PROTEOMIC ANALYSIS OF NUCLEAR HBV rcDNA ASSOCIATED PROTEINS
IDENTIFIES UV-DDB AS A HOST FACTOR INVOLVED IN cccDNA FORMATION

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DEDICATION

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IDENTIFIES UV-DDB AS A HOST FACTOR INVOLVED IN cccDNA FORMATION

Despite the lifecycle of the hepatitis B virus (HBV) being extensively investigated and described, there remains a significant gap in our knowledge of arguably one of the most crucial steps in the HBV lifecycle, the formation and maintenance of a covalently closed circular DNA (cccDNA) reservoir. Advancements in our understanding of host factors and pathways involved in cccDNA formation have been made through hypothesis driven studies and shRNA/siRNA screenings. We sought to create a targeted-unbiased assay to directly observe host factor-rcDNA interactions. This was achieved through an rcDNA Co-Immunoprecipitation paired Mass Spectrometry (rcDNA-CoIP/MS) assay. We created a DNA oligo complimentary to the open portion of the HBV rcDNA, labeled with biotin, to facilitate easy precipitation of nuclear rcDNA and complexed proteins. Proteins precipitated were analyzed through liquid chromatography paired mass spectrometry (LC/MS). Along with previously reported host factors, several factors of DNA damage repair pathways/complexes were also identified. A component of the UV-DDB complex, DDB1, surfaced as a hit. UV-DDB/rcDNA binding was confirmed through ChIP-qPCR. DDB2, the DNA damage binding component of the UV-DDB complex was knocked out in HepG2-NTCP and HepAD38 cells. This resulted in a significant decrease in the formation of cccDNA in DDB2 knockout cell lines following infection or induction. The subsequent reduction of downstream indicators of cccDNA formation such as viral RNA and proteins, HBcAg and HBeAg, showed a consistent decrease with cccDNA levels. Ectopic expression of DDB2 in the knockout cell lines rescued HBV phenotypes of cccDNA levels and its downstream indicators. Inactive mutant DDB2 plasmids were also transfected into the DDB2 K/O cell lines and failed to rescue cccDNA indicators. We therefore showed through a novel assay that we can discover novel viral rcDNA-host interactions, such as the UV-DDB complex recruiting DNA repair pathways to “repair” rcDNA to cccDNA.

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LIST OF ABBREVIATIONS

HBV	Hepatitis B Virus
HBsAg	Hepatitis B Surface Antigen
L-HBsAg	Large Hepatitis B Surface Antigen
M-HBsAg	Medium Hepatitis B Surface Antigen
S-HBsAg	Small Hepatitis B Surface Antigen
HBc	Hepatitis B Core Antigen
rcDNA	Relaxed Circular DNA
HBpol	Hepatitis B polymerase
ORF	Open Reading Frame
C	Core Gene
P	Polymerase Gene
S	Surface Protein Gene
X	X protein Gene
AA	Amino Acid
ER	Endoplasmic Reticulum
HBeAg	Hepatitis B E Antigen
TP	Terminal Protein Domain
pgRNA	Pregenomic RNA
RT	Reverse Transcription Domain
RH	RNase H Domain
cccDNA	Covalently Closed Circular DNA
NTCP	Sodium Taurocholate Co-Transporting Polypeptide
HNF	Hepatocyte Nuclear Factor
HCC	Hepatocellular Carcinoma
HBx	Hepatitis B X Gene Protein
DR	Direct Repeat
DR1	Direct Repeat 1
DR2	Direct Repeat 2
dsIDNA	Double Stranded Linear DNA

MVB	Multivesicular Bodies
CTD	C-Terminal Domain
NLS	Nuclear Localization Signal
dsl-ltr-DNA	Double Stranded Linear Long Terminal Repeat DNA
LTR	Long Terminal Repeats
NHEJ	Non-Homologous End Joining
DP-rcDNA	Deproteinized Relaxed Circular DNA
PF-rcDNA	Protein Free Relaxed Circular DNA
DP-dslDNA	Deproteinized Double Stranded Linear DNA
DHBV	Duck Hepatitis B Virus
CM-rcDNA	Closed Minus Relaxed Circular DNA
DDR	DNA Damage Response
ATM	Ataxia-Telangiectasia Mutate Protein Kinase
ATR	Ataxia-Telangiectasia and Rad3-related Protein
NUCs	Nucleos(t)ide Analogues
PFA	Phosphonoformic Acid
APH	Aphidicolin
FEN1	Flap Endonuclease 1
ChIP	Chromatin Immunoprecipitation
qPCR	Quantitative Polymerase Chain Reaction
PTPD	3-hydroxy-5-methyl-1-phenylthieno[2,3-d]pyrimidine-2,4(1H,3H)-dione
LIG	Ligases
TOP	Topoisomerases
TDP2	Tyrosyl-DNA-Phosphodiesterase
PCNA	Proliferating Cell Nuclear Antigen
RFC	Replication Factor Complex
Pol	Polymerases
PRPF31	Pre-mRNA Processing Factor 31
CDK	Cyclin Dependent Kinase
SAMHD1	SAM and HD Domain Containing Deoxynucleoside Triphosphate Triphosphohydrolase 1

IFN	Interferon
APOBEC	Apolipoprotein B mRNA Editing Enzyme, Catalytic Polypeptide-Like Protein
MXA	Interferon-Induced GTP-Binding Protein
ISG20	Interferon Stimulated Gene 20
CHB	Chronic Hepatitis B
ALT	Alanine Aminotransferase
DMEM	Dulbecco's Modified Eagle Medium
FBS	Fetal Bovine Serum
WT	Wild-Type
Ndel	N-terminal Deletion
TEG	Triethylene Glycol Spacer
Tet	Tetracycline
LC	Liquid Chromatography
MS	Mass Spectrometry
PBS	Phosphate Buffered Solution
crRNA	CRISPR RNA
PAM	Protospacer Adjacent Motif
CRISPR	Clustered Regularly Interspaced Palindromic Repeats
HMM	Hepatocyte Maintenance Media
PMM	Primary Hepatocyte Maintenance Media
ELISA	Enzyme-Linked Immunosorbent Assay
PSAD	Plasmid Safe ATP-Dependent DNase
CoIP	Co-Immunoprecipitation
POI	Protein Of Interest
DDB1	DNA Damage Binding Protein 1
DDB2	DNA Damage Binding Protein 2
UV-DDB	Ultra Violet DNA Damage Binding Complex

CHAPTER ONE

INTRODUCTION

1.1 The Hepatitis B Virus

The Hepatitis B virus (HBV) is a human hepatotropic pathogen of the *Hepadnaviridae* family. It remains one of the major etiologic agents of infectious cirrhosis and liver cancer in the world (2-4). The infectious particle of HBV, AKA the HBV Dane particle, consists of an outer lipid membrane implanted with three isoforms of the Hepatitis B surface antigen (HBsAg): Large (L-HBsAg), Medium (M-HBsAg), and Small (S-HBsAg) (5, 6) (**Fig.1A**). Within the outer membrane lies a viral capsid consisting of the Hepatitis B core protein (HBc) which holds the HBV relaxed circular DNA (rcDNA)(7-9). The HBV rcDNA is unique in that it is covalently bound to the viral polymerase (HBpol) as well as consisting of an incomplete and unligated circular episome with redundant sequence flap on the minus (negative sense) strand. The 5' end of the plus (positive sense) strand also contains a vestigial RNA primer, a remnant of the reverse transcription step in rcDNA synthesis.

