected to immunoblotting with Dako B0586 antibody at 1:1,000. An antibody directed against 13-actin (Sigma-Aldrich, catalog no. A1978) diluted at 1:5,000 was used for the normalization of results.

Immunofluorescence staining. dHepaRG cells were fixed with 2% formaldehyde in 1X phosphate-buffered saline (PBS) for 20 min at room temperature and permeabilized/blocked with 0.1% (vol/vol) Triton X-100 (Sigma), 1% (wt/vol) bovine serum albumin (BSA; Euromedex), and 10% (vol/vol) fetal bovine serum (Thermo Scientific) in 1X PBS at room temperature for 30 min. Primary antibodies against HBeAg (Ab8637; used at 1:500), which only recognizes assembled capsid, promyelocytic leukemia protein (PML; Abcam, catalog no. ab31101; used at 1:200), and the proteasomal 20S subunit (Pierce, catalog no. PA1-977; used at 1:200) were diluted in 1X PBS and incubated 1 h at room temperature. Cells were washed four times with wash buffer (1X PBS with 0.1% Triton X-100) and subsequently incubated at room temperature for 1 h with either Alexa Fluor 488-conjugated goat anti-mouse antibody (Invitrogen) for HBeAg detection or Alexa Fluor 594-

conjugated goat anti-rabbit antibody (Invitrogen) for PML and 20S subunit detections, both diluted at 1:1,000 in 1X PBS. Counterstaining for the detection of nucleic acid was performed using Hoechst 33258 (Life Technologies) diluted at 1:5,000 in the Alexa Fluor mix. Cells were washed four times with wash buffer, and coverslips were mounted using mounting medium (Dako). Images were obtained with a confocal microscope Leica TSC-SP5X.

Statistical analysis. Statistical analyses were conducted using nonparametric Mann-Whitney tests using GraphPad Prism software 6.0. Significance is denoted in the figures as follows: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; and ****, $P < 0.0001$.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/AAC.00835-18>.

SUPPLEMENTAL FILE 1, PDF file, 1.3 MB.

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T.L., A.F., F.Z., and D.D. have nothing to declare. J.M.B., K.V., K.V., and F.P. are employees of Janssen.

Author contributions were as follows: research design, J.M.B., F.P., F.Z., and D.D.; supervision, J.M.B. and D.D.; experiments, T.L., K.V., A.F., K.V., and D.D.; data analysis, T.L. and D.D.; statistics, T.L.; writing of the manuscript, T.L. and D.D.; and editing and approval of the final manuscript, all authors.

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Supplementary figures and figure legends

Novel potent capsid assembly modulators regulate multiple steps of the Hepatitis B virus life-cycle

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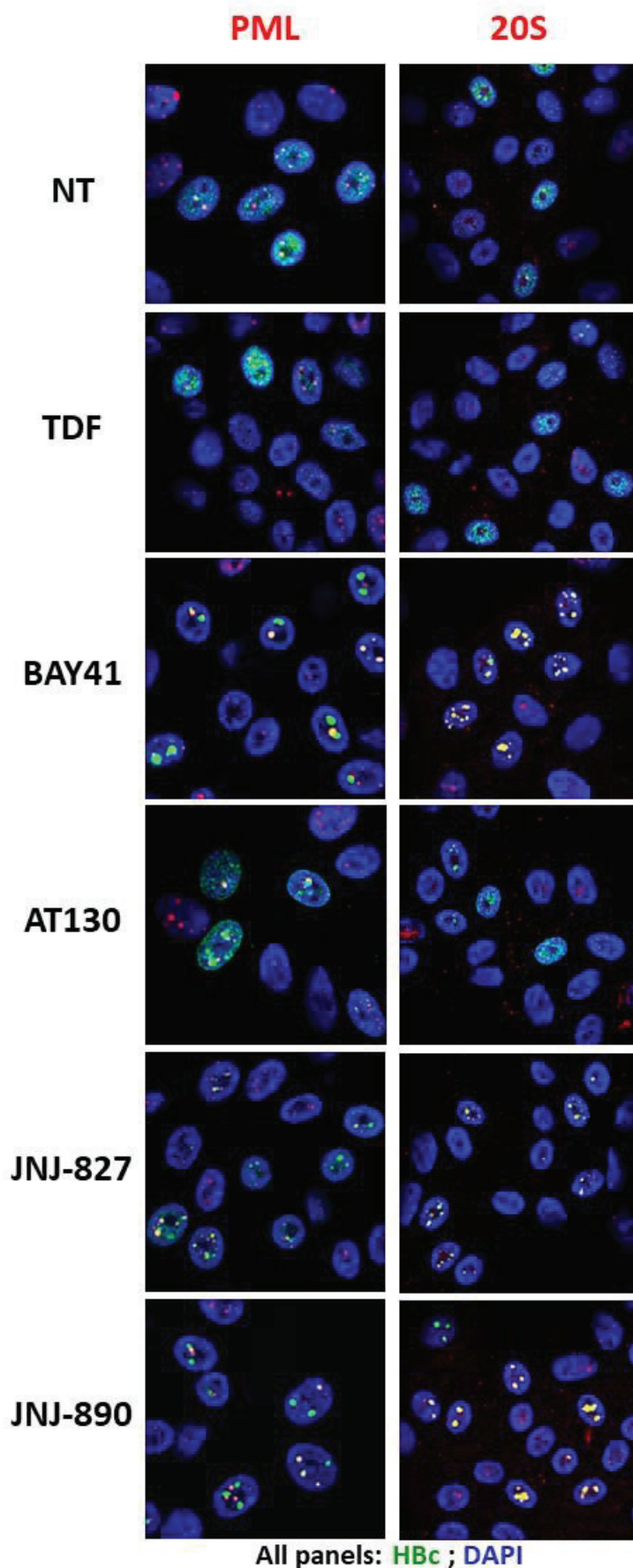
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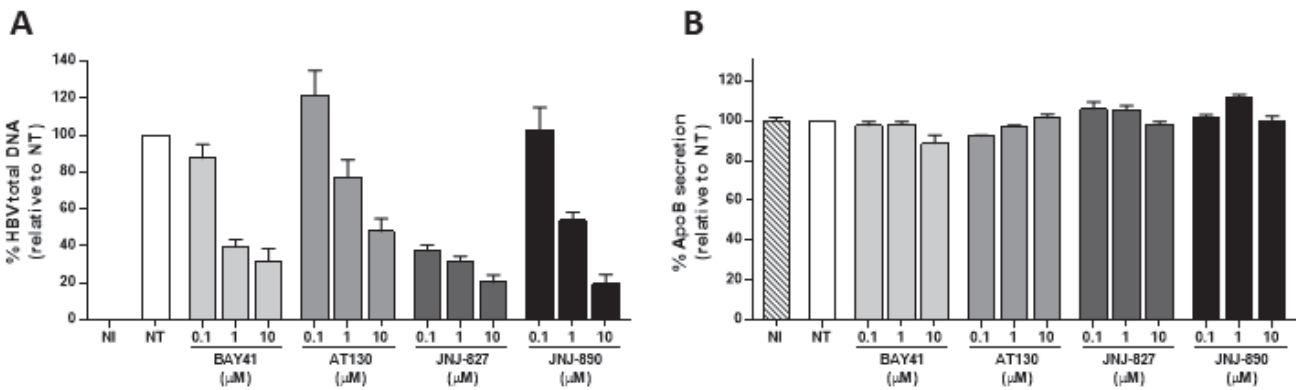
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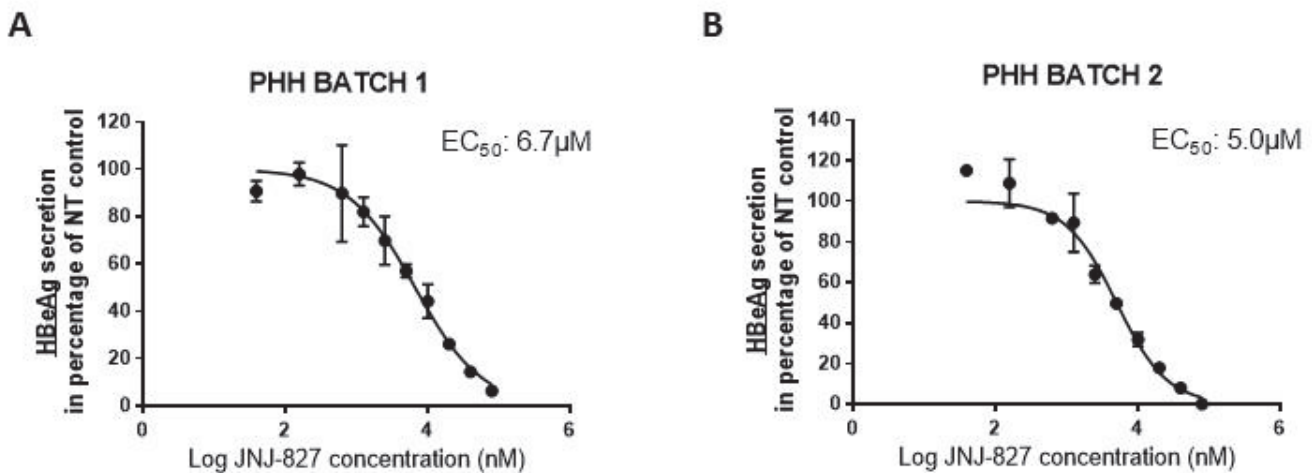


Supplementary figure 1. Effect of JNJ-827 and JNJ-890 on subnuclear localisation of capsids. Differentiated HepaRG were infected with HBV (500 vge/mL), then treated starting at day 7 post-infection (p.i.) for 7 days every two or three other days (3 treatments) with 10 μ M of indicated compounds. Protein subcellular distribution was evaluated by immuno- fluorescence. At the end of the treatment procedure, cells were fixed by 2% PFA and stained with indicated primary antibodies and with fluorescently labeled secondary antibodies. An antibody recognizing only assembled capsid was used (ab8637). Green: HBc; red: PML (Promyelocytic Leukemia) protein or 20S (proteasomal 20 subunit) ; blue: DAPI. The pictures are representatives of 3 independent experiments.



2 **Supplementary figure 2. Dose-response effect of JNJ-827 and JNJ-890 in infected dHepaRG**
 3 **cells.** dHepaRG cells were infected with HBV (200 vge/cell), cultured for one week then
 4 treated 7 days (treatment each 2/3 days) with 0.1, 1 or 10 μM of indicated compound. (A)
 5 Intracellular HBV total DNA level was quantified by qPCR. (B) ApoB secretion levels were
 6 assessed by ELISA. Results are the mean + SEM of n=3.

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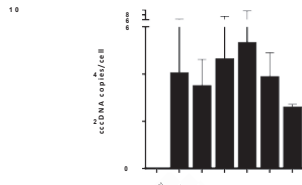
13 **Supplementary figure 3. Determination of JNJ-827 EC₅₀ on HBeAg secretion.** PHH were
 14 infected with HBV (200 vge/mL), cultivated for 5 days post infection, treated three times for
 15 7 days (treatment each 2/3 days) with increasing amount of JNJ-827 ranging from 40 nM to
 16 80 μM. HBeAg levels in cell culture supernatant were monitored by ELISA. Results obtained
 17 with 2 different PHH donors/batches are represented independently.

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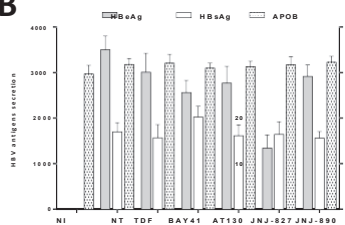
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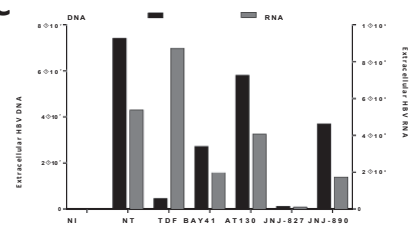
BATCH 1



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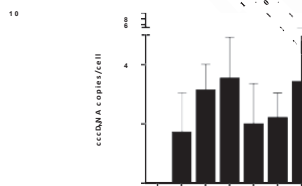


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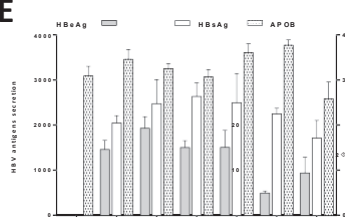


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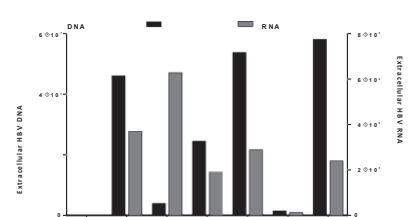
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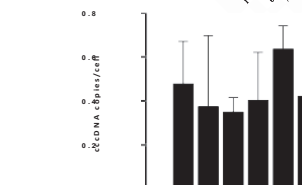


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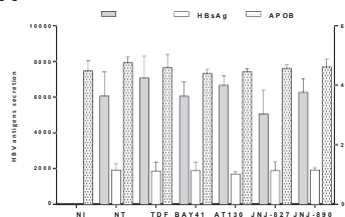


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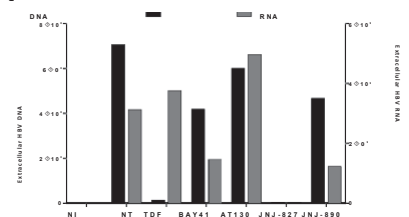
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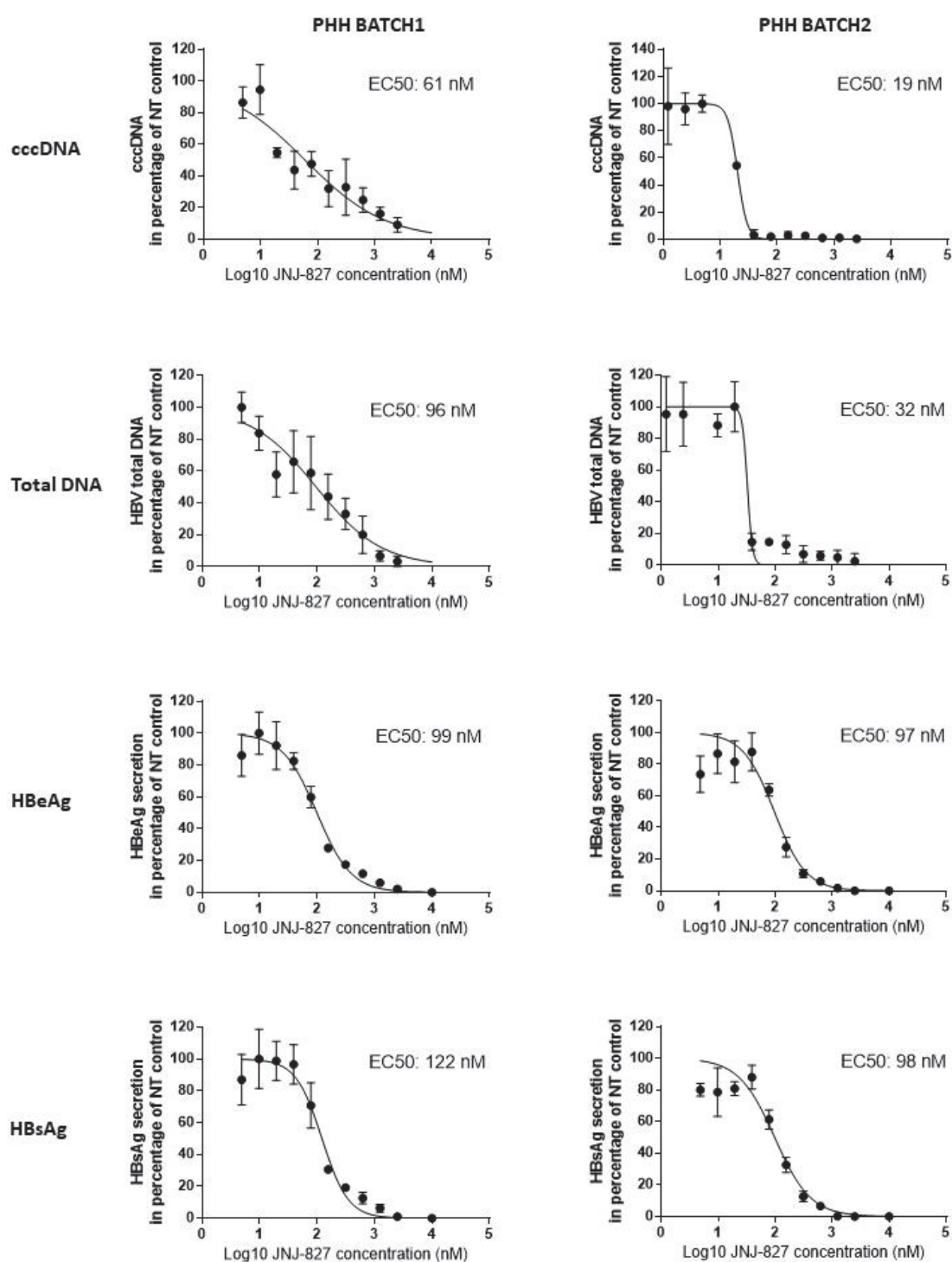


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25 **Supplementary figure 4. Antiviral properties of JNJ-827 and JNJ-890 in primary human**
 26 **hepatocytes.** PHH were mock (NI condition) or infected with HBV (200 vge/mL), cultivated
 27 during 5 days post infection, then non-treated (NT condition) or treated three times for 7
 28 days (treatment every 2/3 days) with 10 μ M of indicated compound. (A, D, G) CccDNA, was
 29 quantified by qPCR. (B, E, H) HBeAg, HBsAg and ApoB levels in cell culture supernatant were
 30 monitored by ELISA. (C, F, I) Extracellular HBV DNA and RNA levels were quantified by qPCR
 31 or RT-qPCR after nucleic acid extraction from supernatant. Results obtained with 3 different
 32 PHH donors/batches are represented independently. Results are the mean + SD of technical
 33 replicates (n=2 or 3).