1.1.1 The HBV Genome

The HBV genome, within the infectious viral particle, is a 3.2 kilobase (kb) semi-double stranded circular DNA molecule with several aberrant terminal structures (10, 11). The viral episome expresses four open reading frames (ORFs), the Core (C), Polymerase (P), Surface (S), and X (12). Due to the compact nature of the HBV genome, all four ORFs overlap and share the same terminal poly-adenylation site (12-15) (**Fig.1B**). The C ORF encodes for the 21-kDa viral capsid structural protein, HBc as well as a 25-kDa Pre-core (Pre-C) protein. The 25-kDa Pre-C protein contains an additional 29 amino acid (AA) from

the small Pre-C extension of the C gene. A 19 AA portion of this extension constitutes a signaling peptide marking the 25-kDa protein for secretion through the endoplasmic reticulum (ER) (16-18). This 19 AA signaling peptide is cleaved within the ER lumen producing a 22-kDa precore protein (p22). The p22's carboxy terminus is further digested by a furin protease within the Golgi, resulting in the Hepatitis e antigen (HBeAg) which is then finally secreted from the cell. Though not directly essential to HBV replication, the HBeAg is believed to have important immunomodulatory functions, allowing for persistent and chronic infections (19-22).

Figure 1. HBV Virion and Genome. HBV structure and genome. A) The envelope of HBV virion consists of lipids, and the large, medium, and small viral surface proteins. Within the lumen of the virus, core protein forms an icosahedral capsid around the rcDNA genome which has the viral polymerase covalently attached to the 5' end of the (-) strand and a capped RNA primer (red) attached to the 5' end of the (+) strand. B) The 3.2kb HBV genome encodes four overlapping ORFs (Core, Surface, Polymerase, and X) and four mRNA transcripts with an overlapping polyadenylation site (3.5kb, 2.4kb, 2.1kb, and 0.7kb).

The viral polymerase, HBpol, is the largest protein expressed by HBV, coming in at 750 AA; it is a DNA polymerase capable of conventional DNA synthesis as well as reverse transcription and RNase H activity (8, 23-27). The terminal protein (TP) domain of HBpol (AA: 1-177) triggers the encapsidation of the HBV pregenomic RNA (pgRNA) as well as priming and initiating reverse transcription along the ε structure of the pgRNA (26, 27). The polymerase/reverse transcriptase (RT) domain facilitates the reverse transcriptase catalytic activity of HBpol and is necessary for the reverse transcription of pgRNA into rcDNA (28, 29). The RNaseH (RH) domain is responsible for the digestion of pgRNA as it undergoes reverse transcription (30-32). Interestingly, despite the presence of HBpol in the infectious virion as it infects host hepatocytes, it has not been shown to be necessary for repairing rcDNA terminal aberrations to form covalently closed circular DNA (cccDNA). Instead HBpol must be removed for the critical lifecycle step of cccDNA formation to take place (8, 33, 34).

The HBV S ORF contains 3 sub-regions allowing for the expression of the three isoforms of HBV surface protein: L-HBsAg, M-HBsAg, and S-HBsAg (35, 36). The Pre-S1 region of the S ORF allows for the transcription of the 2.4 kb HBV mRNA, coding for L-HBsAg. The 2.1 kb HBV mRNA can include both the Pre-S2 and S region resulting in the expression of M-HBsAg or S-HBsAg (36). L-HBsAg has the crucial role of binding to the liver specific HBV receptor Sodium Taurocholate Co-Transporting Polypeptide (NTCP) (37-39). The pre-S1 2-47 AA sequence, present only in the L-HBsAg, was shown to be indispensable for the binding of NTCP and therefore the infectivity of HBV (40-45). The S-HBsAg has also been shown to have significant roles in the HBV lifecycle including virion morphogenesis, secretion, and immunomodulatory functions through B- and T-cell

epitopes(36, 46, 47). The general function of the M-HBsAg has been difficult to ascertain and remains uncertain. It appears the M-HBsAg is not necessary for viral entry or replication, however, pre-S2 deletions were shown to be prevalent in hepatocellular carcinoma (HCC) postoperative intrahepatic recurrence, suggesting possible immunomodulatory/oncogenic effects (35, 36, 48). The S-HBsAg is the most prevalent of the HBs antigens and makes up a majority of the surface protein content of infectious HBV particles as well as non-infectious subviral particles (49, 50).

The HBV X ORF (465 bp) encodes the final and smallest of the HBV proteins, the 16.5-kDa X gene protein (HBx). The HBx is also the sole product of the 0.7kb HBV mRNA transcript and carries out an extensive array of lifecycle and pathogenic functions within host cells. The HBx is known to be intricately involved in host signal transductions, transcriptional regulation through epigenetic modifications of host and viral genomes, DNA repair, and selective degradation/preservation of host factors (51-54). Although not included in the Dane particle, the HBx has been shown to be essential to an active HBV infection by facilitating the expression of cccDNA in an infected nucleus (55, 56). Furthermore, the HBx has been thoroughly linked to the long-term pathogenesis and oncogenesis of HBV leading to the development of HCC. HBx achieves this through a multifaceted approach via the inhibition of DNA repair mechanisms and lifecycle check points such as p53 inhibition, activation of Wnt signaling, Notch 1 signaling inhibition, NF- κ B activation, and interactions with host siRNAs (57-64).

While, in large part, the hepatotropic nature of HBV is mediated by its specific binding to the hepatocyte unique surface protein, NTCP, liver-enriched genetic promoters play an essential role in HBV genome expression. The HBV genome contains two enhancer

regions, EnhI and EnhII that can be bound by several host and viral factors to enhance HBV gene activity. The EnhI region resides between the S and X ORF and is around 300 nucleotides long (65). While EnhII is located upstream of the core promoter and overlaps with the core regulatory sequence. Several hepatocyte nuclear factors (HNFs) interact with these enhancer regions as well as other locations along several ORF regions (66). HNF1 α is reported to bind to and activate the PreS1 and EnhII regions (67, 68). HNF3 β interacts with STAT3 to bind and promote the EnhI region, as well as directly binding and activating EnhII in the HBV genome (69, 70). HNF6 and C/EBP, both liver enriched transcription factors, bind and inhibit the PreS2 and EnhI region respectively (71, 72). C/EBP, while inhibiting the PreS2 regions simultaneously activates the EnhII region (73, 74). The promoting/inhibiting effect of these host nuclear transcriptions factors have significant and often clinically important roles in the expression of the HBV genome.

1.1.2 The Hepatitis B Virus Lifecycle

As a bloodborne pathogen the Hepatitis B virus begins its infection by infiltrating the host vascular system for the most part through sexual contact, contaminated syringes, or contaminated blood transfusion (3, 4). Vertical transmission, from mother to child, following birth is also a common mode of viral transmission (75). Once in the liver vasculature, the virion begins binding to hepatocytes through low affinity binding between host heparan sulphate proteoglycans to positively charged structures on the virion surface (76). Upon the binding of the pre-S1 domain of the viral large surface protein to the liver-specific receptor, NTCP, the virion is endocytosed (**Fig.2**) (37, 77-79). Within the endosome the viral particle fuses to the endosomal membrane, releasing the viral capsid into the cytoplasm, where it unfolds to expose the nuclear localization signal (NLS) of the

Figure 2. HBV Lifecycle. HBV virion binds to the hepatic NTCP protein and is endocytosed. The viral membrane then fuses with the endosome, releasing the capsid into the cytoplasm, followed by nuclear import. The rcDNA is released into the nucleus where it uses host DNA repair mechanisms to form cccDNA. Viral RNA's are transcribed from the cccDNA and are translated to the viral proteins. In the cytoplasm, the viral polymerase binds to the epsilon structure of pgRNA and is encapsidated by core proteins. Within the nucleocapsid the pgRNA undergoes reverse transcription to form rcDNA. The mature capsid is enveloped and secreted as virion particle through the MVB secretory pathway. The mature capsid can also be shuttled to the nucleus where it will amplify cccDNA through a process termed the rcDNA recycling pathway.

core protein (9, 80, 81). Endosomal pH has been shown to be the most important fusion inducing factor with enveloped viruses however the exact site of viral fusion nor the fusion triggers have been identified for HBV endosomal fusion (82-85). Once in the cytoplasm, the viral capsid, is shuttled to the nuclear pore complex where the rcDNA is imported into the nucleus (86-88). Prior to nuclear import, it has been shown the viral polymerase is removed from the rcDNA structure forming the deproteinated rcDNA (DP-rcDNA) (8).

Within the nucleus rcDNA undergoes several enzymatic steps to repair its inhibitory terminal structures, resulting in the formation of cccDNA. Following its formation, cccDNA proceeds to transcribe its 3'-end overlapping mRNAs, including the 3.5kb precore mRNA, pgRNA, 2.4kb and 2.1kb surface mRNA, and 0.7kb X mRNA (89). In the cytoplasm, pgRNA translates viral core proteins and polymerase. The viral polymerase binds to the epsilon structure on the pgRNA and is encapsidated. Within the viral capsid, the viral polymerase begins to reverse transcribe the pgRNA into the (–) strand DNA (**Fig.3**). The reverse transcription is initiated via polymerase's protein priming activity against the epsilon structure, resulting in the covalent attachment of polymerase to (–) strand DNA. Next, three sequential template switches take place to produce rcDNA: (1) the Polymerase-primed nascent DNA translocates to direct repeat (DR) 1 at the 3' end of pgRNA to continue the full-length (–) strand DNA synthesis, and the pgRNA template is degraded simultaneously by the RNase H activity of the viral polymerase; (2) the undigested DR1-containing sequence at 5' end of pgRNA translocates to DR2 at the 5' end of (–) strand DNA and serves as a primer to start (+) strand DNA synthesis; (3) the elongation of (+) strand DNA rapidly reaches the 5' end of (–) strand DNA, then switches over to the 3' end of (–) strand DNA for further synthesis and thus circularizes the DNA into a rcDNA format (**Fig.3**). The 2nd template switch can fail to occur in a low frequency, resulting in an in situ priming of (+) strand DNA and a double-stranded linear DNA (dslDNA) product. During the (+) strand DNA synthesis, the capsid is enveloped at the multivesicular body (MVB) (90, 91) and the nucleotide supply is perhaps exhausted prior to (+) strand completion(92). The enveloped virus is then transported to the cell membrane and secreted as progeny virion. In addition, the cytoplasmic mature capsid can be redirected

Figure 3. HBV rcDNA Synthesis. Within the viral capsid, following pgRNA **(A)** export to the host cytoplasm **(B)** HBV polymerase binds to the ϵ structure of the pgRNA and begins priming with 3 nucleotides. **(C)** The first template switch occurs and the polymerase along with the covalently attached primed DNA move to the direct repeat 1 (DR1) motif at the 3' end of the pgRNA. **(D)** The polymerase continues to synthesize the (-) strand DNA and digest the read RNA until it reaches the 5' end of the pgRNA **(E)**. **(F)** The second template switch occurs when the uncleaved 5' DR1 region of pgRNA moves to the DR2 region of the newly synthesized (-) strand DNA. **(G)** Plus strand DNA synthesis then begins and undergoes a 3rd template switch to the 3' end of the (-) strand, circularizing the novel DNA and forming the mature rcDNA **(H)**. **(J)** In a low frequency, the second template switch fails to occur and the RNA primer undergoes an in situ priming to synthesize the HBV dsIDNA. Adapted from Flint et al. (1)

into the nucleus to supply more rcDNA for cccDNA formation, which is called intracellular rcDNA recycling or cccDNA amplification (8, 24, 34, 92).

1.2. HBV cccDNA Formation

Although the rcDNA in virion particles is infectious, it cannot replicate itself or express its open reading frames without “repairing” the inhibitory terminal structures inherent to the HBV rcDNA structure first. Therefore, to kick off cccDNA formation, the cytoplasmic viral capsid containing the mature rcDNA, either from the incoming virus or the *de novo* viral DNA replication, needs to prepare for the nuclear transportation *via* a conformational change to expose its C-terminal Domain (CTD) NLS for karyopherin binding (9, 93, 94). The viral and host mechanism(s) regulating HBV capsid NLS exposure is unclear, however, previous studies suggest that the rcDNA maturation, deproteination, and/or the binding of karyopherin with capsid could be a trigger (9, 95-98). The entire capsid is then imported through the nuclear pore complex into the nucleus where it then releases the rcDNA into the karyoplasm (93, 94). From cytoplasm to nucleus, HBV rcDNA must undergo a series of enzymatic reactions to repair/remove the previously described obstructive terminal modifications: the viral polymerase must be removed presumably by unlinking the tyrosyl-5'-DNA phosphodiester bond, RNA primer needs to be removed, (+) strand DNA needs to be completed, one copy of the terminal redundant sequence on the (-) strand DNA must be removed and, finally, the (+) and (-) strands must be ligated close to form cccDNA (**Fig.4**). While the dsDNA, a byproduct of rcDNA synthesis, can be converted into cccDNA through non-homologous end joining or integrated into the host genome and still have active transcription for certain viral mRNAs, the only source of genuine novel infectious HBV particles is cccDNA expression (99-102). Despite the virus' complete reliance on cccDNA for reproduction, none of its proteins have been shown to

have an essential role in rcDNA to cccDNA conversion in the nucleus (8, 33, 34, 56, 103-108). Instead, the virus relies heavily, if not completely, on nuclear host factors for converting rcDNA into cccDNA (100, 109-112).

Figure 4. Proposed Molecular Pathway of HBV cccDNA Formation. The plus (+) and minus (-) strands of rcDNA are labeled, and the direct repeat (DR) 1 and DR2 are indicated by rectangles. The terminal modifications of rcDNA are indicated as follows: the dotted line portion of the plus-strand DNA indicates strand incompleteness; the capped RNA primer at the 5' end of (+) strand is shown as a curved line; HBV pol covalently attached to the 5' end of (-) strand is illustrated as a filled oval; the terminal redundancy of (-) strand is labeled with *r*. The *bona fide* cccDNA ought to be converted from rcDNA through a series of DNA repair processes that fix the peculiarities on the termini of rcDNA as indicated. The identified DP-rcDNA and CM-rcDNA are putative transitional intermediates during rcDNA-to-cccDNA conversion. The lines drawn between the listed DNA species correspond to the certainty (solid lines) and uncertainty (dotted lines) of their direct relationships, and the cellular DNA repair factors that have been implicated in each DNA transitions are indicated. See text for more details.

1.3 Intermediates/Precursors of HBV cccDNA

The order in which these catalytic events occur is not completely understood. Due to the complexity of rcDNA to cccDNA conversion, it is expected that DNA intermediate(s) may exist during cccDNA formation.

1.3.1 dsl-ltr-DNA

More than two decades ago, a hypothetical linear hepadnavirus genome, termed double stranded linear HBV DNA with long terminal repeats (dsl-ltr-DNA), which

contains terminal duplication of the cohesive region (between DR1 and DR2) from rcDNA by displacement synthesis through the cohesive overlap, has been proposed to be a cccDNA precursor. Through homologous recombination of these LTRs, the dsl-ltr-DNA could bypass the removal of the terminal structures of rcDNA and form cccDNA directly (113). However, although the integrated viral DNA copies appeared to have the sequence of this linear DNA species, its extrachromosomal form has not been directly detected and thus remains mysterious (113). Instead, cccDNA species with extensive insertion/deletions (indels) have been discovered and would somewhat indicate that a dsl-ltr-DNA intermediate may occur but is transient and is ligated through non-homologous end joining (NHEJ). However, it is now well acknowledged that the viral dsDNA replicative intermediate is the predominant precursor for indels-containing cccDNA formation through NHEJ (34, 114-117).