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35 **Supplementary figure 5. Determination of JNJ-827 EC₅₀ on cccDNA establishment.** PHH

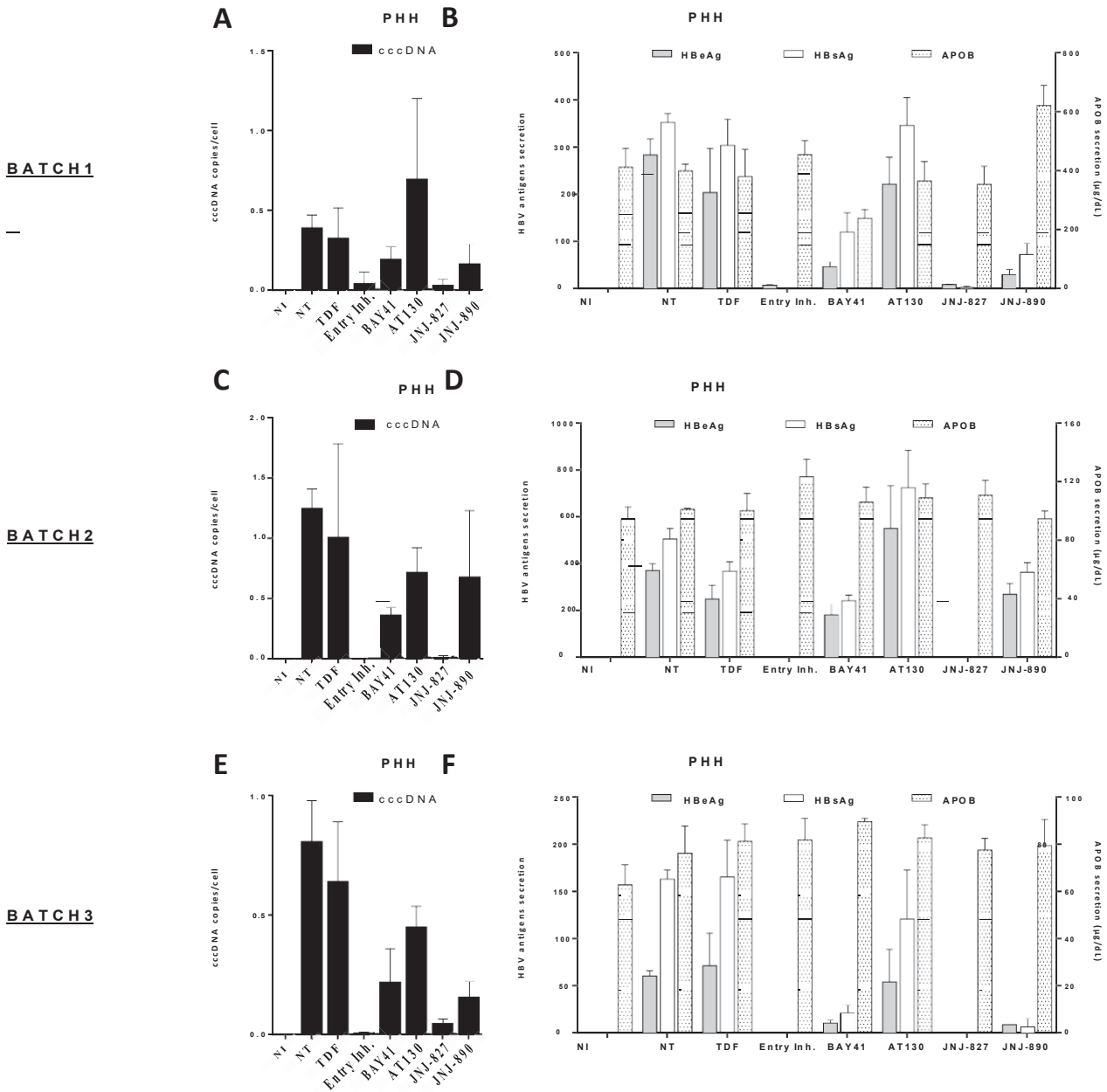
36 were pre-treated for 24h with increasing amount of JNJ-827 ranging from 5 nM to 10 μ M

37 then inoculated with HBV (200 vge/mL) in presence of drug for 24h. One day after infection,

38 medium was changed and cells were cultured for one week. Intracellular cccDNA and HBV

39 total DNA levels were quantified by qPCR FRET and qPCR respectively. HBeAg and HBsAg

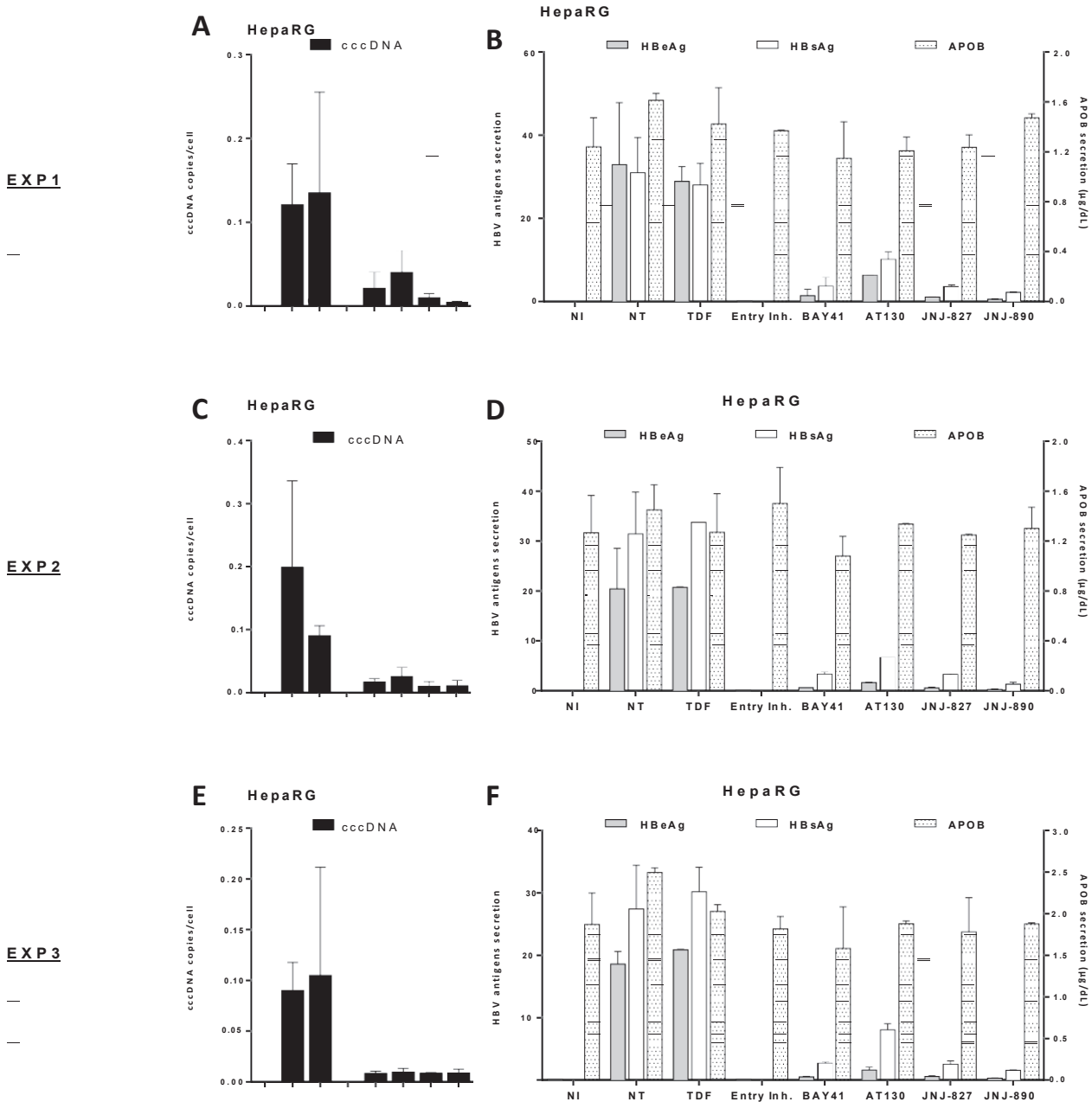
40 levels in cell culture supernatant were monitored by ELISA. Results obtained with 2 different
 41 PHH donors/batches are represented independently.



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 43 **Supplementary figure 6. Effect of JNJ-827 and JNJ-890 on HBV infection establishment in**
 44 **PHH.** PHH cells were pre-treated for 24h with 10 μ M of indicated CAMs or 100 nM of preS1
 45 peptide entry inhibitor, then inoculated with HBV (200 vge/mL) in presence of drug for 24h.
 46 One day after infection, medium was changed and cells were cultured for one week. For the
 47 TDF control, cells were treated twice after infection. A non-infected (NI) control was
 48 included. (A, C, E) CccDNA, was quantified by qPCR. (B, D, F) HBeAg, HBsAg and ApoB levels
 49 in cell culture supernatant were monitored by ELISA. Results obtained with 3 different PHH

50 donors/batches are represented independently. Results are the mean + SD of technical
 51 replicates (n=2 or 3).

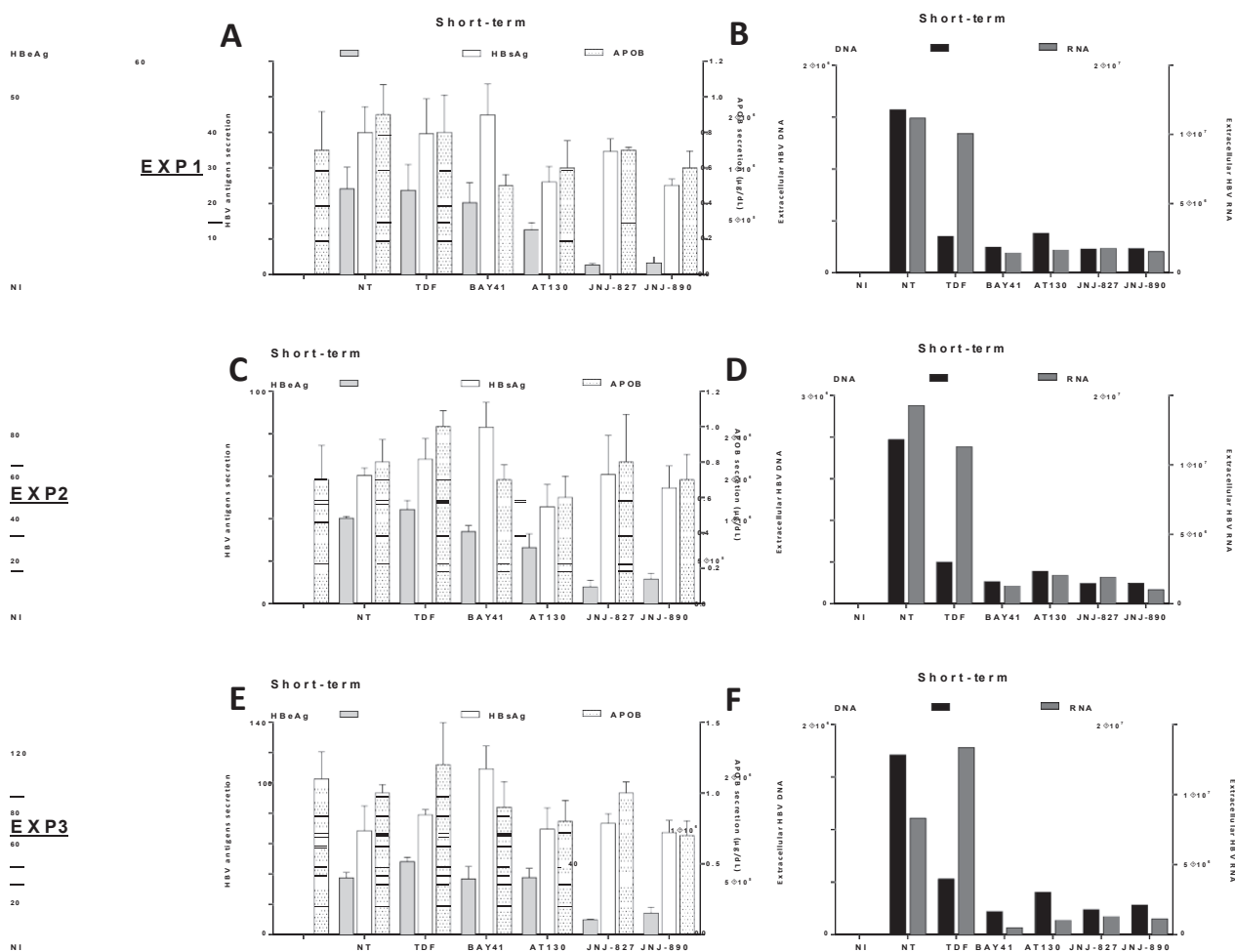
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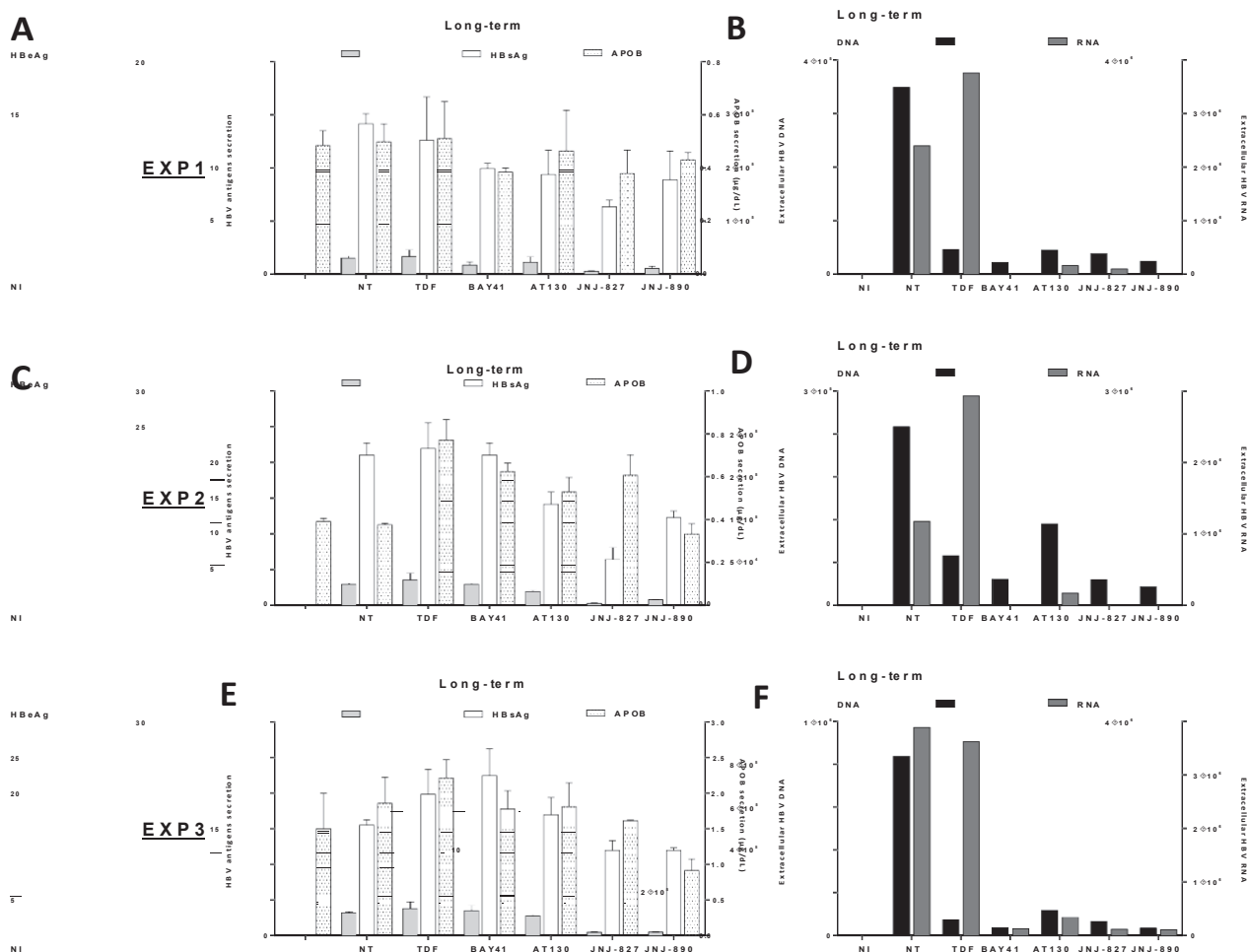
56 **Supplementary figure 7. Effect of JNJ-827 and JNJ-890 on HBV infection establishment in**
 57 **dHepaRG.** Differentiated HepaRG cells were pre-treated for 24h with 10 µM of indicated
 58 CAMs or 100 nM of preS1 peptide entry inhibitor, then inoculated with HBV (200 vge/mL) in
 59 presence of drug for 24h. One day after infection, medium was changed and cells were

60 cultured for one week. For the TDF control, cells were treated twice after infection. A non-
 61 infected (NI) control was included. (A, C, E) CccDNA, was quantified by qPCR. (B, D, F)
 62 HBeAg, HBsAg and ApoB levels in cell culture supernatant were monitored by ELISA. Results
 63 obtained with 3 different HepaRG differentiations are represented independently. Results
 64 are the mean + SD of technical replicates (n=2 or 3).



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 66 **Supplementary figure 8. Antiviral properties of JNJ-827 and JNJ-890 in dHepaRG: short**
 67 **versus long-term treatment; Part 1: short-term results.** dHepaRG were infected with HBV
 68 (200 vge/mL), cultured during one week then treated for 7 days (treatment each 2/3 days; 3
 69 treatments in total) with 10 μ M of indicated compound. The antiviral effects of the
 70 compounds were monitored at end point. (A, C, E) HBeAg, HBsAg and ApoB levels in cell
 71 culture supernatant were monitored by ELISA. (B, D, F) Extracellular HBV DNA and RNA
 72 levels were quantified by qPCR or RT-qPCR after nucleic acid extraction from supernatant.
 73 Results obtained with 3 different HepaRG differentiations are represented independently.
 74 Results are the mean + SD of technical replicates (n=2 or 3).

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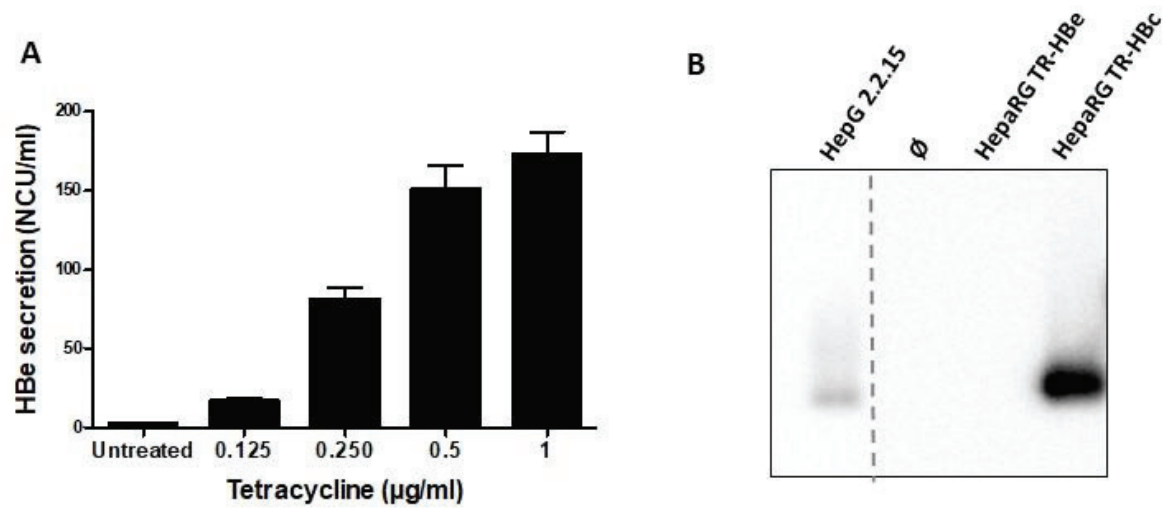
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83 **Supplementary figure 9. Antiviral properties of JNJ-827 and JNJ-890 in dHepaRG: short**
84 versus long-term treatment; Part 2: long-term results. dHepaRG were infected with HBV
85 (200 vge/mL), cultured during one week then treated for one month (treatment each 2/3
86 days; 15 treatments in total) with 10 μM of indicated compound. The antiviral effects of the
87 compounds were monitored at end point. (A, C, E) HBeAg, HBsAg and ApoB levels in cell
88 culture supernatant were monitored by ELISA. (B, D, F) Extracellular HBV DNA and RNA
89 levels were quantified by qPCR or RT-qPCR after nucleic acid extraction from supernatant.
90 Results obtained with 3 different HepaRG differentiations are represented independently.
91 Results are the mean + SD of technical replicates (n=2 or 3).

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Supplementary figure 10. Characterization of the HepaRG-TR-HBe cell line. (A) The secretion of HBeAg by cells was done by ELISA on supernatant recovered 48 hours after induction of gene expression by the indicated concentration of tetracycline. (B) Capsid migration assay. HepaRG TR-HBe and a positive control expressing HBe, HepaRG-TR-HBe, were induced using 1 µg/mL of tetracycline for 48hrs. Cell lysates were loaded in a native agarose gel for visualization of intracellular capsid formation. Capsid formation was monitored by immunoblotting using an anti-HBe antibody.

Virion-associated Hepatitis B core (HBc) protein is an early negative regulator of hepatocyte Interferon (IFN) response.

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*contributed equally

In preparation.

Summary

Hepatitis B virus (HBV), as many other viruses, has evolved different strategies to evade immune responses to establish and maintain viral persistence. In particular, HBV does not seem to induce any measurable IFN and pro-inflammatory responses during acute infection. The absence of innate immune response could be due to an impaired detection of HBV by the host innate sensors or could result from an active evasion via the prompt establishment of inhibitory mechanisms.

In a previous work, we showed that HBV virion could trigger a very early inhibition of the hepatocyte-mediated IFN response immediately after infection. Here, we demonstrate that HBV core protein (HBc) is the main viral component involved in this inhibitory process. After HBV entry and migration of nucleocapsids to the nuclear pore, HBc enters the nucleoplasm, where, after binding to target promoters, it impairs the activation of specific innate immunity gene expression upon dsRNA sensors engagement. The recruitment of EZH2 histone methyltransferase is responsible for the establishment of repressive epigenetic histone marks (H3K27) at specific ISG-related HBc-bound-promoters, affecting IFN gene response immediately after the onset of infection. This inhibitory mechanism is maintained during persistent infection thanks to the recycling of nucleocapsids to the nucleus. Interestingly, we also found that this inhibition is at work in non-parenchymal cells (Kupffer and liver endothelial sinusoidal cells) into which HBV does penetrate and deliver HBc to the nucleus compartment.

HBV has evolved a strategy to impair IFN response immediately after the onset of infection by using the incoming virion structural protein HBc to regulate gene expression by epigenetic mean.

Introduction

Around 250 million individuals are chronically infected with hepatotropic viruses (WHO), which represent, if left untreated, a high risk of developing liver cirrhosis and hepatocellular carcinoma (Arzumanyan et al., 2013; El-Serag, 2012). Hepatitis B virus (HBV), a DNA virus belonging to the *Hepadnaviridae* family, is highly endemic in some area of the globe where parenteral transmission was until recently possible. The natural history of HBV infection is quite complex with distinct physiopathology and therapeutic stages. The natural course of HBV infection consists of four phases: i) the previously called "immune-tolerance" phase (IT), now referred as "low-inflammatory" phase, during which the virus replicates at very high levels without immune control and apparent liver damage, ii) a chronic-active-hepatitis (CAH) phase, iii) an inactive-carrier phase, and iv) potential reactivation episodes (Busch and Thimme, 2015; Fattovich et al., 2008; McMahon, 2010).

To establish and maintain a chronic infection, HBV has evolved various strategies to evade the host innate and adaptive immune responses (Ait-Goughoulte et al., 2010; Bertoletti and Ferrari, 2012; Dandri and Locarnini, 2012; Ferrari, 2015; Tan et al., 2015). Several HBV proteins, including those expressed within the infected hepatocytes (e.g. HBx, HBV polymerase) or the HBV antigens released in the bloodstream (i.e. HBsAg and HBeAg), have been shown or proposed to bear immune-modulatory functions within infected hepatocytes or the liver micro-environment, with subsequently a lack of immune cell cross-talk and antigen presentation, as well as a general HBV-specific T cell exhaustion (Ait-Goughoulte et al., 2010; Bertoletti and Ferrari, 2012; Dandri and Locarnini, 2012; Ferrari, 2015; Tan et al., 2015). To restore/favour an immune control of the virus, virus-mediated inhibitory mechanisms would have to be defeated/unlocked in an interventional therapeutic perspective. To this end, a better knowledge of the underlying molecular mechanisms responsible for the blockade of host immune responses is crucial before envisaging such strategies.

The interferon response is a central component of host antiviral strategies (de Weerd and Nguyen, 2012; Randall and Goodbourn, 2008; Wang and Fish, 2012). This response, which involves the production of a wide range of IFNs (type-I, II and III), may occur within the infected cells or in the microenvironment by the activation of professional innate immune cells (i.e. IPC; IFN producing cells such as pDC or mDC2-BDCA3+). This response is crucial to hold the early spreading of viruses, to reduce the intensity of their replication, to finely

tune cellular innate response and to finally enable the orchestration of subsequent adaptive response. In turn, viruses, in particular those that progress to chronicity, have evolved many strategies to evade this IFN response to their own benefit (Bowie and Unterholzner, 2008; Heim, 2013; Manel and Littman, 2011; Randall and Goodbourn, 2008). In this respect, HBV is a peculiar virus, as, in contrast to HIV or HCV, it does not seem to induce a measurable systemic production of IFN neither in the early (Dunn et al., 2009; Stacey et al., 2009) nor in the late phase of human infection (Rehermann and Bertolotti, 2015). If the results obtained with human samples are subjected to caution, because of the difficulty to get robust and homogeneous cohort of patients during the acute phase of infection, the experiments performed in self-resolving HBV-infected chimpanzees have suggested that innate immunity genes were not up-regulated in their liver when HBV viremia was exponentially rising (i.e. few weeks post infection) (Wieland et al., 2004). Since then, HBV was considered as a "stealth" virus, which does not induce IFN response, likely due to a lack of detection or activation, by innate sensors (pattern recognition receptors; PRRs) (Wieland et al., 2005). Corroborating this, a close look at the viral life cycle indeed suggests that viral PAMPs (i.e. pathogen associated molecular patterns), in particular viral nucleic acids, are not highly exposed to PRRs (Seeger et al., 2015). However, a stealth virus can also be a virus that is able to actively inhibit nascent responses.

Substantiating this hypothesis, our recent work, performed *in vitro* in infected primary human hepatocytes (PHH) or closely related in HepaRG cells {Luangsay, 2015 #96}, has shown that HBV could be weakly detected by PRRs in hepatocytes; however, HBV was capable to actively and rapidly counteract nascent innate response. The inhibitory mechanism involved a viral component present in HBV virions, but was neither due to HBsAg nor HBeAg {Luangsay, 2015 #95}. The core protein (HBc) and HBV polymerase, with a stoichiometry of 240 unit to 1 in favour of HBc (Seeger et al., 2015), were potentially the two viral proteins composing the infectious virions that could mediate this inhibitory effect. In this respect, it was shown that over-expression of the viral polymerase could both inhibit RIGI/MDA5 and STING-mediated IFN- α induction by either interfering with DDX3, a regulating component of the IRF3 kinase complex (Wang and Ryu, 2010) or the K63-linked ubiquitination of STING {Liu, 2015 #98}. Other studies have also suggested, using a reporter gene and a non-physiologic expression system, that HBc could inhibit the expression of IFN- α and MxA by directly interfering with

their transcription (Fernandez et al., 2003; Rosmorduc et al., 1999; Whitten et al., 1991). However, detailed mechanisms about this inhibition remained until now largely unknown.

The aim of this work was to identify the HBV-virion associated factor responsible for the early inhibition of IFN response we evidenced in a previous work {Luangsay, 2015 #95}, and to get more insights on the molecular underlying inhibitory mechanism. In this study, HBc was identified as the major viral determinant responsible for this inhibition. This is the first demonstration that a viral structural protein of HBV virions is able to modulate the host innate immune response by epigenetic regulation of type-I IFN gene expression, shortly after viral entry/penetration.

Results

Virion-associated HBc is the viral determinant involved in the early inhibition of hepatocyte IFN response

We recently reported that HBV could actively and promptly, i.e. within minutes/hours after the onset of infection, and in the absence of *de novo* viral synthesis, inhibit PRR agonist-mediated IFN response in both differentiated HepaRG (dHepaRG) cells and primary human hepatocytes (Luangsay et al., 2015b). We extended these results by very early kinetic studies (Figures 1A and 1B). Again, a strong and early inhibition of a dsRNA-mediated IFN response (i.e. prototypic type-I (*IFN- α*), type-III (*IL-29*) IFN, as well as *OAS-1* as a prototypic ISG) in HBV- infected PHH stimulated with poly(I:C) was observed as early as 2h post-HBV exposure, whereas a delayed inhibition, starting at 8h p.i., was observed for *IL-6* (i.e. prototypic pro- inflammatory gene). Interestingly, we showed that a HBV virion/inoculum-associated factor was responsible for this very early inhibition, as the inhibitory phenotype was still maintained with UV-inactivated virus in HBV-infected dHepaRG (Figures 1B). Since HBsAg and HBeAg, the two secreted viral antigens present in the HBV inoculum, were previously ruled out as early negative regulator of IFN response (Luangsay et al., 2015b), we reasoned that HBc, the major protein composing viral nucleocapsid (240 units/capsid) within infectious particles, could be potentially responsible for this inhibition.

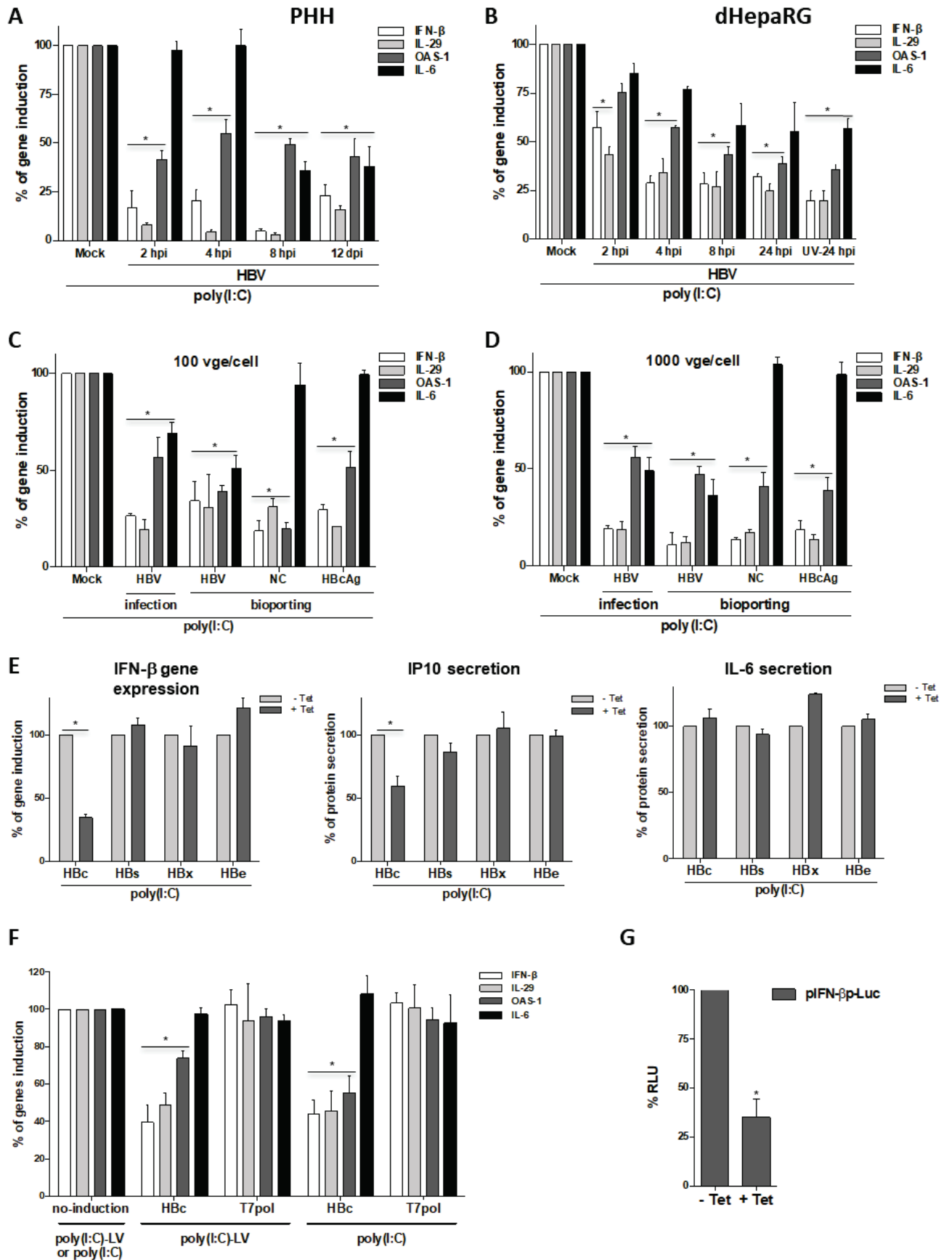
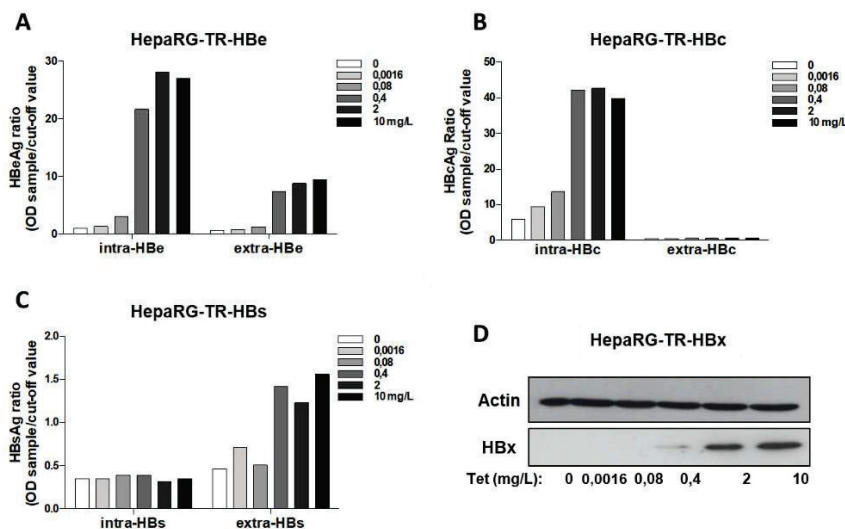


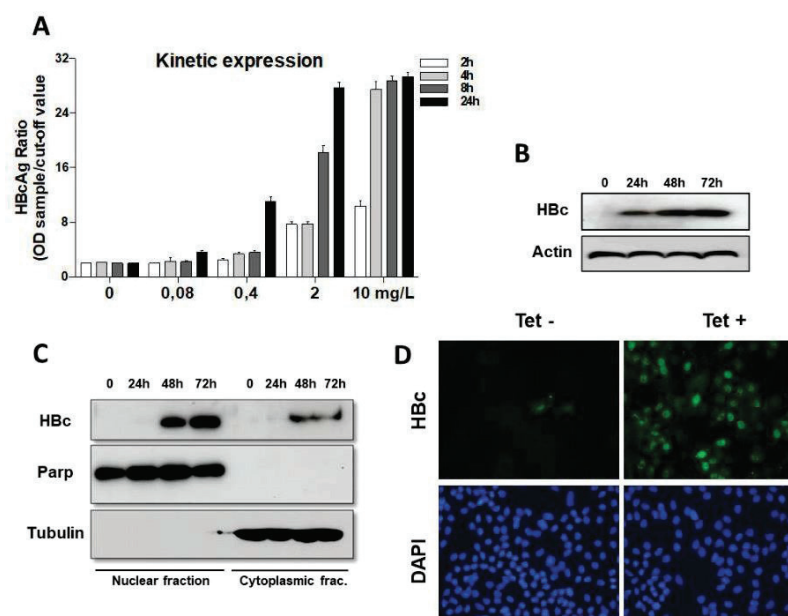
Figure 1. HBc protein is the viral determinant involved in the early inhibition of dsRNA-mediated IFN responses in hepatocytes. (A) PHH or (B) differentiated HepaRG were exposed/infected with 1000 vge/cell of wild type or UV-inactivated HBV for the indicated time, then stimulated with poly(I:C)-LMW (10 µg/mL) for 4h. After total RNA purification, the expression of innate immunity genes (*IFN-3*, *IL-29*, *OAS-1* and *IL-6*) was analysed by RT- qPCR and compared to the mock control normalized to 100%. Proliferative HepaRG cells were either mock- or lipo-transfected for 4 hours with (C) low or (D) high amounts of complete HBV inoculum, nucleocapsids or recombinant HBcAg (100 or 1000 vge/cell and HBcAg at 50 or 500 pg /cell) prior to poly(I:C) stimulation (4h). Expression of indicated genes was analysed by RT-qPCR and compared to the mock control normalized to 100%.