1.3.2 DP-rcDNA (PF-rcDNA)

More than one decade ago, we and others systematically characterized a rcDNA species without the covalently attached viral polymerase, which was termed as deproteinized rcDNA (DP-rcDNA) also known as protein-free rcDNA (PF-rcDNA) (**Fig.5A**) (8, 34). It is worth to note that DP-rcDNA has been shown in even earlier studies but did not draw much attention at that time (118, 119). Deproteinized dsDNA (DP-dsDNA) also exists but protein-free ssDNA does not, multiple reports indicate that deproteinization occurs selectively on mature double stranded viral DNA (8, 9, 34, 116). The DP-rcDNA can be extracted by Hirt DNA extraction method, which is also used to extract cccDNA (120, 121). In the absence of protease digestion, a phenol treatment during Hirt DNA extraction from HBV replicating cells allows for the polymerase covalently

bound rcDNA to become soluble in the phenol fraction leaving behind the DP-rcDNA and cccDNA as protein-free DNA. Cell fractionation showed a significant population of DP-rcDNA in the cytoplasm as well as the nucleus, suggesting that the rcDNA deproteination step occurs prior to nuclear import (8). Further studies on cytoplasmic DP-rcDNA suggested that the completion of viral (+) strand DNA inside the nucleocapsid triggers rcDNA deproteination and nucleocapsid conformational shift, resulting in the exposure of the NLS on the C-terminus of capsid protein, followed by binding of karyopherins and nuclear import of DP-rcDNA containing capsid (9). The conformational change or partial disassembly of cytoplasmic DP-rcDNA-containing capsid was also inferred by the accessibility of encapsidated DP-rcDNA by DNase I (8, 9). In line with this, another study reported that DP-rcDNA was predominantly found in nucleus, which was likely due to a treatment of cytoplasm samples with Turbonuclease before Hirt DNA extraction (34). Further analyses of the cytoplasmic DP-rcDNA demonstrated that the (+) strand DNA is complete or almost complete with the RNA primer being removed from the 5' end, and the viral polymerase is completely removed from the 5' end of (-) strand DNA through unlinking the tyrosyl-DNA phosphodiester bond with the terminal redundant sequence remaining on both ends (**Fig.5A**) (122). In the nucleus, DP-rcDNA is released from the capsid and converted into cccDNA by employing the host DNA repair machinery (8, 9, 116, 123).

The existing evidences supporting DP-rcDNA as a functional precursor of cccDNA include but may not be limited to: 1) it always appears earlier than cccDNA in HBV-transfected or -infected cells (8, 34, 124-126); 2) inhibition of rcDNA deproteinization by compounds or blocking DP-rcDNA nuclear transportation resulted in the accumulation of

cytoplasmic DP-rcDNA but reduction of nuclear DP-rcDNA and cccDNA (9, 127); 3) inhibition of NHEJ DNA repair pathway in cells exclusively replicating duck HBV (DHBV) dsDNA genome resulted in accumulation of nuclear DP-dsDNA but reduction of cccDNA (116); 4) transfection of purified DP-rcDNA into cells resulted in viral DNA replication, suggesting a successful conversion of DP-rcDNA into cccDNA (8). Nevertheless, whether DP-rcDNA is the major precursor for cccDNA remains uncertain. In the HBV stably transfected cells, such as HepG2.2.15, HepAD38 cells and HepDE(S)19 cells, that support cccDNA formation exclusively through the intracellular amplification route, nuclear DP-rcDNA normally accumulate to a much higher level than cccDNA (8, 34, 118, 123, 127, 128), indicating that the majority of nuclear DP-rcDNA may be a dead-end product or there is a rate-limiting mechanism for converting DP-rcDNA into cccDNA. However, the levels of DP-rcDNA are similar to or even less than cccDNA in HBV-infected cells *in vitro* and *in vivo* (107, 126, 129-132), indicating that the production, role, or conversion efficiency of DP-rcDNA in cccDNA formation may be different between HBV transfection and infection systems. The DHBV system is helpful in the study of cccDNA as the viruses are closely related and therefore have similar genomes and lifecycles. One advantage is the DHBV model produces more cccDNA than HBV, which often is difficult to detect due to low copy numbers even in experimental settings. This model is limited in that the minute differences between the viruses carry large real-world discrepancies. For example, DHBV lacks an X gene ORF, which is known to be imperative to HBV infection and genomic expression.

1.3.3 CM-rcDNA

A recent study has reported observing another possible cccDNA intermediate believed to be a closed (-) strand rcDNA (CM-rcDNA) population (**Fig.5A**) (133). In this study, by treating Hirt DNA extraction samples with exonucleases Exo I/III that digest non-circular DNA with unblocked 3' end, the DP-rcDNA was removed but cccDNA was preserved, and a minor DNA species of (-) polarity was evident on the Southern blot below single stranded linear DNA. This minor species was identified as a single stranded circular DNA derived from the CM-rcDNA by removing the open circular (+) strand DNA through Exo I/III digestion. The absence of single stranded circular (+)DNA upon Exo I/III treatment of Hirt DNA implied that the circular (-)DNA was not a result of randomly nicked cccDNA which would have resulted in two equally sized populations. The presence of CM-rcDNA indicates that the peculiarities on the (-) strand DNA are repaired and the termini are ligated earlier than (+) strand of rcDNA during cccDNA formation, though it remains unclear whether CM-rcDNA is a genuine precursor for cccDNA or a byproduct during cccDNA formation. It is also unknown whether CM-rcDNA is a derivative of the aforementioned DP-rcDNA or coproduced together with DP-rcDNA from rcDNA.

1.4. Host DNA Repair Factors Involved in cccDNA Formation

Once the rcDNA is delivered in the nucleus and uncoated, it is expected that the structural peculiarities on the termini of rcDNA will be recognized as DNA damage signals by the host DNA repair apparatus and converted into cccDNA (100, 109, 111, 134). Through a variety of experimental approaches, a handful of cellular DNA repair factors involved in cccDNA formation have been identified as follows.

1.4.1 ATR-Chk1

There are two major high-fidelity cellular DNA damage response (DDR) pathways, the ataxia telangiectasia mutated (ATM) pathway and the ataxia telangiectasia and Rad3-related (ATR) pathway, which are two distinct kinase signaling cascades leading to DNA damage checkpoints (135). To assess whether DDR is involved in rcDNA to cccDNA conversion, Luo et al. treated HBV-infected HepG2-NTCP cells and primary human hepatocytes with ATR and Chk1 inhibitors (ATR/CHK1 inhibitors: AZD6738, VE-821, and CHIR-124) and observed a marked decrease in cccDNA levels, but not under ATM inhibitor treatment (ATM inhibitors: KU-55933 and KU-60019) (136). HBV stable cell lines AML12HBV10 and HepAD38, which support cccDNA formation through the rcDNA recycling pathway, also illustrated a similar inhibition of cccDNA production when treated with the ATR-Chk1 inhibitors or siRNA. Thus, the role of the ATR-Chk1 pathway is not limited to the initial round of cccDNA formation but also the internal cccDNA amplification. Considering that ATM-Chk2 and ATR-Chk1 pathways are activated by double-strand breaks and persistent single-stranded DNA (137), the involvement of ATR-Chk1 in cccDNA formation is likely due to the single-strand gaps on rcDNA. Interestingly, the study found that PF-rcDNA (aka DP-rcDNA) levels increased under the ATR-Chk1 inhibitor conditions, but CM-rcDNA was reduced to a similar degree of cccDNA, indicating that CM-rcDNA may be processed from DP-rcDNA by ATR-Chk1 machinery. Under the condition of ATR-Chk1 inhibition, a smear also appeared between the DP-rcDNA and cccDNA bands on Southern blot, which were determined to be the DP-rcDNA intermediates lacking large portions of their 5' end of (-) strand DNA, indicating that ATR-Chk1 pathway may protect the deproteinated rcDNA from cellular nuclease digestion.