(E) dHepaRG expressing in a tetracycline dependent manner (Tet at 10 µg/mL; 48h of expression of the corresponding protein) either HBc , HBs, HBx, or HBe were stimulated with poly(I:C) for 4h. Then *IFN-3* gene expression as well as IL-6 or IP-10 secretion were monitored by RT-qPCR or ELISA. (F) Differentiated HepaRG-TR- HBc and HepaRG-TR-T7 cells were treated or not for 24h with tetracycline (Tet, 10 µg/mL) prior to stimulation with poly (I:C) for 4h and gene expression analysed by RT-qPCR and compared to the non-tet-treated cells normalized to 100%. (G) HepaRG-TR-HBc cells were transfected with the pIFN-13p-Luc plasmid, induced or not with tetracycline for 48h, then stimulated with poly(I:C) for 4h. Luciferase activity was analysed on cell lysate with a Luminoskan Ascent. All results are represented as the mean ± SEM of at least 3 independent experiments.

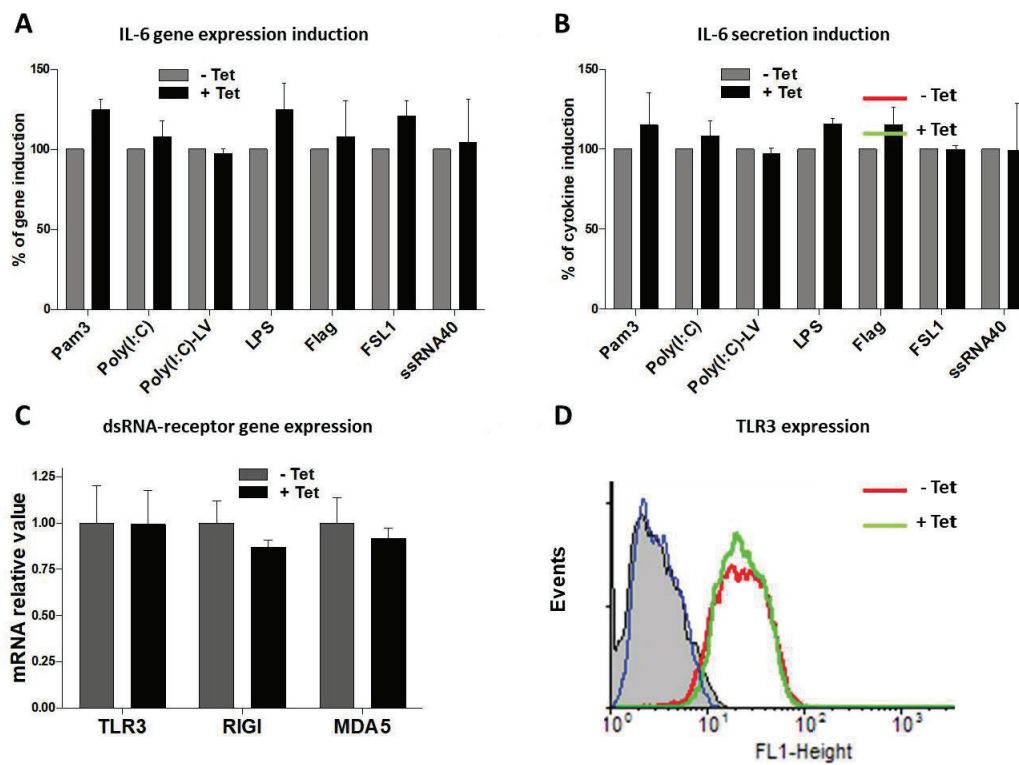


Suppl. Fig. 1. Analysis of HBV protein expression in various cell lines. (A, B, and C) Intra- and extracellular expression of HBeAg, HBcAg, and HBsAg quantified by ELISA in indicated cell lines at 24h post-induction with indicated doses of tetracycline. (D) Intracellular expression of the HBx protein by the HepaRG-TR-HBx cell line at 24h post-induction with indicated doses of tetracycline analysed by western blot with anti-V5 (detection of C- terminally tagged HBx) and anti-actin antibodies.

To test this hypothesis, we exposed dHepaRG cells to standardized amounts (100 or 1000 vge/cell) of HBV complete inoculum (i.e. containing HBV virions and subviral particles (SVPs; HBsAg and HBeAg)), non-enveloped nucleocapsids only (NCs, gradient-purified), or recombinant HBc (rechHBc). To allow their cell penetration, NCs and rechHBc, were delivered by lipo-transfection as previously reported (Rabe et al., 2006). Twenty four hours later, cells were stimulated by poly (I:C) for an additional 4h after washing the excess of viral inoculum, nucleocapsids or core proteins. Transfection of dHepaRG cells with complete HBV inoculum, as for that obtained with a conventional infection, could also lead to a significant inhibition of poly (I:C)-mediated induction of the expression of *IFN- α* , *IL-29*, *OAS-1*, and *IL-6* genes (Figures 1C and 1D). Importantly, similar results were obtained with both transfected NCs and rechHBc, with the exception that induction of *IL-6* gene expression was not affected by these two forms of HBV core protein. This suggests that HBc *per se* is responsible for the specific inhibition of the IFN response, whereas a different type of inhibition of NF- κ B-mediated pro-inflammatory response by other HBV proteins could be at play (Luangsay et al., 2015b).



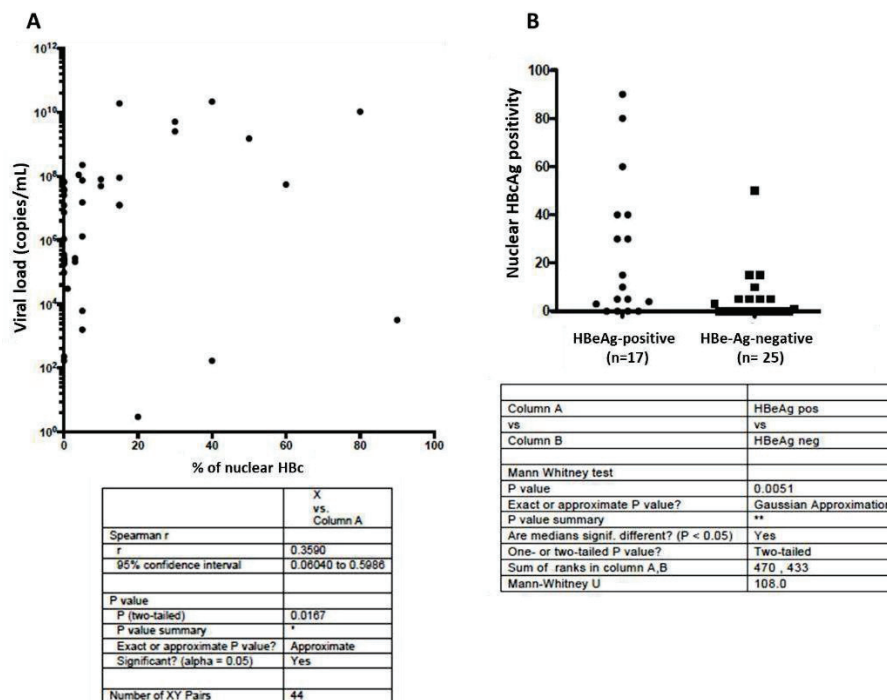
Suppl. Fig. 2. Core expression and subcellular localization in the inducible HepaRG-TR-HBc cell line. (A) Kinetical expression of HBcAg analysed by ELISA in differentiated HepaRG-TR-HBc cells, following treatment with several dose of tetracycline (0.08, 0.4, 2 and 10 µg/ml) for 2h, 4h, 8h and 24h. (B) Kinetic of HBc expression after induction with 10 g/mL of tetracycline for the indicated time analysed by WB. (C) Kinetic and subcellular localization of HBc in tet-induced HepaRG-TR-HBc cells after cytoplasm and nucleoplasm separation analysed by WB. (D) Immunofluorescence analysis of HBc expression (with anti-HBc C1 Abcam) in tet-induced HepaRG-TR-HBc cells at 24h post induction. Cell nucleus were stained with Hoechst reagent.



Suppl. Fig. 3. Core expression and subcellular localization in the inducible HepaRG-TR-HBc cell line. (A) IL-6 cytokine gene expression, and (E) protein secretion were analysed by RT-qPCR and ELISA respectively in dHepaRG-TR-HBc cells treated or not for 24h with prior stimulation with several TLRs ligands (TLR2, Pam3CSK4; TLR3, Poly(I:C); RIGI/MDA5, Lyovec-Poly(I:C); TLR4, LPS; TLR5, Flagellin; TLR6, FSL1; TLR7/8, ssRNA40) for 4 hours and compared to the non-tetracycline stimulated HepaRG-TR-HBc cell line normalized to 100%. (C) Expression of *TLR3*, *RIGI*, and *MDA5* genes analysed by RT-qPCR on total RNA extracted from dHepaRG-TR-HBc cells induced or not with tetracycline (10 µg/mL) for 24h. (D) FACS analysis of TLR3 expression dHepaRG-TR-HBc cells induced or not with tetracycline (10 µg/mL) for 24h.

To further demonstrate the prominent role of HBc in the early inhibition of hepatocyte IFN response, we engineered an inducible HepaRG cell line expressing HBV core protein under the control of tetracycline (i.e.). Similar cell lines were also generated for HBs, HBe and HBx expression as well as a control cell line expressing the T7 polymerase protein (Figure S1). In HepaRG-TR-HBc cells, HBc was detected as early as 2 hours post-induction, was not secreted, reached a plateau of expression by 48-72 h post-induction, and was mainly localized within the nucleus of cells (Figure S2). Following HBc, HBs, HBe, or HBx mock- or tet-induction, cells were stimulated for 4h with TLR3-L to measure *IFN-α* gene expression, as well as IP10 and IL-6 secretion. Among the four inducible cell lines, only that expressing HBc showed a reduced induction of *IFN-α* gene expression and IP10 secretion following stimulation, thus confirming that HBc is the main contributor to this inhibition (Figures 1E).

Similar results were also obtained with other prototypic genes of the IFN response, and with a RIGI/MDA5 ligand (poly (I:C)-LMW-Lyovec) (Figure 1F), or using an *IFN- α* luciferase reporter construct transfected in HBe-expressing cells (Figure 1G). These results corroborated with those obtained in dHepaRG cells exposed to purified HBV nucleocapsids or recHBe (Figures 1C and 1D). Similarly, in this cell line, IL-6 expression or secretion was not affected by HBe and interestingly none of the other viral protein tested (HBs, HBx, HBe) could modulate its secretion as well (Figures 1E and 1F). The fact that, in our experimental conditions, HBe did not seem to regulate HBV-mediated NF- κ B-dependent pro-inflammatory pathways was further confirmed by using other TLR agonists (TLR1/2, TLR3, RIGI/MDA5, TLR4, TLR5, TLR6, and TLR8 agonists) involved in NF- κ B dependent IL-6 induction (RNA and protein levels) (Figures S3A and S3B). Importantly, the inhibition of the poly(I:C)-mediated induction of *IFN* and *ISG* genes, observed in HBe expressing cells, was not due to a modification of expression of dsRNA sensors in the time range of the experiment (Figures S3C and S3D). Altogether, these results suggest that HBe is an early inhibitor of the IFN response in hepatocytes exposed to HBV.



Suppl. Fig. 4. HBe immunohistochemistry in biopsies of HBeAg positive and HBe-Ag negative patients. A total of 42 biopsies from HBe-Ag positive (n= 17) and HBe-Ag negative (n=25) were labeled with an anti-HBe antibody and revealed by immunoperoxidase staining. The percentage of cells with nuclear staining was established and plotted in function of viremia (A) or HBeAg status (B). Statistics were done with the Mann-Whitney test.

Nuclear localization of HBe is required to efficiently impair dsRNA-mediated IFN response

It has been previously proposed that HBe could be mainly localized in the nucleus of hepatocytes in patients with high viremia (i.e. IT/low inflammatory phase or HBeAg+ patients in CAH phase), whereas a cytosolic or mixed subcellular localization was observed in patients well-advanced in CAH phase (HBeAg negative patients). This suggested that the nuclear localization of HBe could be associated with a high viremia and a poor/lack of immune response (Akiba et al., 1987; Chevallier-Queyron and Chemin, 2011; Chu and Liaw, 1987). By performing ISH staining on liver biopsies from a cohort of 45 patients in CAH (18 HBeAg- positive and 27 HBeAg-negative patients), correlations between the viral load and percentage of hepatocytes positive for HBeAg in the nucleus (Figure S4A), and between HBeAg positivity and percentage of hepatocytes positive for HBeAg in the nucleus (Figure S4B) were found. Our results confirmed that high viremia seems to correlate with a higher localization of HBeAg within the nucleus of infected hepatocytes.

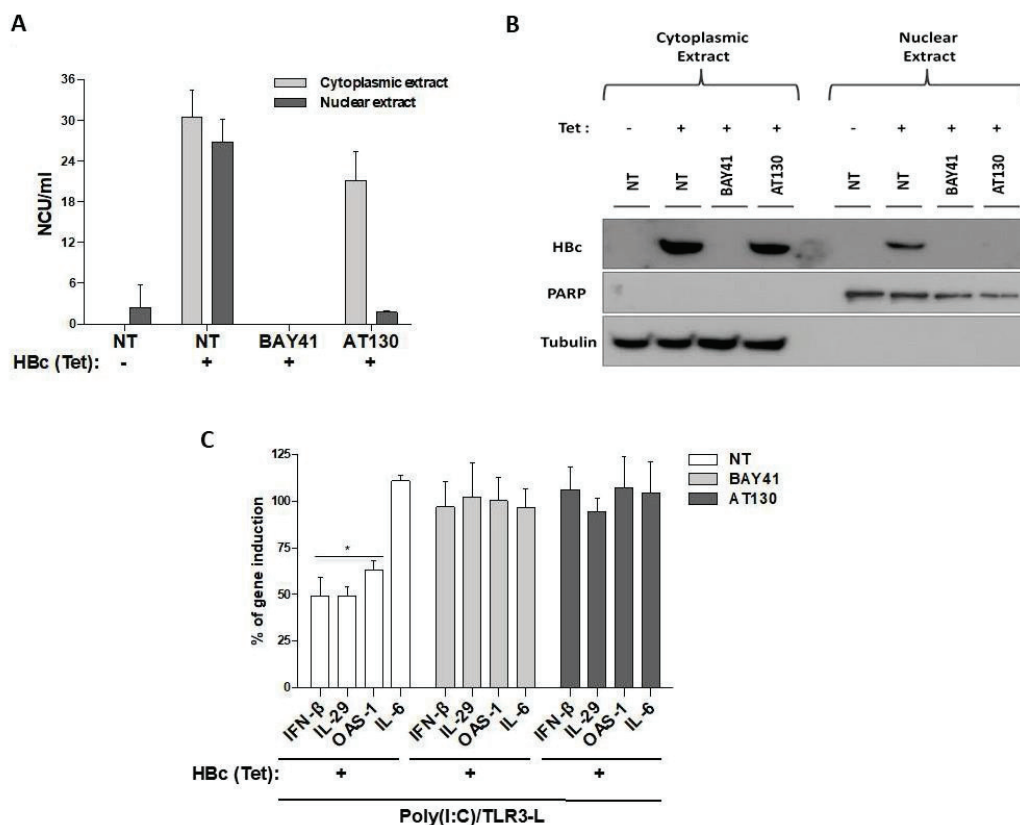


Figure 2: Capsid assembly inhibitor treatment reverts HBe-mediated inhibitory effect. (A and B) Differentiated HepaRG-TR-HBe cells were treated or not for 72h with Bayer41-4109 (1 μ M) or AT130 (3 μ M) prior stimulation with tetracycline (10 μ g/mL) for 24h. Nuclear and cytoplasmic protein extract from dHepaRG-TR-HBe cells

stimulated with tetracycline (10 µg/ml) for 24h were extracted and HBcAg were evaluated by ELISA (A) and western blot (B) using homemade anti-HBc antibody, anti-PARP (nucleus marker), and anti-tubulin (cytoplasmic marker) antibodies. (C) Differentiated HepaRG-TR-HBc cells were treated or not for 72h with Bayer41 (1 µM) or AT130 (3 µM) prior to be stimulated with tetracycline for 24h (2µg/ml) and then treated with Poly (I:C) ligand for 4h. Expression of indicated genes was analyzed by RT-qPCR and compared to the non-tetracycline stimulated HepaRG-TR-HBc cells normalized to 100%. Results in panels A and C are represented as the mean ± SEM of 3 independent experiments.