1.4.2 DNA Polymerases

It has been reported that inhibiting hepadnavirus polymerase does not block the first round cccDNA formation in *in vitro* virus infection, indicating that host polymerase(s) are responsible for repairing rcDNA into cccDNA (33, 106). In one study, a siRNA knockdown screen of 15 cellular polymerases in HepG2-NTCP cells was carried out. HBV 3.5kb RNA and HBeAg were used as markers of cccDNA formation and successful infection. Knockdown of Pol κ , Pol η or Pol λ significantly reduced both 3.5kb RNA and HBeAg with Pol κ knockdown having the most significant reduction (107). Co-inhibition of Pol κ and other polymerases showed enhanced inhibition of infection, but Pol κ was observed to have the most critical role. Pol κ was knocked out using the CRISPR/Cas9 system in HepG2-NTCP cells. Infection assays were performed on the Pol κ knockout cells and cccDNA was observed through Southern blot. There was no observable cccDNA band in the -/- Pol κ cells and a reduced band in the +/- Pol κ cells. A knockout of Pol λ in HepG2-NTCP cells was also made using similar methods and was also observed through Southern blotting to reduce cccDNA formation. While not as significant of a reduction as the Pol κ knockout, Pol λ was still shown to have a role in cccDNA formation. Pol κ , of the Pol Y family, is known for its role in trans-lesion DNA synthesis and the nucleotide excision repair (NER) pathway (138). Although the exact mechanism of its involvement was left unexplored, it is implied by Pol κ 's function that it is perhaps involved in the completion of (+) strand DNA of nuclear rcDNA.

In another study, Tang et al. investigated the role of polymerase alpha in cccDNA formation and uncovered its crucial role in cccDNA formation through the rcDNA recycling pathway (139). Phosphonoformic acid (PFA), a reversible HBV polymerase

inhibitor, was used to arrest HBV replication in HepAD38 cells. Once PFA is removed there is a synchronized mass production of cccDNA from newly synthesized rcDNA, allowing for transient treatment of potential toxic inhibitors of cellular polymerases. Pol B family polymerases α , δ , and ϵ were inhibited using pan-inhibitor Aphidicolin (APH). This resulted in a marked decrease in cccDNA formation. To investigate the individual polymerases further, a CRISPR/Cas9 knockout of Pol δ was made in HepAD38 cells. The knockout caused a modest decrease in cccDNA formation, however, when paired with APH there was a marked decrease in cccDNA levels. Although Pol κ is involved in *de novo* cccDNA formation as above mentioned (107), these results suggest that it is not the main effector in this mechanism. The role of Pol α in cccDNA formation was further inspected by specifically inhibiting it using CD437. The specific inhibition of Pol α showed a large decrease in cccDNA formation without changing cccDNA stability, together with a co-reduction of CM-rcDNA, indicating that Pol α may be involved in the process of rcDNA (-) strand ligation. The requirement for different cellular polymerases in *de novo* cccDNA formation and the intracellular cccDNA amplification is less understood but may be related to the potentially different structures of enveloped capsid compared to intracellular naked capsid or their rcDNA content. Pol B family polymerases are the primary subunits of cellular DNA replication and are most active during the S phase of replicating cells (140). This could suggest that the intracellular cccDNA amplification, through the rcDNA recycling pathway, may occur specifically in dividing cells, lending a plausible explanation of how cccDNA reservoirs are conserved amid mitosis.

1.4.3 Flap Endonuclease 1

When rcDNA or DP-rcDNA is imported into the nucleus, one copy of the terminal redundancies must be removed before the ligation of the (-) strand. Flap endonuclease 1 (FEN1) is a eukaryotic 5'-flap endonuclease involved in DNA replication and repair, and its major function is to remove the 5'-flap structure of Okazaki fragments during lagging strand DNA synthesis (141). In this regard, the terminal redundant sequences at both ends of the rcDNA (-) strand represent the junction of two Okazaki fragments, if a 5'-flap is formed (**Fig.1B** and **Fig4A**). Kitamura et al. performed an *in vitro* FEN1 activity assay using a synthetic DNA substrate mimicking a putative 5'-flap structure of the terminal redundancy of HBV rcDNA and demonstrated FEN1's ability to bind and cleave the synthetic HBV rcDNA 5'-flap (142). Two catalytically defective FEN1 proteins were unable to cleave the 5'-flap and a FEN1 inhibitor, 3-hydroxy-5-methyl-1-phenylthieno[2,3-d]pyrimidine-2,4(1H,3H)-dione (PTPD), inhibited the synthetic HBV DNA cleavage. CRISPR/Cas9 knockdown and PTPD inhibition of FEN1 in HepAD38.7 cells reduced cccDNA levels, suggesting FEN1's enzymatic activity is involved in the formation of cccDNA. The chemical inhibition of FEN1 through PTPD in HepG2-NTCP cells showed a consistent decrease in cccDNA formation in an infection model cell line. A FEN1-HBV ChIP qPCR confirmed FEN1 binds to HBV DNA in cells. The proposed role of FEN1 in removing the 5' terminal redundancy of HBV rcDNA is consistent with a previous study indicating that the 5' terminal redundancy of DHBV rcDNA is preferentially removed during cccDNA formation (143). Considering that FEN1 recognizes the free 5'-end of the flap and threads the ssDNA strand through its helical arch and enzymatic site to create a configuration for cleavage (141), this threading requirement

suggests that FEN1 cannot directly cleave the rcDNA with polymerase attached, and DP-rcDNA can serve as the substrate for FEN1. However, since a complete inhibition of HBV cccDNA formation was not achieved through FEN1 inhibition, it remains unknown whether other 5'-flap endonucleases, including Dna2 and XPG, or even exonucleases, are playing an overlapping role in cccDNA formation.

1.4.4 DNA Ligases

Our previous study investigated possible DNA repair factors involved in cccDNA formation through a large shRNA screen of DNA repair related proteins in HepDES19 cells (117). The screen revealed host ligases as potential host factors for cccDNA formation. This result was investigated further in an *in vitro* cell-free cccDNA formation assay. DHBV rcDNA was collected and incubated with nuclear extracts and cccDNA formation was measured through a sensitive PCR assay. When the system was treated with ligase inhibitors the cccDNA amplicon was completely blocked suggesting the ligases were involved in forming the cccDNA. The pan ligase inhibitor, L189, inhibited DHBV cccDNA formation in a DHBV inducible cell line, HepDG10, consistent with the results of the *in vitro* assay. Specific knockouts of LIG1 and LIG3 were made with CRISPR/Cas9 in HepDG10 cells. The depletion of LIG1 and LIG3 significantly reduced cccDNA formation but did not seem to effect rcDNA levels, suggesting they have a specific role in cccDNA formation. Similar knockouts of LIG1 and LIG3 were made in the HBV inducible cell line, HepDES19, and showed a similar reduction in cccDNA formation. This phenotype was also replicated in infection assays with HepG2-NTCP cells when LIG1 and LIG3 were individually knocked down by shRNA. Therefore, the study indicated that LIG1 and LIG3 play an overlapping role in HBV cccDNA formation, likely acting on the last step of

rcDNA to cccDNA conversion by ligating the ligatable ends of rcDNA. It remains unknown whether LIG1 and LIG3 have DNA strand-specificity on rcDNA ligation, including the formation of CM-rcDNA. In addition, LIG4, a component of NHEJ pathway, has been shown to be responsible for noncanonical cccDNA formation from dsDNA (117).

1.4.5 DNA Topoisomerases

Topoisomerase (TOP) 1 and 2 transiently nick/cut DNA to release torsion built up during DNA replication occurring in splitting cells or DNA repair(144), which were recently shown to be involved in cccDNA formation (145). A PFA arrest and synchronized release in HepAD38 cells similar to that previously described was also used in this study to allow short-term treatment of normally toxic TOP inhibitors. Interestingly, inhibition of TOP1 by Topotecan or Camptothecin reduced cccDNA and CM-rcDNA levels. On the other hand, when TOP2 was inhibited with Idarubicin and Doxorubicin, it only affected cccDNA levels. This study suggests that TOP1 and 2 have potentially separate roles in cccDNA formation, which TOP1 is potentially involved in the repair/ligation of the rcDNA (-) strand during CM-rcDNA formation if CM-rcDNA is indeed a precursor for cccDNA. However, the detailed molecular mechanism underlying TOP1/2-mediated cccDNA formation remains elusive, including how and where TOP1/2 interact with rcDNA/cccDNA, how TOP1/2 cooperate with DNA ligases to close rcDNA, and whether other topoisomerases play overlapping role in cccDNA formation.