To further investigate whether the nuclear localization of HBc could significantly impact on the hepatocyte innate immune responses *in vitro*, HepaRG-TR-HBc cells were treated, prior to tetracycline induction, with two different capsid assembly inhibitors, an heteroaryldihydropyrimidine (i.e. BAY41-4109) or a phenylpropenamide derivative (i.e. AT-130), which both impair nucleocapsid assembly (Deres et al., 2003; Feld et al., 2007). These two treatments, which could prevent both HBc trafficking and translocation into the nucleus (Figure 2A and 2B), reverted the phenotype of HBc-mediated inhibition of expression of *IFN-6*, *IL-29*, *OAS-1* genes (Figure 2C). These results suggest a correlation between the nuclear localization of HBc and the inhibition of IFN and ISG gene expressions upon engagement of TLR3 or RIGI/MDA5 signaling pathway.

Upon HBV infection, HBc can rapidly interact with hepatocyte innate gene promoters to control IFN response

In conditions of overexpression of HBc in hepatoma cells, it has been suggested that HBc could interact with host promoters to regulate gene expression. For instance, *IFN-α* and *MX-A* promoters were shown to be repressed by HBc (Rosmorduc et al., 1999; Whitten et al., 1991), whereas HBc was shown to directly bind to ISRE-containing DNA in EMSA experiments (Fernandez et al., 2003). More recently, using liver biopsies from HBV-infected patients and chromatin immunoprecipitation (ChIP) microarrays, it was also shown that HBc could bind *in vivo* to a broad spectrum of CpG rich human gene promoters and potentially modulate their expression, even though functional analyses to validate the results were only performed *in vitro* with few genes (Guo et al., 2012).

To determine whether HBc could interact with our studied gene promoters, we performed ChIP-qPCR experiments on tet-induced dHepaRG-TR-HBc cells using an anti-HBc Ab (and

control Ab) and primers specific for *IFN- α* , *IL-29*, *OAS-1* and *IL-6* promoters, or control gene promoters (i.e. *HKG*, *GAPDH*, or *DHFR*). HBc was found to interact with *IFN- α* , *IL-29* and *OAS-1* promoters, but not with *IL-6* one, as early as 4 hours post-induction (Figure 3A). Interestingly, the blockade of the nuclear localization of HBc by capsid assembly inhibitor treatments that prevent HBc entry into the nucleus resulted in a marked decrease of the interaction between HBc and gene promoters in HBc-expressing cells (Figure 3B).

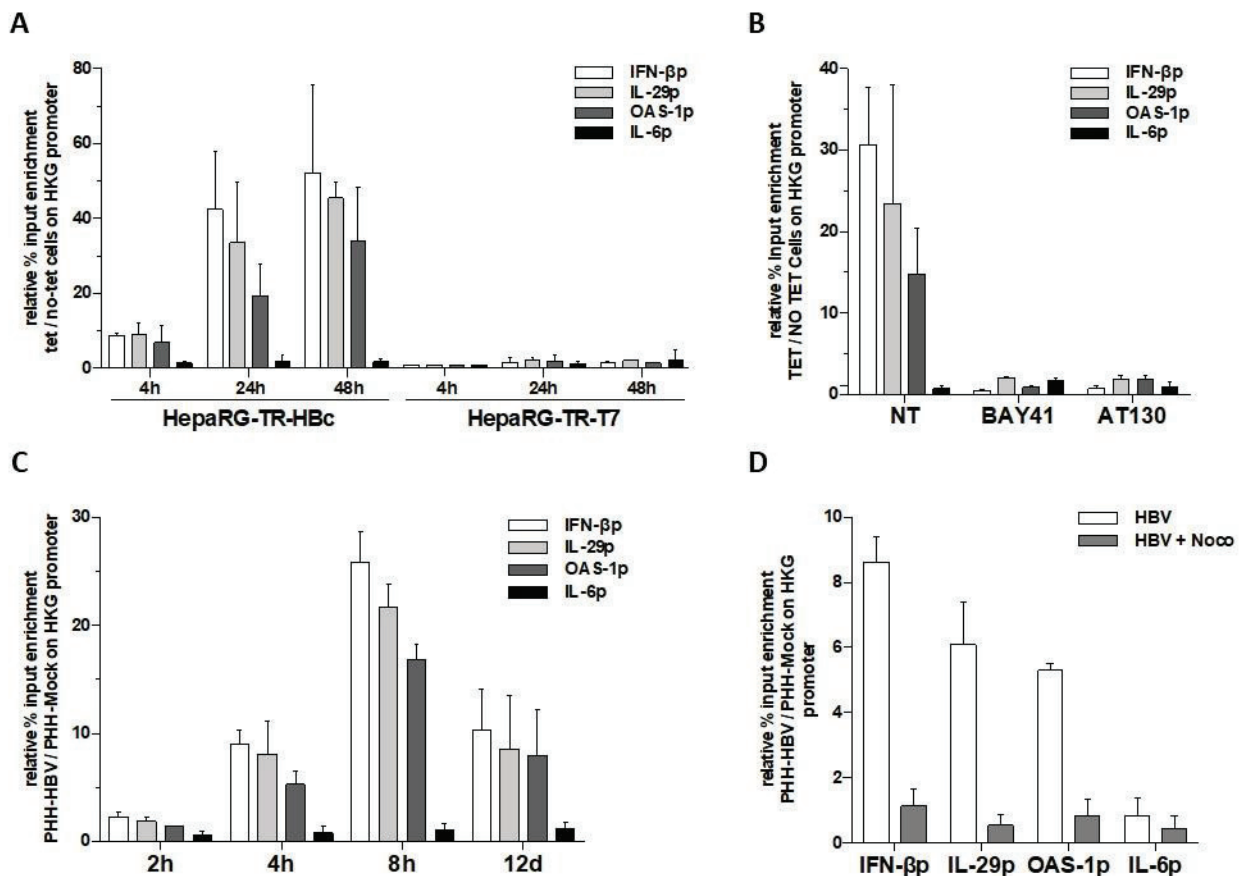


Figure 3: HBc protein interacts rapidly with innate gene promoters to inhibit dsRNA-mediated IFN response in hepatocytes. (A) Differentiated HepaRG-TR-HBc and HepaRG-TR-T7 cells were treated for 4h, 24h and 48h with tetracycline (10 μ g/mL), or (B) dHepaRG-TR-HBc cells were treated or not for 72h with Bayer41-4109 (1 μ M) or AT130 (3 μ M) prior to be stimulated with tetracycline (10 μ g/mL) for 24h, and ChIP-qPCR was performed with homemade anti-HBc (or isotopic antibodies) and specific primers for *IFN- β* , *IL-29*, *OAS-1* and *IL-6* promoters. The percentage of input enrichment was normalized on the non-tetracycline stimulated HepaRG-TR-HBc cells and on HKG promoter. (C) PHH were infected with a complete HBV inoculum for 2h, 4h 8h, or 12 days and ChIP-qPCR performed as in (A and B). (D) PHH were infected with a complete HBV inoculum for 8h in the presence or absence of nocodazole, and ChIP-qPCR was performed as in (A and B). All results are represented as the mean \pm SEM of 3 independent experiments.

ChIP analysis was also performed in HBV-infected PHH. The binding of HBc to *IFN- α* , *IL-29* and *OAS-1* promoters was evidenced, whereas no binding to *IL6* one at any of the time points was observed (Figure 3C). These results are consistent with the observation that *IFN- α* , *IL-29* and *OAS-1* gene expressions were inhibited by HBc, while the expression of *IL6* was not (Figure 1A). Of note, the binding of HBc was long lasting, as at day-12 pi, HBc could be still "ChIPed" on promoters. Importantly, no binding of HBc on *IL-6* promoter was again observed, thus confirming that HBc did not regulate *IL-6* transcription in our experimental conditions. We previously showed a correlation between HBc nuclear localization and gene expression modulation. To support this observation, we used short-term and non-cytotoxic treatment with nocodazole, which inhibits trafficking and translocation of HBc to nucleus (Rabe et al., 2006), and in turn prevents the binding of HBc to target promoters evidenced by ChIP-qPCR experiment (Figure 3D). Altogether, our results suggest that in the very early phase of infection, HBc from "incoming" virions could, after trafficking through the cytosol and translocation into the nucleus, negatively modulate the IFN response by binding to the promoters of some innate genes and, thereby, interfering with their expression.

HBc induces the recruitment on target genes of EZH2, a histone methyl-transferase involved in the establishment of repressive epigenetic marks

Several viruses were shown to manipulate the epigenetic state of the host genome notably by modifying the histone methylation status (Berger, 2007; Ferrari et al., 2012; Fonseca et al., 2012; Niller et al., 2009). We postulated that HBc-mediated repression of innate genes could be due to its binding to targeted promoters and further epigenetic modulations. To demonstrate this, we performed ChIP studies on HBc-expressing cells using antibodies directed either against hyper-methylated or acetylated histone H3 or against EZH2, a methyl-transferase involved in histone H3 methylation at position K27 (Czermin et al., 2002; Wu et al., 2011). Results showed significant enrichment of the repressive H3K27me3 mark on *IFN- α* , *IL-29* and *OAS-1* promoters, as early as 4h post-HBc expression (Figure 4A). Mirroring the pattern of HBc expression after tet-induction and defining a dose response, the enrichment of this repressive mark further increased at 24h and 48h post-induction, while an active mark (H3K27Ac) remained low throughout the kinetic. Confirming this result, we also found EZH2,

which contributes to the establishment of the H3K27me3 modification, on HBC-regulated promoters (Figure 4A).

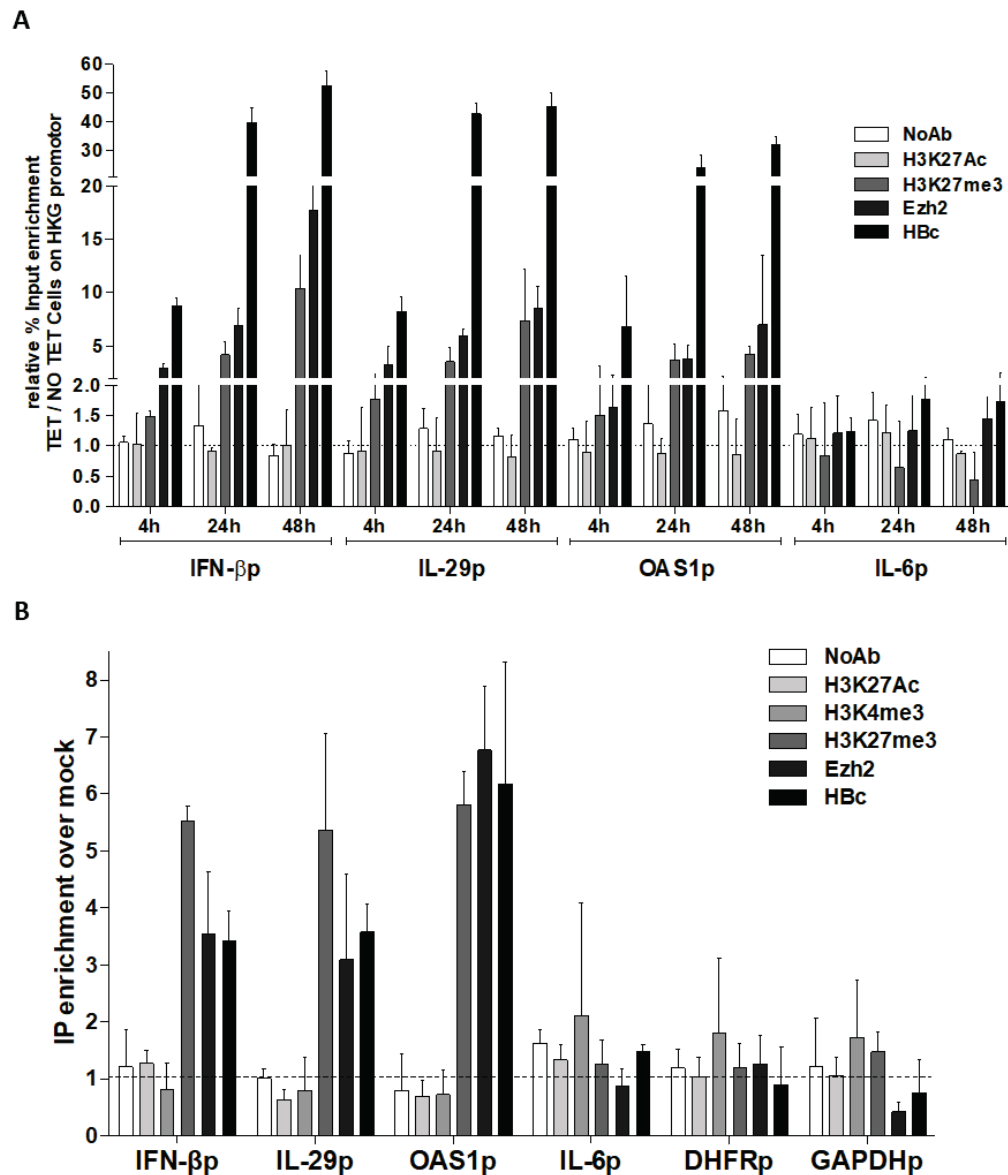


Figure 4: HBC affects host gene epigenetic status to inhibit IFN response in hepatocytes. (A) Differentiated HepaRG-TR-HBC cells were treated for 4h, 24h, or 48h with tetracycline (10 $\mu\text{g}/\text{mL}$), or (B) PHH were infected by 100 vge/cell of HBV for 24h, then histone modifications, recruitment of the histone methyl-transferase EZH2, and binding of HBC to *IFN-6*, *IL-29*, *OAS-1*, and *IL-6* promoters were assessed by ChIP-qPCR, using either none, control, anti-H3K27Ac, anti-H3K27me3, anti-EZH2, or anti-HBC antibodies. The percentage of input enrichment was normalized either to the non-tetracycline stimulated HepaRG cell lines (A) or to mock infected PHH (B) and on H3K promoter. All results are represented as the mean \pm SEM of 3 independent experiments.

ChIP experiments were also performed on HBV-infected PHH at 24h post-infection and showed a concomitant occupation of *IFN- α* , *IL-29*, and *OAS-1* promoters by HBC, EZH2, and K27me3 histone H3, while no active marks (H3K27Ac and H3K4me3) were found (Figure 4B).

This result is consistent with the fact that the binding of HBc to specific innate gene promoters could result in a direct or indirect recruitment of EZH2, which in turn contributed to the establishment of epigenetic repressive marks by tri-methylation of H3 at position K27. Therefore, these results strongly suggest that the inhibition of IFN response is due to a very early epigenetic reprogramming of innate immunity genes caused by the active transport into the nucleus and binding of incoming HBc to their promoters.

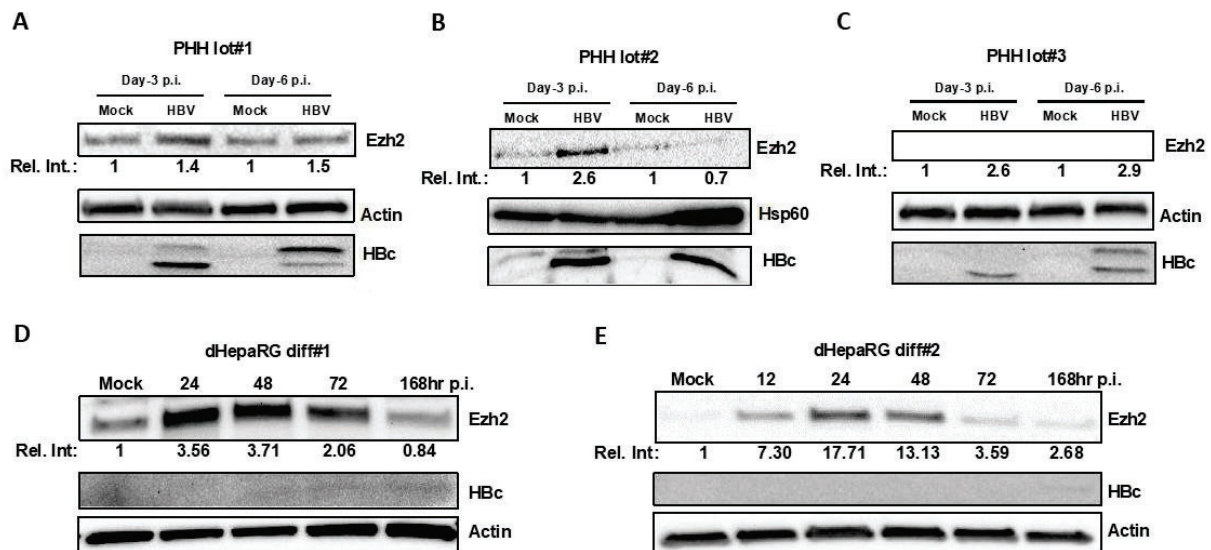
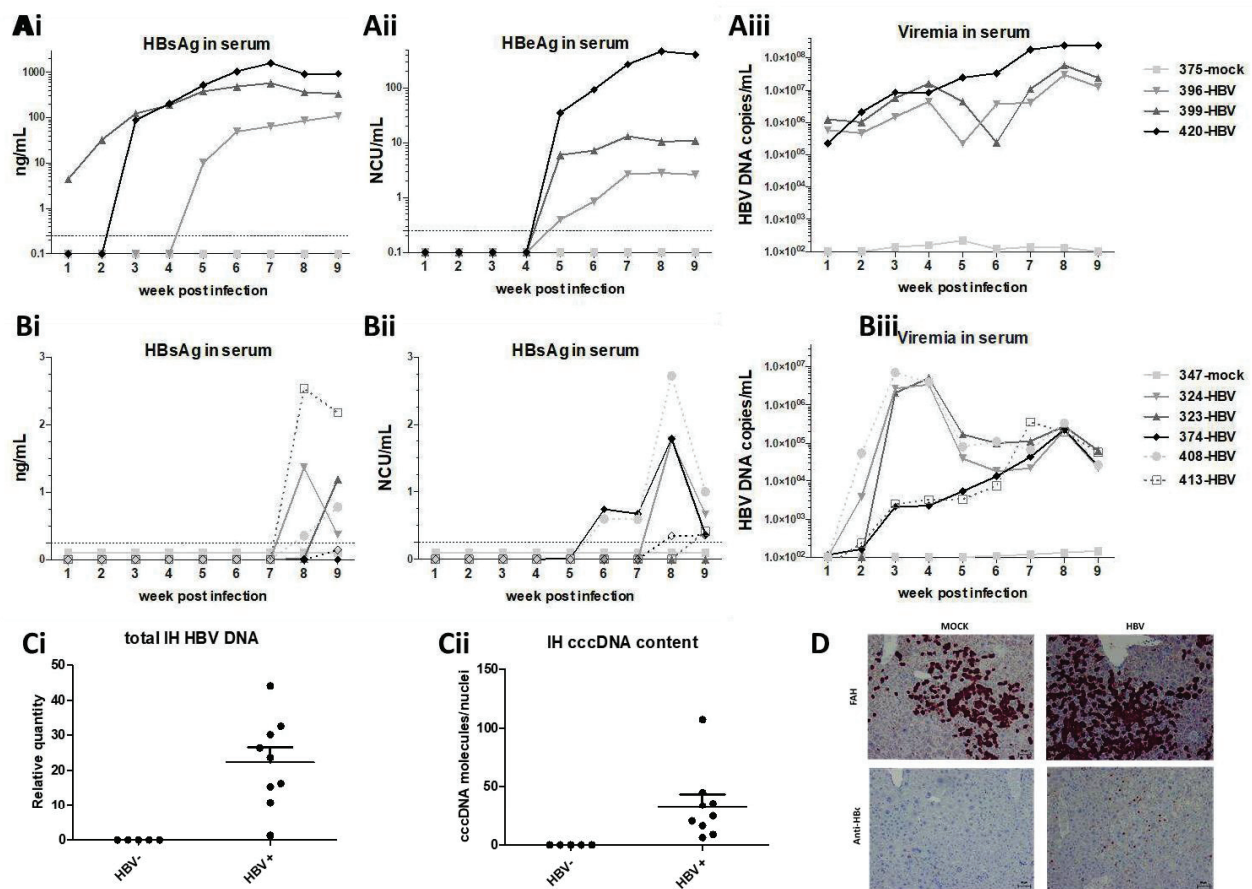


Figure 5: HBV induces EZH2 expression very early after the onset of infection. Three independent batches of PHH (A to C) and two different lots of differentiated HepaRG (D and E) were infected with 500 vge/cell of HBV and protein harvested at indicated times. Cell protein extracts were subjected to western blotting using anti- EZH2, anti-HBc and anti-Actin or anti-HSP60 antibodies.

In order to determine whether HBc could be directly responsible for the recruitment of EZH2 on target promoters, we attempted to co-immunoprecipitate HBc and EZH2 using specific antibodies, but failed to detect interaction between these proteins in HepaRG-TR-HBc cell lines, as well as in HBV-infected PHH. We also analyzed the effect of HBV infection on EZH2 expression. In both HBV-infected PHH (Figure 5A-5C) and dHepaRG (Figures 5D and 5E), we found an increased expression of EZH2, as compared to kinetically-paired and non-infected hepatocyte. It is worth noting that the weak/lack of HBc detection in HepaRG cells is due to very low level of HBV replication in this model as compared to PHH.

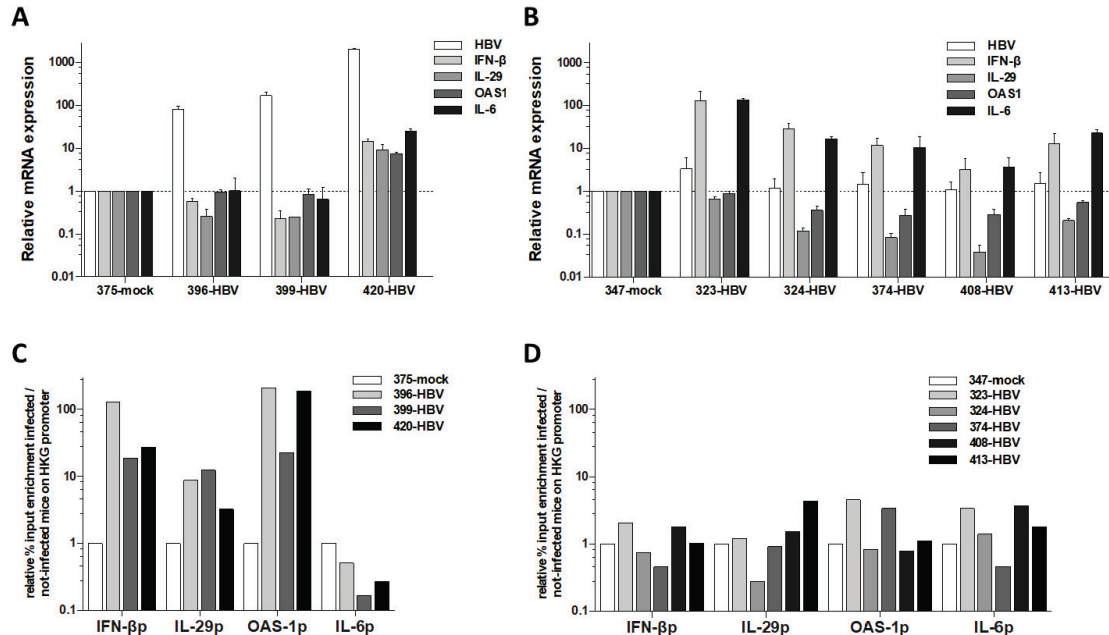
Long-term *in vivo* maintenance of the HBe-mediated inhibition of IFN response



Suppl. Fig. 5. Viremia, antigenemia, and intra-liver virologic parameters in HBV-infected FRG mice. (A and B) Viremia and antigenemia (HBeAg and HBsAg) was monitored every week for 9 weeks after HBV ($5.10e8$ vge/mouse for panels A; $5.10e7$ vge/mouse for panels B) infection by qPCR and ELISA. (C) Intra-liver total HBV DNA and cccDNA levels were obtained by qPCR and cccDNA specific qPCR {Werle, 2004 #88}. (D) Immunohistostaining was performed on paraffin embedded mouse liver samples with anti-HBc (Dako) and human-specific anti-FAH (Cliniscience) antibodies. Representative picture of immune-staining in mock- and HBV- infected mice are shown.

The next step was to determine whether HBe could play the same role *in vivo*. We first investigated the sub-cellular localization of HBe in human hepatocytes engrafted in liver-humanized FRG mice (Bissig et al., 2010), which were chronically infected with HBV. In mice showing persistent infection and high levels of HBV DNA and viral proteins in the serum and liver (Figure S5A and S5C, i.e. mice 396, 399, and 420 versus mock infected 375), we assessed for the cellular distribution of HBe in hepatocytes and found it mainly in the nucleus (Figure S5D, lower right panel). These results were consistent with the data obtained with HBe-

expressing HepaRG cells (Figure S2) and in liver biopsies of chronically HBV infected patients during the immune-tolerance phase (Akiba et al., 1987; Chevallier-Queyron and Chemin, 2011; Chu and Liaw, 1987). Then, ChIP experiments were performed in the liver of these chronically HBV-infected mice to measure Hbc binding to innate gene promoters, as well as the association of EZH2 and related epigenetic marks (Figure 6). Results showed that these highly HBV replicative mice featured concomitant similar enrichment to IFN-related gene promoters for Hbc, EZH2 and the repressive H3K27me3 mark, whereas active marks (H3K27Ac and H3K4me3) were not changed. In addition, in these mice the epigenetic profile was associated with a low expression of innate genes (Figure S6A and S6C). Moreover, on the *IL-6* promoter, neither Hbc, nor EZH2 and the associated repressive mark H3K27me3 were found enriched, whereas positive marks (H3K27Ac and H3K4me3) were found with a low level of enrichment, thus confirming the absence of Hbc effect on *IL-6* promoter. Interestingly, in mice with low viremia and antigenemia (Mice 323, 324, 374, 408 and 413; Figure S5B), Hbc was not found associated to *IFN- α* , *IL-29*, and *OAS-1* promoters (Figure S6D), and *IFN- α* and *IL-6* genes were found significantly up-regulated (Figure S6B), which nicely correlated with the low replicative status.



Suppl. Fig. 6. Innate immune gene expression and ChIP analysis with mouse liver samples. Expression of indicated innate immune genes and level of HBV RNA in high (A) and low (B) viremia mice as measured by RT-qPCR. Results of ChIP-qPCR experiments done with anti-Hbc and primers specific to indicated promoters in high (C) and low (D) viremia mice.

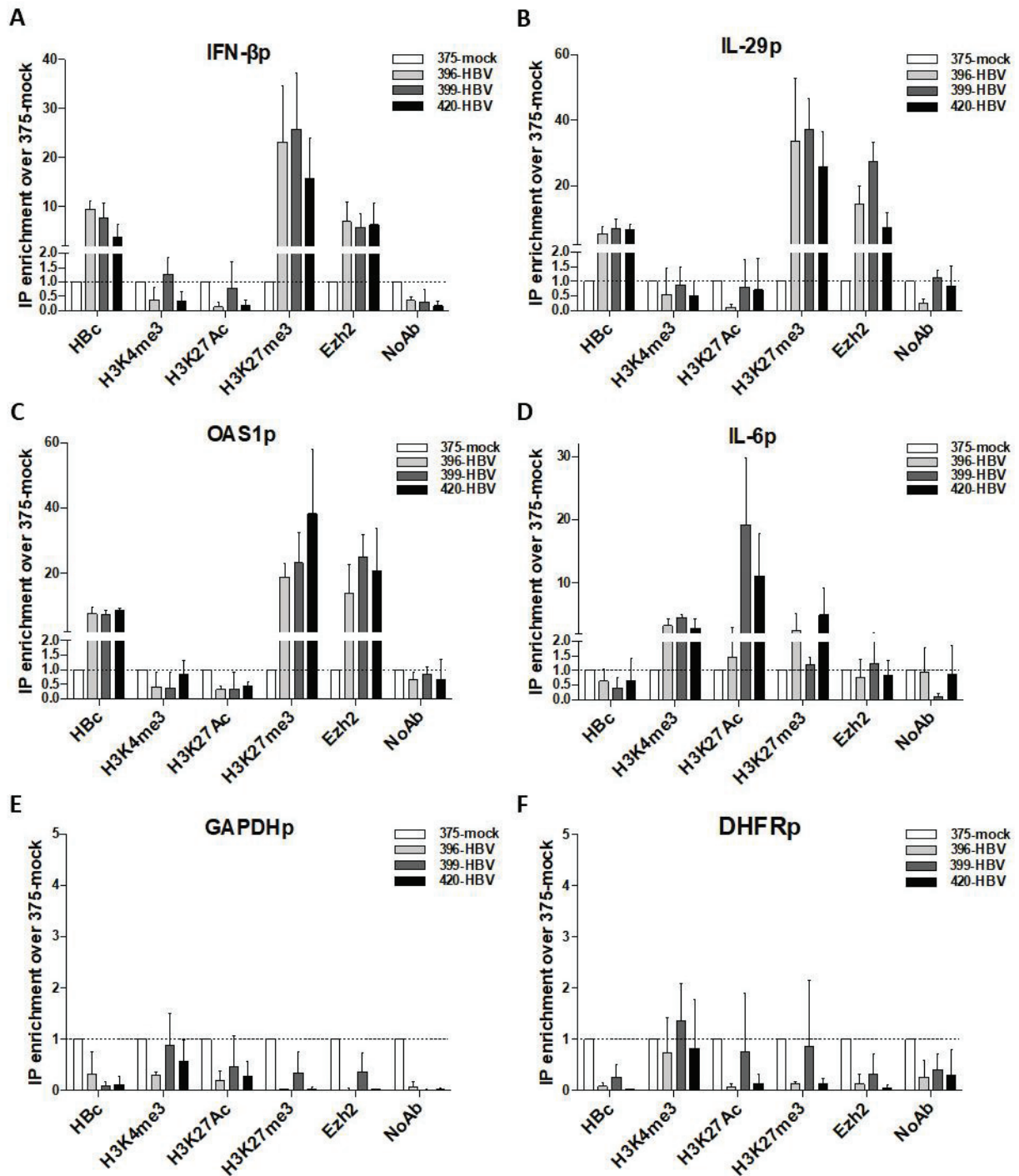
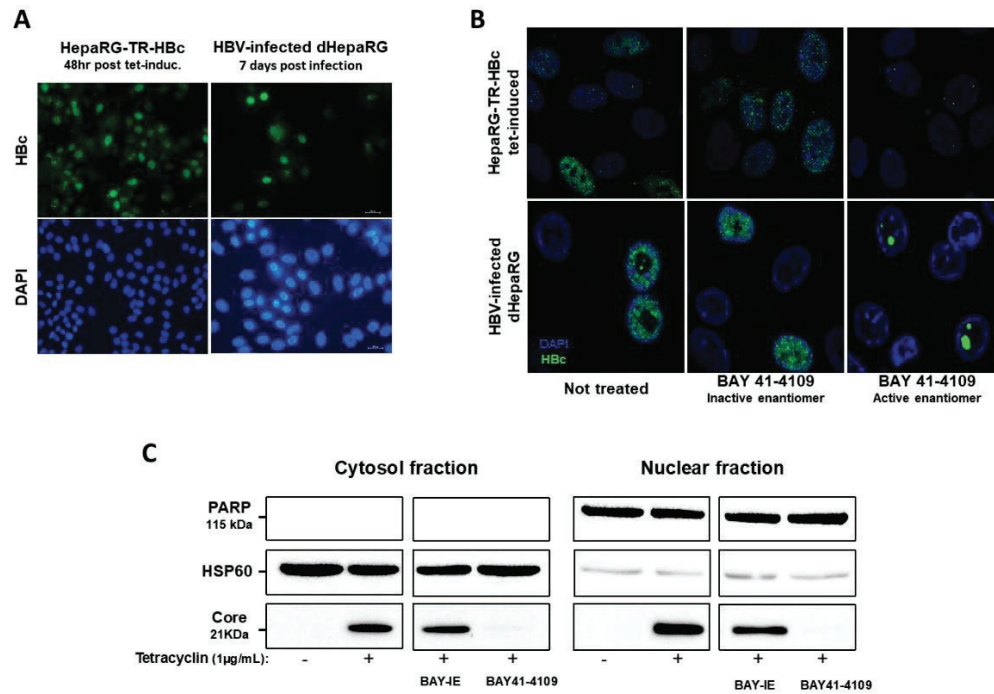


Figure 6: Long-term inhibition of hepatocyte innate response by HBV core protein. Persistently HBV-infected liver-humanized FRG mice were sacrificed 8 weeks post-infection and livers store at -80°C after snap freezing in liquid nitrogen. ChIP-qPCR was performed using none, control, antiH3K4me3, anti-H3K27Ac, anti-H3K27me3, anti-EZH2, or anti-HBc antibodies and specific primers for *IFN-6*, *IL-29*, *OAS-1*, *IL-6*, *GAPDH* and *DHFR* promoters. The percentage of input enrichment was normalized to the non-infected mice. All results are represented as the mean \pm SEM of 3 independent experiments performed with 3 independent extraction of the same liver samples.

Capsid assembly inhibitors increase the anti-HBV effect of an IFN-response inducer

As Hbc interfere with the dsRNA-mediated induction of IFN response, we wondered whether administration of capsid assembly inhibitors, interfering with Hbc localization in infected hepatocyte prior to dsRNA receptor stimulation, could potentiate IFN-mediated antiviral effect. To this end, we used the active enantiomer of BAY41-4109 (Deres et al., 2003), which is capable to block trafficking of Hbc to nucleus in our models (Figure S7) and has an efficient concentration 50% (EC_{50}) of around 320 nM (see panel CII of Figure 7). Persistently HBV-infected dHepaRG cells were treated twice (at day-7 and 10 p.i.) with various concentrations (between 0 to 5 μ M) of the active enantiomer of BAY41-4109, then co-treated once (day-13) with the same concentrations of BAY41-4109 and suboptimal antiviral concentrations (0, 0.33, 1, and 3 μ g/mL; see Figure S8 for the data on antiviral effect) of a TLR3 ligand (i.e. poly (I:C)-LMH). While the effect of combined treatments on HBeAg secretion was minor (Figure 7A), the inhibition of the intracellular HBV DNA accumulation by poly(I:C)-LMW was potentiated by increasing amounts of the capsid assembly inhibitor (Figure 7B and Ci); and vice versa, the inhibition of the intracellular HBV DNA accumulation by BAY41-4109 was also potentiated by increasing amount of poly(I:C)-LMW, with an EC_{50} for the former going from 0.321 nM to 0.078 nM (Figure 7Cii). The removal of Hbc from the nucleus by specific inhibitors could therefore represent an interesting strategy to restore IFN responsiveness of infected hepatocytes.