1.4.6 Tyrosyl-DNA Phosphodiesterase 2

At times topoisomerases can fail to release the cut and untwined DNA and remain covalently bonded to the strands. To avoid the potentially lethal consequences, tyrosyl-DNA phosphodiesterases are recruited to cleave the covalent phosphor-tyrosyl bond,

allowing the DNA to be ligated shut (146, 147). Tyrosyl-DNA phosphodiesterase 2 (TDP2) specifically cleaves the 5' bound TOP2 (148, 149), which resembles the 5' tyrosyl-DNA phosphodiester bond between HBV polymerase and rcDNA (**Fig.1**). It was for this similarity, researchers investigated TDP2's potential to be involved in the removal of the viral polymerase from rcDNA and subsequently the formation of HBV cccDNA. The in vitro biochemical assays demonstrated that human TDP2 exhibited an ability to cleave the 5'-tryosyl bound DHBV and HBV Polymerases from newly primed nascent ssDNA or mature rcDNA(150-153). A knockdown of TDP2 was then carried out with shRNAs in DHBV-transfected Huh7 cells, the result showed that a knockdown of TDP2 was associated with a delay in cccDNA formation, and ectopic expression of TDP2 in the TDP2 knockdown cells restored cccDNA formation kinetics (153). This potential role of TDP2 in cccDNA formation, however, has been refuted by other studies. Cui et al. established a HepG2/NTCP TDP2 knock out cell line and observed the cells to be permissive to HBV infection (151). The study also showed that TDP2 knockdown in HepAD38 *via* siRNA resulted in a modest increase of cccDNA, and overexpression of TDP2 resulted in a modest decrease in cccDNA formation in HBV transiently transfected cells. However, when a DHBV replication plasmid was transfected into TDP2 knockout HepG2 cells, the group observed a moderate decrease in DHBV cccDNA formation. Furthermore, the chemical inhibitors of TDP2 failed to inhibit HBV infection in a hepatic co-culture system (154). Our recent study on mapping the termini of cytoplasmic DP-rcDNA suggested a role of tyrosyl-DNA phosphodiesterase in removing viral polymerase from rcDNA, however, knockout of TDP2 in HepAD38 cells did not markedly reduce the levels of DP-rcDNA and cccDNA (122). These observations cast doubt on whether TDP2 is essentially involved in

the cleavage of the viral polymerase on rcDNA during the formation of DP-rcDNA and/or cccDNA, or there is a cellular compensatory activity from a TDP2-like protein in the absence of TDP2.

1.4.7 Lagging Strand Synthesis Machinery

As previously mentioned, the terminal structures on HBV rcDNA can resemble Okazaki fragments, the phenomena resulting from 5' to 3' DNA synthesis on the “lagging” strand DNA. It takes the coordination of multiple proteins to repair these fragments. Due to their similarity, one group recently investigated if the lagging strand synthesis machinery was involved in the repair of rcDNA (108). First a synthetic DNA substrate mimicking the structure and terminal modifications of rcDNA was created. When incubated with yeast and human cellular extracts, the synthetic recombinant rcDNA (RrcDNA) was able to form cccDNA. Five core components involved in lagging strand synthesis, including PCNA, RFC, FEN1, Pol δ , and LIG1, were then immunodepleted in yeast extracts, resulting in a complete or significant inhibition of cccDNA formation. The absence of any one of these proteins completely inhibited the formation of cccDNA *in vitro*. This research corroborates and enhances the evidence showing certain DNA repair factors having important roles in cccDNA formation. While *in vivo* cell culture assessment did not show the complete loss of cccDNA compared to these yeast and hepatic cell extract *in vitro* assays, they still lend themselves to these host factors potentially crucial roles in the virus's lifecycle.

1.4.8 Non-Homologous End Joining Pathway

While NHEJ is the most readily available form of DNA repair to host cells, it is unlikely the main pathway utilized by the virus to repair its rcDNA due to the error-prone propensity of NHEJ (155). However, as previously mentioned, it has been reported to be

an accessory pathway for cccDNA formation from aberrant or unusual precursors.

Figure 5. Modern cccDNA and dsIDNA Pathway Model. Proposed pathway of cccDNA formation. A) Genuine cccDNA would be classified as cccDNA from which progeny virus can be synthesized and would therefore have to undergo a relatively high-fidelity DNA repair. The host factors listed have been heavily implicated in the formation of genuine cccDNA. It is important to note the exact temporal/mechanistic relationship these factors have with cccDNA formation is yet to be fully explored or explained. DP-rcDNA and CM-rcDNA have been thoroughly shown to be downstream of rcDNA. Though there is credible evidence to suggest DP-rcDNA, or an unclassified subset of DP-rcDNA, is necessary for cccDNA formation, it remains uncertain if these populations including CM-rcDNA are actual genuine intermediates to cccDNA. The lines drawn between the HBV DNA populations correspond to the certainty (solid lines) and uncertainty (dotted lines) of their relationships, and the host DNA repair enzymes that have been implicated in each DNA transitions are indicated. B) Through non-homologous end joining (NHEJ) mechanism, the HBV dsIDNA can be integrated into the host genome or circularized to a pseudo cccDNA with substantial indels.

Precursors such as dsl-ltr-DNA and dsIDNA can be converted into cccDNA utilizing

NHEJ mechanism (101, 113-115). Previous studies have explicitly demonstrated the

essential role of NHEJ core components, including Ku80 and LIG4, in DHBV cccDNA

formation from viral dsIDNA precursor (**Fig.5B**) (116, 117). Indels between DR1 and

DR2 are common in this DNA repair pathway and have been observed in dsIDNA-

derived cccDNA (34, 114, 117). It is therefore a dead end for rcDNA replication

purposes due to the gross indels, though illegitimate dsDNA replication may still occur *via* subsequent rounds of NHEJ-mediated cccDNA formation from the newly synthesized dsDNA mutants(114). In addition, NHEJ is also responsible for dsDNA integration into the host chromosomes at the double-strand break site (101, 156).

1.5 Non-DNA Repair Factors Involved in cccDNA Formation

DNA repair factors have the most direct relationship with HBV cccDNA formation, however, many complicated and indirect interactions with host machinery and HBV cccDNA are being discovered.

1.5.1 Pre-mRNA Processing Factor 31

A spliceosome component, Pre-mRNA Processing Factor 31 (PRPF31) was discovered to be involved in cccDNA formation through a siRNA screening of 1000 genes involved in DNA damage response, epigenetic and nucleic acid binding pathways (157). The study demonstrated that PRPF31 associates with cccDNA and colocalizes with HBx in the nucleus, but whether PRPF31 interacts with nuclear rcDNA was not investigated in this study. When overexpressed together, PRPF31 and HBx, showed a marked increase in cccDNA formation that is not observed when either protein is overexpressed on their own. the exact mechanism of how a PRPF31-HBx complex enhances cccDNA formation remains unknown.

1.5.2 Cyclin-Dependent Kinase 9

The cyclin-dependent kinase (CDK) family proteins are largely responsible for the regulation of cell division or gene transcription (158). CDK9, specifically is known to be involved in the transcription or replication of several viruses (159-161). A CDK9-specific inhibitor, FIT-039, was able to reduce HBV replication in a dose dependent manner without

cytotoxicity (162). Pan-inhibitors of the CDK family resulted in high toxicity and subsequent cell death. A time-to-addition treatment of infected HepG2-NTCP cells with FIT-039 showed the compound inhibited an early step in cell infection. Inhibition of NTCP was ruled out through an *in vitro* preS1 binding assay while Southern blot and qPCR analysis of cccDNA formation confirmed FIT-039 reduced the level of cccDNA. HBV-infected chimera mice showed a greater reduction of serum HBV DNA when treated with a combination of entecavir and FIT-039 compared to entecavir alone. These results imply that CDK9 may play a role in HBV cccDNA formation or stability.

1.5.3 SAM and HD Domain Containing Deoxynucleoside Triphosphate

Triphosphohydrolase 1

SAM and HD Domain Containing Deoxynucleoside Triphosphate Triphosphohydrolase 1 (SAMHD1), is stimulated by interferon (IFN) and acts as a dNTPase to inhibit viral replication (163). Vpx from the SIV_{SMM}/HIV-2 lentiviruses was used to target and degrade SAMHD1 in infected HepG2-NTCP cells and resulted in a decrease of cccDNA levels (164). Exogenous dNTPs were provided indicating the reduction of cccDNA levels was not dependent on dNTP levels. A CRISPR/Cas9 knockout of SAMHD1 resulted in a similar phenotype that was reversed by ectopic wild type SAMHD1 but failed with expression of a mutated SAMHD1 lacking the nuclear localization signal. It has been reported that SAMHD1 can bind ssDNA and act as a scaffolding protein to promote both homologous recombination and DNA resection (165), indicating that the nuclear SAMHD1 may be involved in repairing rcDNA into cccDNA, but this study did not provide any direct evidence (164). On the other hand, mRNA levels of APOBECs, MXA, and ISG20 were all observed to be higher in the SAMHD1 knockout

cells when compared to the control, inferring a role of SAMHD1 in restricting host innate immunity. APOBEC3A/B, in particular, were shown to degrade cccDNA through cytidine deamination and apurinic/apyrimidinic site formation (132, 166). In this regard, SAMHD1 may also indirectly maintain the stability of cccDNA.

1.6 Hepatitis B

Hepatitis B continues to be a global health burden with nearly 300 million people living with chronic hepatitis B (CHB), the primary disease causing around a million HBV-related deaths annually (167-169). The horizontal transmission of HBV is achieved, for the most part, through sexual contact, the use of contaminated syringes, or contaminated blood transfusion. The disease in this context is, for the most part, realized through an acute self-limiting infection with only 5-10% of individuals progressing to a chronic infection (4, 170). Vertical transmission, in stark contrast, is the major source of CHB carriers (90% of infants; 50% of children, ages 1-5 years old who contract the virus will develop CHB), who will likely live with the disease their entire lives and without treatment will in all likelihood succumb to the disease and its complications, including fulminant hepatitis, cirrhosis, and hepatocellular carcinoma (170-173)

1.6.1 Acute Hepatitis B

Acute Hepatitis B primarily results in an asymptomatic manor and will commonly remain undetected to the host (174). The virus can incubate for 1-6 months following initial infection. HBV DNA and HBsAg are commonly discovered for the first time in the patient during the incubation stage from routine health checkups (175). Following incubation, the disease progresses to a transient (a few days to a week) preicteric or prodromal period in which one third of patients will feel the onset of constitutional symptoms, such as fever, fatigue, anorexia, nausea, and body aches (176). The preicteric phase transitions to the

icteric phase and coincides with an onset of jaundice and/or dark urine usually lasting 1-2 weeks and is characterized by an increase of viral levels. Finally, 90-95% of acute Hepatitis B carriers will progress to convalescence, in which HBsAg levels decrease below detection levels subsequently followed by the disappearance of HBV DNA (4, 174, 176). In approximately 1% of acute Hepatitis B patients, acute liver failure can occur(177). Fulminant hepatitis is commonly indicated by a sudden onset of fever, abdominal pain, vomiting, and jaundice and superseded by delirium and coma; likely requiring a liver transplantation (178).

1.6.2 Chronic Hepatitis B

Chronic Hepatitis B varies wildly between patients as it is influenced significantly by host and viral factors. However, chronic hepatitis b can be defined as HBsAg being detected twice at least 6 months apart following an acute infection (179). It is typically asymptomatic and diagnosed following routine health examinations or screenings such as doctor's visits or blood donations. Throughout CHB, it is common for patients to shift between phases of immunologic activity and inactivity. Extended and repeated cycles of immunologic activity are likely to lead to fibrosis and eventually hepatocellular carcinoma in CHB patients. The stages of CHB do not have unique clinical presentations and therefore serological markers are essential to determine which of the four stages of CHB patient is in. The four stages include, the immunotolerant stage, HBeAg positive immunoactive disease, HBeAg negative inactive disease, and the HBeAg negative immunoreactive disease. There are three important factors to understanding the CHB phase of a patient, HBV active population (HBV DNA levels), the HBeAg status of the infection (HBeAg levels), and the immunoactivity of the infection, (alanine aminotransferase [ALT], a

sensitive marker for liver inflammation and damage). As opposed to acute infections in which HBeAg, HBV DNA, ALT, and HBsAg subside and give way to Hepatitis B s antibodies (HBsAb) and convalescence, in patients with CHB HBsAg and ALT is lower than in acute infection but maintains a baseline presence throughout the infection. HBeAg is an important marker of CHB progression from HBeAg positive CHB and HBeAg negative CHB. The loss of HBeAg coincides with the production of HBeAb and a decrease in HBV DNA levels and could represent a partial immune control of the infection. Though this is positive in a sense that HBV DNA is decreased, these HBeAg conversions events are often marked by transitory increase in ALT and liver fibrosis from the immune system becoming more reactive to the HBV chronic infection. The brief flare up is followed by a cessation of liver inflammation and damage; the HBeAg seroconversion does not necessarily occur at a specific timepoint following infection but by the 4th decade of CHB infection up to 80% of CHB hosts undergo the HBeAg seroconversion. On very rare occasions, 0.5%-2%, CHB hosts can spontaneously clear the viral infection without treatment, described as loss of HBsAg without HBsAb and HBV DNA below detection levels in the peripheral blood. With treatment of pegylated α -interferon (α -IFN) and reverse-transcription inhibitors such as nucleos(t)ide analogues (NUCs), the rates of HBeAg and HBsAg seroconversion can increase and a functional cure of CHB can be achieved, with continued treatment.

1.6.3 In Search of a Hepatitis B Cure

As previously mentioned, unabated HBV infection, particularly CHB infection, will inevitably lead to liver disease progression to fulminant hepatitis and/or HCC. The most successful reduction of CHB is achieved through the stringent implementation of a

HBV vaccination program, as evidenced by the highly successful HBV vaccination program put in place in 1992 in China, which reduced to the presence of HBsAg in children less than 5 years old to only 1% (180). However, the vaccine fails to help the over 300 million people worldwide suffering from CHB. There are three categories of HBV cures: functional, partial, and virological. A functional cure to HBV is considered as the loss of HBsAg and undetectable levels of HBV DNA in peripheral blood. A partial cure is similar in that peripheral HBV DNA is below detection levels, however, differs from functional cure in that HBsAg is still detectable albeit in low levels. A partial cure is also further defined as HBeAg loss and normal ALT levels. To be categorized as these cures, these conditions must be maintained following cessation of treatment. Though these cures are possible, they are rare and occur in 3-11% of IFN treated patients. Unfortunately, even in functionally cured patients it is common, up to 23.8%, for the HBV infection to be reactivated (179). The cause of HBV reactivation in CHB patients and functionally cured individuals is the persistent chronic HBV factor, cccDNA. Therefore, a virological, or genuine, cure for HBV is defined as the complete removal of HBV DNA from the peripheral blood and liver. Following infection, a reservoir of HBV cccDNA is established in the liver. The highly stable nature of cccDNA and its intimate relationship with the host genome, make it extremely difficult to target for destruction, pharmacologically, and immunologically. Despite the difficulty surrounding targeting HBV cccDNA for eradication, it remains the “holy grail” of HBV research. Though the eradication of cccDNA is highly sought after, our understanding of HBV cccDNA formation and interaction with the host remains fragmentary. It is critical to further our understanding of

how HBV cccDNA is formed and how it interacts with the host genomic mechanisms, in order to achieve a genuine, virological cure for HBV chronic infections.