Suppl. Fig. 7. Effect of capsid assembly inhibitors on HBc trafficking and subcellular localization. (A) Immunofluorescence analysis with anti-HBc (C1; Abcam) in tetracycline-induced HepaRG-TR-HBc and HBV-infected dHepaRG cells. (B) Immunofluorescence analysis, as in panel A, on cell treated or not with inactive and active Bay41-41-09 {Deres, 2003 #66}. (C) Western blot analysis with anti-HBc, anti-HSP60, and anti-PARP antibodies using cell lysates from mock- or tetracycline-induced HepaRG-TR-HBc, which have been fractionated into cytosol and nuclear fraction.

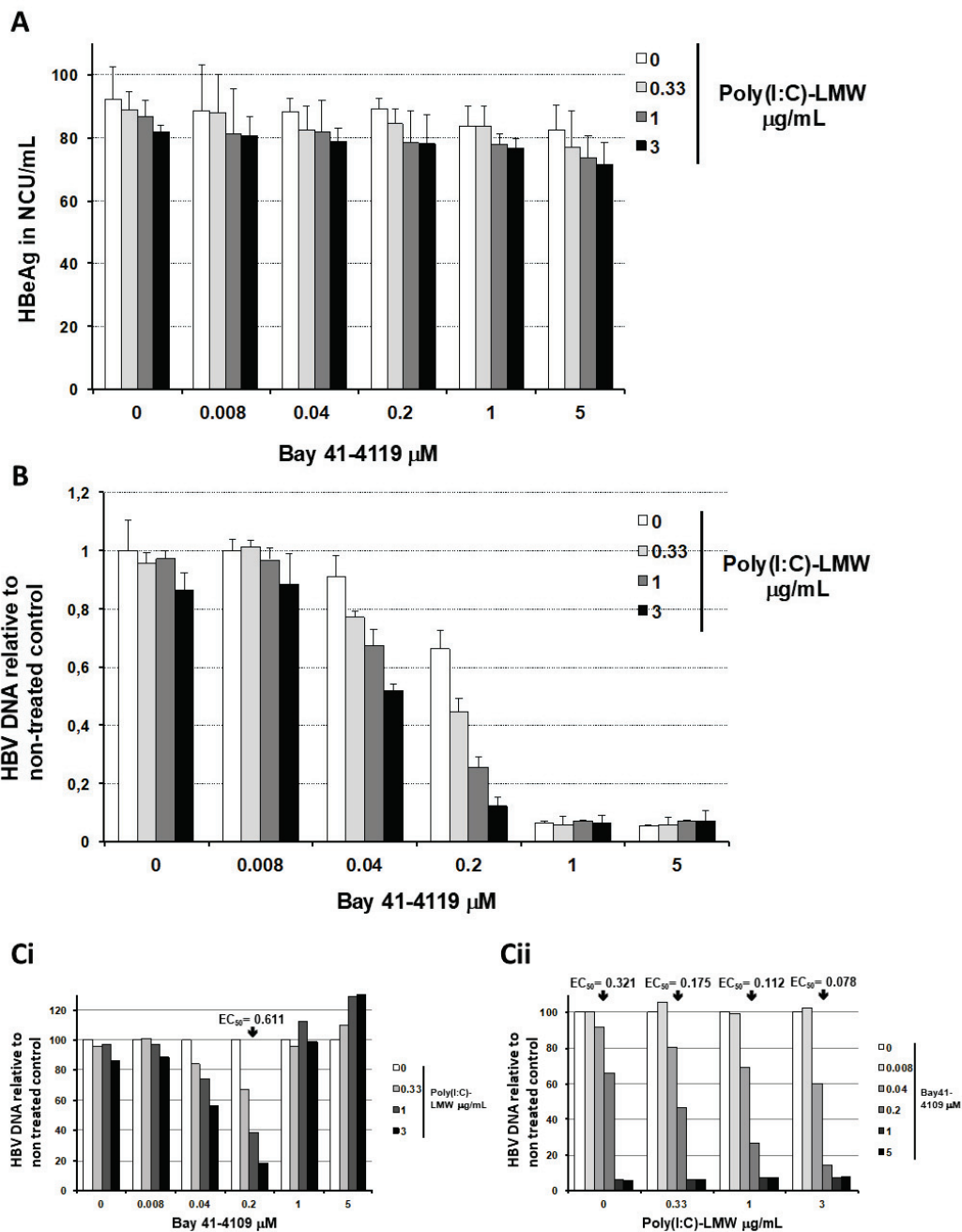
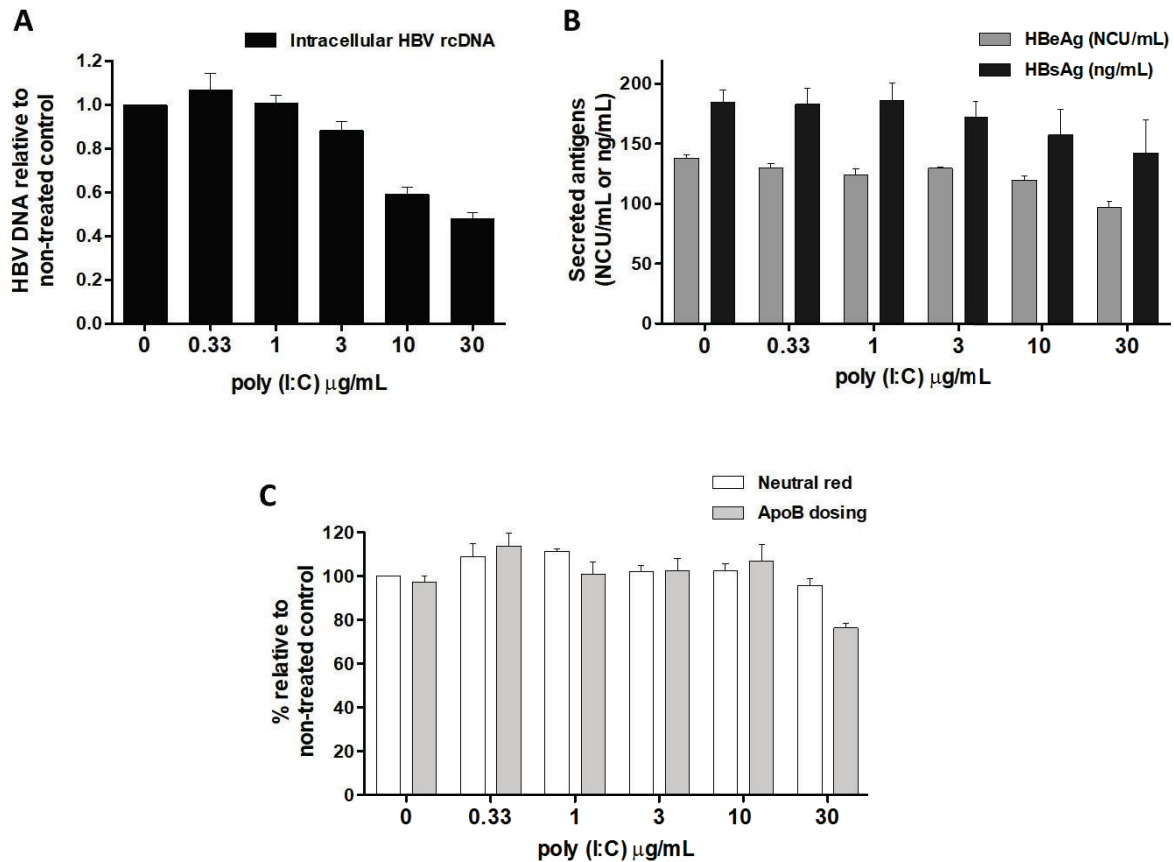


Figure 7: Hbc inhibitor treatment potentiates the anti-HBV effect of TLR3-L. Differentiated HepaRG cells were infected with HBV (100 vge/cell) and left untreated for 7 days. Infected cells were treated twice (at days 7 and 10 p.i.) with various concentration of an Hbc inhibitor (BAY41-4109) as indicated, then co-treated (day 13) for 72 h with various concentrations of BAY41-4109 and poly (I:C). Secretion of HBeAg was monitored in the supernatant of cells at the end of treatment (day 16) (A), and intracellular HBV DNA accumulation by qPCR (B). All results are represented as the mean \pm SEM of 3 independent experiments. On panels (Ci and Cii), the same data as in (B) were re-analyzed in order to standardized in a reciprocal manner to 100% for the no drug condition of a given drug while the other is varying in concentration. This could enable the visualization of EC_{50} that were calculated using the "polynomial equation of individual curves" for each experiment. The precise EC_{50} is given at the top of the graph.

HBc can also inhibit dsRNA-mediated IFN response in non-parenchymal cells exposed to HBV

So far we have shown that HBc is capable to inhibit IFN response in HBV-infected hepatocytes (i.e. liver parenchymal cells). But other cells can be exposed to the virus in the liver, including liver-resident macrophages (i.e. Kupffer cells) and liver endothelial sinusoidal cells (LSEC), the two main scavenger cell types that are also involved in foreign antigen presentation and have innate immune functions in the liver (Crispe, 2011; Knolle, 2016). Moreover, both cell types could also contribute to the active delivery of HBV (or related virus) to hepatocytes by transcytosis mechanisms (Breiner et al., 2001b) (Esser et al., unpublished data), thus suggesting that HBV could penetrate into and deliver HBc to the nucleus of these cells, without replicating in them. To check whether HBV and HBc could also inhibit dsRNA-mediated IFN response in these cell types, we used purified KC from human liver resections, as previously reported (Zannetti et al., 2016), and an immortalized LSEC cell line we previously generated (called iLSEC here) (Parent et al., 2014), exposed them for 24h to 1000 vge/cell of HBV or 500 pg/cell (i.e. equivalent of 1000 vge/cell) of recombinant HBc, then stimulated them with poly(I:C) for 4h. An immunostaining with an anti-HBc at 24h post exposition to HBV (1000 vge/cell) is shown to evidence viral penetration into iLSEC cells (Figure 8A); auto fluorescence of KC did not allow to generate such data in this cell type. A significant reduction of the induction of *IFN- α* , *OAS-1*, and *IL-6* was observed in both cell types with HBV, whereas HBc was only able to inhibit induction of *IFN- α* and *OAS-1* (Figure 8B and C), which is consistent with results obtained in hepatocytes (Figure 1). CHIP experiments were also performed on HBV-exposed iLSEC and KC to measure HBc binding to innate gene promoters. As previously

shown in infected hepatocytes or in the liver of infected mice, HBc was found on *IFN- α* and *OAS-1* promoters, and not on *IL-6* one in both cell types (Figure 8D).



Suppl. Fig. 8. Antiviral effect of TLR3-L on HBV replication in infected dHepaRG. Persistently HBV-infected dHepaRG (day-7 p.i.) were treated for 3 days with indicated concentrations of poly (I:C)-LMW. (A) Intracellular HBV DNA was monitored by qPCR on total DNA. (B) HBV secreted antigens were quantified by ELISA in the supernatant of treated cells. (C) Toxicity was evaluated by neutral red staining on cell and dosing of secretion of apolipoprotein B in supernatant with ALerCHEK ELISA.

Discussion

Neither acute human (Dunn et al., 2009; Stacey et al., 2009) or chimpanzee (Wieland et al., 2004) HBV infections, nor chronic HBV infections (Tan et al., 2010) are accompanied by the production of IFN in the bloodstream of patients and the induction of innate immunity gene expression in the liver. This is in sharp contrast with chronic HCV or HIV infections, in which some interferon-stimulated genes are up-regulated and an uncontrolled IFN response could contribute to immune-pathogenesis {Heim, 2014 #90;Doyle, 2015 #89}.

As for others viruses (Bowie and Unterholzner, 2008; Heim, 2013; Manel and Littman, 2011; Randall and Goodbourn, 2008), HBV has likely evolved mechanisms to prevent and/or escape IFN response. Using rather non-physiological cell culture models and levels of viral protein expression, several groups have pointed out the role of HBx, HBV polymerase, and HBc as potential intracellular regulator of the induction of the expression of type-I IFN (Jiang and Tang, 2010; Wang and Ryu, 2010; Wang et al., 2010; Wei et al., 2010; Wu et al., 2007b; Yu et al., 2010). Moreover secreted HBV proteins, i.e. HBsAg and HBeAg, were also shown to inhibit TLRs-mediated innate immune responses, yet by an ill-defined mechanism (Wu et al., 2007a; Wu et al., 2009).

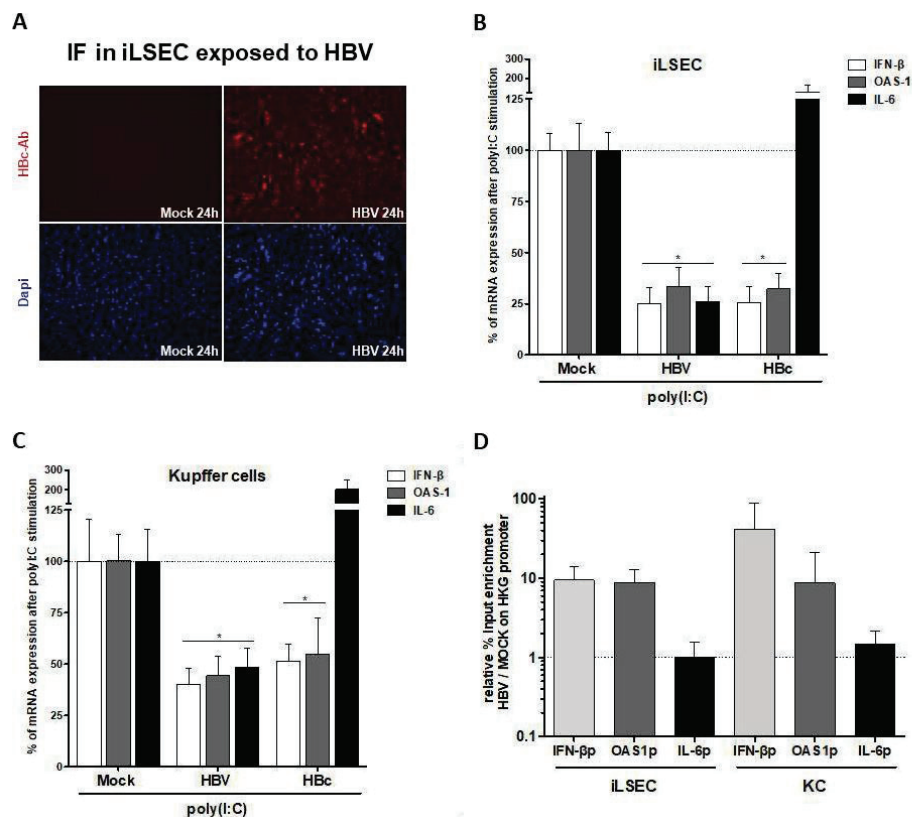


Figure 8: HBc also inhibit TLR3-L-mediated IFN response in primary Kupffer and immortalized liver endothelial sinusoidal cells. (A) Immortalized LSEC were mock- or exposed to HBV (1000 vge/cell), and 24h later cells were fixed and permeabilized (2% PFA; 0.5% triton), then immunostained with an anti-HBc (C1; Abcam). (B and C) Immortalized LSEC or primary KC were either mock-, or exposed to HBV (1000 vge/cell) or recombinant HBc (500 pg/cell; i.e. equivalent of 1000 vge/cell) for 24h, and stimulated by poly (I:C) (10 g/mL) for 4h. Expression of indicated gene was analyzed by RT-qPCR and compared to the mock control normalized to 100%. (D) ChIP experiment was performed on HBV-exposed (1000 vge/cell) iLSEC and KC, with an anti-HBc on indicated promoters as in Figure 3. All results are represented as the mean \pm SEM of at least 2 independent experiments.

In this study, using more relevant models, i.e. proper infection of primary human hepatocytes (PHH) or closely related cells (dHepaRG), we demonstrate that HBc is a key very early negative regulator of the IFN response. Indeed, we have shown that the HBc protein associated to "incoming" HBV virions can inhibit IFN response after trafficking of nucleocapsids (NCs) through the cytosol and translocation of HBc into the nucleus, where it is able to transcriptionally repress IFN-response gene expression. In a previous study, using mainly HepaRG cells, we previously showed (Luangsay et al., 2015b), and we confirmed here using PHH, that this inhibition could be seen as early as 2 hours after the onset of infection, and showed that HBc can be found on the promoter of target genes after only 4h of exposure. Others have found that, after experimental lipo-transfection of NCs to cells, the delivery of HBV genomic DNA into the nucleus takes only few minutes (Rabe et al., 2006), thus confirming that HBc can be rapidly found in the nucleus of neo-infected cells, where it can play its immune-modulatory role.

HBc is capable to bind to dsDNA, including cccDNA as well as host genes (Bock et al., 2001; Guo et al., 2012), and it likely does it via the same domain required for RNA binding, which is a protamine like domain found in mammalian DNA binding protein located at the C-terminus of the protein (Seeger et al., 2015; Zheng et al., 1992). The biophysical aspect of this interaction is yet to be defined, but could involve a dimeric forms of the protein. It is worth noting that all the promoters of targeted genes contained ISRE motifs. EMSA experiment also showed that HBc could bind to ISRE-containing dsDNA (Fernandez et al., 2003).

The early action of HBc could explain why a nascent innate response, as evidenced previously (Luangsay et al., 2015b), could be stopped and why no IFN would not be produced upon infection. We have also shown that when HBV life cycle is launched via a baculoviral vector, therefore in the absence of HBc during inoculation, a strong interferon response, accompanied by eradication of infection, could be observed {Lucifora, 2010 #91}. Altogether our observation show that HBV is indeed a "stealth" virus, not because of absence of detection, but rather because of a very early inhibition of innate immune responses. Such an early inhibition of innate immune responses via a structural protein embarked into virions and readily mobilized into the newly penetrated cells has been shown, to our knowledge, only in the case of the tegument protein (ORF52) of KHSV. This protein was indeed very recently

shown to prevent cGAS DNA sensing just after entry of virions within cells, and in the absence of viral replication and protein neo-synthesis (Wu et al., 2015).

In the nucleus of newly infected cells, we have shown that HBc binds to promoters of IFN response genes, and somehow induce the recruitment of the histone methyl-transferase EZH2, which is in turn responsible for the establishment of the H3K27me3 repressive mark, leading to the impairment of their inducibility upon dsRNA sensors stimulation. The binding of HBc to IFN response gene promoters was also found in liver-humanized HBV-infected FRG mice, thus suggesting that this early-established mechanism, set with incoming HBc, would also at work in persistent infections. The accumulation of HBc within nucleus is possible throughout the life cycle of the virus thanks to the recycling of neo-synthesized nucleocapsid to nucleus in infected cells. In chronically infected patients, HBc was also found in the nucleus of hepatocytes (Akiba et al., 1987; Chevallier-Queyron and Chemin, 2011; Chu and Liaw, 1987; Nakayama et al., 2001; Ng et al., 2009), in particular in patients who are in the high-replicative, low-inflammatory phase. Here, we show immune-staining data that further support the idea of a correlation between high viremia and nuclear localization of HBc in the liver of patients. The correlation between the nuclear localization of HBc and the low-inflammatory phase might be explained, at least in part, by the inhibitory mechanism we described here.

As we have shown that the virus does not need to replicate to deliver HBc to the nucleus of hepatocytes, HBc could be delivered in other cell types to set similar inhibitory effect. Indeed in the immune tolerance phase, a high amount of infectious particles, containing HBc, circulates in the blood of patients (between 10^7 and 10^{10} HBV-genome-copies/mL) (Seeger et al., 2015), and this concentration may be even higher in the liver microenvironment, thus potentially out numbering liver cells. It has been suggested that HBV could penetrate into LSEC (liver sinusoidal endothelial cells) (Breiner et al., 2001a), Kupffer cells (Esser et al., 2012), and pDC (Vincent et al., 2011), which are three important cell types to mount an efficient innate response. Our data suggest that indeed HBc could be delivered to the nucleus of primary KC and immortalized LSEC, and lead to impairment of IFN gene expression. Therefore, the new concept of IFN response inhibition we described for hepatocytes may also be valid for many other non-parenchymal and/or liver resident immune cells.

Finally, we have shown that prevention of the migration of HBc to the nucleus with

nocodazole or CpAMs, restore the IFN response to dsRNA ligands. Does this mean that core assembly modulators could be used *in vivo* to restore immune function in infected hepatocytes and help reinforced immune response against the virus? As HBc possesses both nuclear localization (NLS) and export (NES) sequences (Li et al., 2010), as it has been proposed that the pool of nuclear cccDNA could be constantly replenished by NCs recycling (Seeger et al., 2015), and despite its long-half life in the nucleus (our data not shown), it is tempting to speculate that a long-term treatment with core inhibitors could lead to such a purge. To substantiate this hypothesis, we have finally shown in this study that a capsid assembly inhibitor, leading to nuclear HBc removal (or aggregation), could potentiate the anti-HBV effect of a TLR3-L.

Many core/capsid inhibitors, also called CpAM (core protein allosteric inhibitor) (Zlotnick et al., 2015), are under current development. Selected on their "canonical functions", i.e. inhibition of the packaging of HBV pregenomic RNA and subsequent inhibition of capsid reverse transcription, CpAMs could also have additional antiviral effect due to their impact on HBc nuclear function, and should therefore be also selected on this criteria. Our results suggest that CpAMs could be favorably combined to immune stimulators to obtain a greater anti-HBV effect. Among immune-stimulator in current clinical evaluation is the TLR7 agonist GS-9620, which has shown very interesting antiviral effect, leading in particular to the degradation of cccDNA in animal models (Lanford et al., 2013; Menne et al., 2015). As TLR7 is not expressed in infected human hepatocytes (Luangsay et al., 2015a), and TLR7-L likely acts via pDC-expressed IFN- α , it would be of interest to develop PRR agonist that could both stimulate innate cells and induce expression of IFN- α and related ISGs within infected hepatocytes. In this respect, our study makes a case for a combinatory approach with CpAMs and PRR agonists in order to progress toward the rupture of tolerance to HBV.

Material and Methods

Antibodies and others reagents. Most of chemicals and control antibodies were purchased from Sigma Aldrich. Poly (I:C) low-molecular weight (LMW), poly (I:C) (LMW)/Lyovec, and other ligands were from Invivogen. Antibodies for CHIP experiments were purchased from Diagenode (anti-H3K27me3 pAb-069-050, anti-H3K4me3 pAb-003-050, anti-H3K27Ac pAb-

196-050, anti-EZH2 pAb-039-050, and control antibodies), or homemade (polyclonal against HBc; (Petit and Pillot, 1985)). A polyclonal rabbit anti-HBc from Dako, as well as a monoclonal from Abcam (Clone C1) were used for immunostainings and Western blots.

Production of HBV inoculums, Dane particles and non-enveloped HBV nucleocapsids.

HBV viral stocks were prepared and tittered as previously reported (Luangsay et al., 2015b). Non- enveloped nucleocapsids (density of 1.25 g/mL) were purified from NP40-treated HepG2.2.15. supernatant and characterized, as previously reported (Rabe et al., 2006; Luangsay et al., 2015b). All preparations were tested for the absence of endotoxin (Lonza Verviers, Belgium). *Pichia pastoris*, GMP-produced recombinant HBc (VTI610) was purchased from Meridian Life Science. To standardize the various inocula, the same quantity of HBc was used, and a dose of 100 vge/cell for a full HBV inoculum corresponded to 50 pg of HBc/cell.

Isolation of primary human cells and cell line culture conditions. Primary human hepatocytes (PHHs) and Kupffer cells were prepared from surgical liver resections after informed consent of patients (kindly provided by Pr. Rivoire (CLB, Lyon)) as previously described (Lecluyse and Alexandre, 2010; Zannetti et al., 2016), and cultured in complete William's medium supplemented with 1.8 % of DMSO for PHH, and complete RPMI medium for KC. The human liver progenitor HepaRG cells (Gripon et al., 2002) were cultured and infected as previously described (Luangsay et al., 2015a; Luangsay et al., 2015b). Immortalized liver endothelial sinusoidal cells TRP3/iLSEC cells were cultured as previously reported. (Parent et al., 2014).

Generation of HBV core and other HBV protein- expressing HepaRG cells. To generate recombinant cell line we used lentiviral vectors and procedures from Invitrogen (ViraPower™ T-REx™ Lentiviral Expression System). Stable overexpression of the tetracycline repressor (TR) in HepaRG cells was obtained after transduction with a lentivirus (m.o.i. of 5) produced upon cotransfection of HEK-293 cells with pLenti6-TR plasmid and packaging vectors according to manufacturer's instructions. Blastidicin-resistant HepaRG-TR cells were then transduced with a second lentivirus conferring zeocin resistance and containing either HBc, HBs, HBx, HBe (genotype D, serotype ayw, Galibert's strain) or a T7 phage polymerase transgene, and produced upon cotransfection of HEK-293 cells with packaging vectors and with corresponding pLenti4/TO plasmids, in which transgene are under the control of a tetracycline-regulated promoter. HepaRG-TR-HBc, HepaRG-TR-HBs, HepaRG-TR-HBe,

HepaRG-TRx and HepaRG-TR-T7pol were derived by co-selection with blasticidin and zeocin.

HBV infection and lipo-transfection of recombinant HBcAg and non-enveloped nucleocapsids. Differentiated HepaRG cells and PHH were infected with HBV or its control mock (depleted from HBsAg, HBeAg and infectious Dane particles) in complete William's medium supplemented with 4% PEG-8000 at 37°C as previously described (Hantz et al., 2009). Recombinant HBcAg and non-enveloped nucleocapsids were lipo-transfected into HepaRG cells using "Pro-Ject Protein Transfection Reagent" kit according to manufacturer's instructions (Thermo Scientific).

Humanized FRG mouse model and HBV infection. All animal studies were reviewed and approved by a local ethical comity. The highly immunosuppressed FRG mice ($Fah^{-/-}/Rag2^{-/-}/Il2rg^{-/-}$), deficient for T-, B- and NK-cells were used (Azuma et al., 2007) and maintained in a specific pathogen-free facility. High quality, cryopreserved human hepatocytes were purchased (BD, Biosciences) and injected via an *intra-splenic* route into 2 to 3 month old mice as described previously. Liver humanized $Fah^{-/-}/Rag2^{-/-}/Il2rg^{-/-}$ mice featuring a seric production of human albumin of at least 5mg/mL were infected with 200 μ l of HBV inoculums (1.10^8 veg to 1.10^9 veg in PBS) via *intra-peritoneal* route (Bissig et al., 2010). Serum was harvested every week by retro-orbital bleeding and stored at -80°C in aliquots for further antigenemia and viremia analysis. Mice were sacrificed 9 weeks post-infection and hepatic tissues were processed for RT-qPCR and CHIP analysis or fixed in formalin and embedded in paraffin for immune-staining.

PRRs stimulation. Cells were stimulated with TLR or RLR agonists and harvested for the analysis of the innate gene expression (after 4h) and cytokines production (after 6h). TLR and RLR agonist were: TLR1/2 (Pam3CSK4, 0.8 μ g/ml), TLR3 (Poly (I:C)-LMH, 10 μ g/ml), TLR4 (LPS, 0.4 μ g/ml), TLR5 (Flagellin, 0.1 μ g/ml), TLR6 (FSL-1, 0.1 μ g/ml), TLR7/8 (ssRNA40, 10 μ g/ml), RIGI/MDA5 (Poly (I:C)-LMH-Lyovec 0.2 μ g/ml).

RNA extraction and qRT-PCR. Total RNA were purified with the Nucleospin RNA II kit according to the manufacturer's protocol (Macherey Nagel). cDNA was obtained after reverse transcription using the Superscript® III Reverse Transcriptase (Life technologies) and qPCR was performed using the Express SYBR GreenER qPCR Supermix Universal according to the manufacturer's instructions (Life technologies) and run on the MyiQ Biorad machine. The

relative mRNA expression was analyzed with q-base software (Biogazelle, Belgium) using the comparative cycle threshold ($2^{-\text{titiCt}}$) method with 2 housekeeping genes (RPLP0 and α -Actin). The relative HBV mRNA level was quantified using the same primer pairs used for the HBV PCR quantification. The sequences of primer pairs were listed on **Table 1**.

Table 1. Primer pairs for RT-qPCR

GENE	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')
α-actin	TGGCATTGCCGACAGGATGC	TCTGCTGGAGGTGGACAGCGA
RPLP0	CACCATTGAAATCCTGAGTGATGT	TGACCAGCCCAAAGGAGAAG
IFN-α	GCCGCATTGACCATGTATGAGA	GAGATCTTCAGTTTCGGAGGTAAC
IL-29	GTGACTTTGGTGCTAGGCTTG	GCCTCAGGTCCCAATTCCC
OAS-1	AGGTGGTAAAGGGTGGCTCC	ACAACCAGGTCAGCGTCAGAT
IL-6	ACCCCTGACCCAACCACAAAT	AGCTGCGCAGAATGAGATGAGTT

Western Blotting. Cells were lysed in TTB buffer (10 mM Tris, 150 mM NaCl, 2 mM EDTA, 0.5% Triton X-100, pH 7.5) for total proteins extraction or in NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific) for nuclear and cytosolic proteins separation. All lysis buffers were complemented with inhibitor cocktail of proteases (Complete EDTA-free tablets, Roche). Extracted proteins were separated electrophoretically on a 10% SDS-PAGE gel and "semi-dry" transferred onto hybond-ECL membranes (Amersham, biosciences). Membranes were blocked with 5% non-fat milk in PBS 0,1% Tween for 1 hr. at RT. Polyclonal rabbit anti- HBc (Petit and Pillot, 1985), anti-actin (13E5, Cell Signaling), anti-tubulin (MAB3408, Millipore) and anti-PARP (Zymed, Invitrogen) antibodies were applied overnight at 4°C under gentle agitation. After washing, HRP conjugated secondary antibodies from Sigma Blots were applied. After extensive washing, blots were revealed using the SuperSignal West Pico Chemiluminescent substrate (Thermo Scientific).

ELISA. Commercial immunoassay kits for HBs antigen (Autobio Diagnostics Co., China), HBe/HBc antigens (Autobio Diagnostics Co., China), and IL-6 and IP-10 cytokine (R&D system) detections were used following manufacturer's instructions.

Chromatin immunoprecipitation (ChIP) assays. Cells were fixed with formaldehyde 1% at room temperature with rocking to capture the DNA-protein interaction and cross-linking was stopped with Glycine 0.125M (Euromedex). Cells were washed 3 times with ice-cold PBS 1X then scrapped and lysed in 500µl of lysis buffer per 10^7 cells (50 mM Tris HCl pH8, 10 mM EDTA, 1% SDS and protease inhibitor cocktail (Complete EDTA-free tablets, Roche)) for 5 min in ice. Cell lysates were sonicated to reduce DNA length to 200 bp using a Bioruptor system (Diagenode). The sheared chromatin extract was then frozen in aliquots at -80°C until use. Sheared chromatin (100 µl) was diluted 10-fold in RIPA buffer (10 mM Tris HCl pH5, 140 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 1% Triton, 0.1% SDS, 0.1% sodium deoxycholate, protease inhibitor cocktail) and then pre-blocked with protein A sepharose 4B (Sigma) for 2 hours at 4°C under gentle agitation. Pre-blocked chromatin was incubated overnight with rotation at 4°C with 10µg of specific antibodies. An additional sample of lysate containing no antibody was also incubated overnight at 4°C (no antibody control). Chromatin was then immunoprecipitated with magnetic beads (Dynabeads Protein G, Novex) for 2 h at 4°C with rotation. After several washes with RIPA buffer, cross-linking was reversed at 68°C for 2h under shaking in elution buffer (20 mM Tris HCl pH7.5, 5 mM EDTA, 50 mM NaCl, 1% SDS, 50 µg/ml proteinase K (Eurobio) and inhibitor cocktail proteases). DNA from elution buffer was extracted with Phenol/Chloroform/Isoamyl-Alcohol and precipitated with ethanol supplemented with NaAc 3M and Glycogen 20mg/ml (Sigma) overnight at -20°C . Extracted DNA from non-immunoprecipitated fraction of no antibody control was used as internal control (total input). Extracted DNA was used as template for qPCR specific on IFN- α p, IL-29p, IL-6p, OAS-1p and GAPDHp. The percentage of input enrichment ($2^{\Delta\text{t}i\text{Ct}}$ (total input - antibody input)) was normalized on the Mock condition depending of the experiments (no tetracycline stimulation or no HBV infection) and also on GAPDH housekeeping gene promoter. Results are representative of 3 independents experiments. The list of primer pairs is given on **Table2**.

Table 2. Primer pairs for ChIP-qPCR

GENE	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')
<i>GAPDHp</i>	CTTCTCCCCATTCCGTCTTC	CCCCAGCTACAGAAAGGTCA
<i>IFN-6p</i>	CTTTCGAAGCCTTTGCTCTG	CAGGAGAGCAATTTGGAGGA
<i>IL-29p</i>	GCCCAGGGAGTTCTAAGGAT	CTGATGAGGGAACAGGTGTG
<i>OAS-1p</i>	TGAAATTCAGCACTG GGATCAC	GGAGGAGCTGTCTTTGCACTT
<i>IL-6p</i>	GCCCAGGGAGTTCTAAGGAT	CTGATGAGGGAACAGGTGTG

Statistical analysis. Statistical analysis was performed by two-way ANOVA, non-parametric Mann-Whitney, or t-tests using the GraphPad Prism software. For all tests, p-values <0.05 (*), <0.01 (**), and <0.001 (***) were considered as significant.

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MicroRNA inhibitors rescuing RNA helicase DDX5 as HBV antiviral and anti-HCC tools

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Chronic hepatitis B virus (HBV) infection is linked to liver cancer pathogenesis. Despite the HBV vaccine, 250 million people globally are chronically infected with HBV. Current treatments are ineffective. New approaches are needed targeting mechanisms that regulate both virus biosynthesis and liver cancer pathogenesis.

We have identified an epigenetic mechanism hijacked by HBV and linked to poor prognosis HBV-associated liver cancer¹, involving the chromatin modifying PRC2 complex, the RNA helicase DDX5, and the lncRNA HOTAIR². Loss of PRC2 function in a subpopulation of HBV replicating cells de-represses expression of a hepatic cancer stem cell (hCSC)-like gene signature (EpCAM, BAMBI, CD44, CD133, Oct4, Nanog, and Sox2)³. Furthermore, DDX5 which facilitates HOTAIR/PRC2-mediated transcriptional repression of these genes, is downregulated during HBV infection. Significantly, re-expression of these hCSC genes and downregulation of DDX5 are linked to poor prognosis HBV-associated liver cancer.

Our ongoing studies have generated data which show: 1) DDX5 is a roadblock to hCSC formation. Specifically, downregulation of DDX5 allows hepatosphere formation, expression of Wnt-induced pluripotency genes, decreased sensitivity to cisplatin and Sorafenib, and abrogation of anti-tumor immunity. 2) Downregulation of DDX5 during HBV infection occurs via induction of oncomiRs miR17~92 and miR106b~25. Interestingly, downregulation of DDX5 enhances virus biosynthesis via an unknown mechanism. 3) Inhibitors of miR17~92 and miR106b~25 reverse loss of DDX5 and other known tumor suppressor targets (PTEN, SMAD7, LKB1), and preliminary studies show these miR inhibitors (anti-miRs) reduce HBV replication. These observations suggest development of these anti-miRs as anti-viral and anti-tumor molecular tools.

To effect targeted delivery of these anti-miRs to infected hepatocytes, we determined the expression of the folate receptor in HBV replicating cells (HepAD38 cells and PHHs). Interestingly, HBV infection increases expression of the folate

receptor, providing a specific handle for delivering the anti-miRs via covalent conjugation to folate, as recently described⁴.

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