1.7 Hepatitis B Therapeutics

Since a genuine virological cure is not currently feasible the goal in HBV and CHB therapies is to delay or completely avoid liver failure and hepatocellular carcinoma. Treatment is normally initiated during the immunoactive stages of CHB where ALT and HBV DNA levels are elevated or if fibrosis or liver inflammation is detected. Treatment is always initiated in patients with cirrhosis, regardless of ALT or HBV DNA levels. The 7 currently available treatments for HBV are separated into 2 categories, interferons and nucleos(t)ide analogues.

1.7.1 Interferons

Interferons are a family of innate immune system response molecules, called cytokines, triggered by viral infections. Each have immunomodulatory effects that are specifically inhibitory to common viral replication pathways, such as reverse transcription and abnormal RNA structures. The exact mechanisms of which are not completely understood, but it is apparent they can act directly, possibly by destabilizing cccDNA, or indirectly through general immunomodulatory effects, stimulating immune response against HBV infection, to suppress common viral infectious or replication pathways. In particular interest to the medical community are Interferon alpha and beta whose immunomodulatory effects appear to be the most potent against general viral infections. These have been adapted to injectable formulations of pegylated interferon alpha (pegylated interferon alpha-2a and alpha-2b). Injected weekly for 48 weeks, INF-alpha is associated with higher rates of HBeAg loss (30%) and HBsAg loss and a reduction in

cirrhosis and hepatocellular carcinoma compared to untreated controls (181-184). Despite the success of INF- α therapy, it is insufficient as the benefits of the therapy are only manifested in approximately a third of patients.

1.7.2 Nucleos(t)ide Analogues

Nucleos(t)ide analogues (NUCs) come in 5 varieties in the United States as: adefovir, entecavir, lamivudine, tenofovir disoproxil, and tenofovir alafenamide. They all act similarly in that they mimic the structure of nucleotides or nucleosides and inhibit the viral RNA dependent DNA polymerase reverse transcriptase and inhibit the formation of novel HBV rcDNA from pre-genomic RNA. This can be achieved through multiple pathways including blocking the 3' hydroxyl group in the NUCs, resulting in the early termination of the synthesizing DNA. Other NUCs function as negative enantiomers (L-enantiomers) of the naturally occurring D-form nucleosides, causing inhibitory steric hinderance in the viral polymerase and ceasing the DNA polymerization. All five drugs showed histological improvement in more than 50% of patients and a significant reduction in HBV DNA in randomized trials compared to placebos after one year of treatment (185-193). Overall disease progression in CHB patients with advanced liver disease was reduced from 17.8% to 7.8% in lamivudine treated individuals (187). Similarly in the same study incidence of HCC was reduced from 7.4% to 3.95%. Fetal transmission of HBV from infected mothers was also greatly reduced from 18% to 5% (194). The central issue facing the earlier developed NUCs, Lamivudine and adefovir, is their high instance of emergence of resistant HBV variants. However, this issue has been mostly resolved in the development of modern NUCs such as entecavir and tenofovir disoproxil with a 1.2% probability of developing a resistant strain after 5 years and no resistant variants detected up to 7 years

after initiation of treatment, respectively (195, 196). The latest NUCs like entecavir and tenofovir also show an improvement in efficacy with a suppression of HBV DNA in 80-93% of patients when compared to 39-63% in lamivudine or adefovir treated patients. Combination of IFN therapy with NUCs like tenofovir or entecavir, showed an enhanced response to therapy in previously untreated CHB patients. Combination therapy enhanced HBeAg loss rates after 72 weeks of therapy when compared to either therapy on its own (197). Evidence thus suggests a combination therapy approach has a greater benefit to first time patients HBeAg seroconversion and HBV DNA suppression. Despite the high success rate of NUCs and IFN-NUC combined therapy they at the end of the day do not directly or effectively target the source of CHB, cccDNA. Though IFN therapy can destabilize cccDNA, NUCs altogether miss cccDNA, and only inhibit the reverse transcription step downstream of cccDNA formation. This allows for HBV to establish a cccDNA reservoir, even throughout treatment and mostly inhibits the creation of progeny virus. This explains the relatively high rate of HBV infection reactivation following treatment cessation.

1.8 Questions and Hypothesis

In reflection of our current understanding of the HBV lifecycle and its interaction with host mechanisms to form cccDNA we have proposed several questions in which we hope to answer, and in doing so elucidate several longstanding gaps in our knowledge of HBV biology.

1.8.1 Questions

Question I: What other Host DNA repair factors are involved in cccDNA formation?

Question II: Can we develop an assay to specifically precipitate cccDNA

precursors and identify DNA repair factors co-precipitated along with said precursors?

1.8.2 Hypothesis

Hypothesis I: By targeting the open minus strand HBV rcDNA with a complimentary biotinylated oligo, we can precipitate nuclear rcDNA along with host factors associated with the nuclear rcDNA.

Hypothesis II: Specific proteins precipitated along with rcDNA that have known DNA repair activity or repair signaling abilities are targeting nuclear rcDNA as damaged protein and actively repairing it or signaling for its repair into cccDNA.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Cell Lines

HepAD38 cells, a tetracycline-inducible HBV stable cell line, were maintained in DMEM/F12 medium (Corning) and supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 100 µg/mL streptomycin, and 1 µg/mL tetracycline (tet) (8, 198). To induce HBV pgRNA transcription, DNA replication, and cccDNA formation in confluent HepAD38 cells, tet was withdrawn from the culture medium and cells were cultured up to 14 days. HepG2-NTCP cells, a cell line permissive to HBV infection, were cultured in the same medium as HepAD38 cells without tet (129). Primary Human Hepatocytes were purchased from BioIVT as LIVERPOOL 5-Donor Human Cryoplateable Hepatocytes Mixed Gender (BioIVT, X008052-P, Lot# SML). PHH cells were cultured in HMM media (Lonza, CC-3197) supplemented with HMM SingleQuot (Lonza, CC-4192) according to manufacturer's instructions. All cell lines were cultured in the standard 37°C, 5% CO₂ conditions.

2.2 Plasmids and Cell Transfection

The N-terminally Flag-tagged wild type human DDB2 expression plasmid Flag-DDB2 was purchased from Sino Biological (HG14460-NF). The T7-tagged wild type DDB2 plasmid WT (UIC), and plasmids expressing DDB2 naturally occurring mutants (K244E and R273H) and WD deletions (Δ WD, 1-320, and 1-380) were provided by Dr. Pradip Raychaudhuri (University of Illinois Chicago) (199, 200). The HA-tagged wild type DDB2 plasmid WT (Kobe), and plasmids expressing N-terminal deletion (Ndel) and seven N-terminal lysine-to-arginine mutants (N7KR), were provided by Dr. Kaoru Sugasawa

(Kobe University) (201). The HDV genome replication-competent plasmid pSVL(D3) was provided by Dr. Jinhong Chang (Baruch S. Blumberg Institute) (202). Plasmid pLMS expressing the HBV large (L), middle (M), and small (S) envelope proteins, was provided by Dr. Youhua Xie (Fudan University) (203). Plasmid DNA transfections of cells were conducted by Lipofectamine 3000 according to manufacturer's directions (Invitrogen). DDB2 siRNA was purchased from Santa Cruz (sc-37799) and cell transfections were performed with Lipofectamine RNAiMAX reagent (Invitrogen).

2.3 Nuclear rcDNA Pulldown and Mass Spectrometry

A 52-mer oligonucleotide complementary to the HBV minus strand DNA sequence (nt 1540-1591, GenBank No. U95551) was synthesized by IDT, and a biotin tag was ligated to the 5' end of the oligonucleotide by means of a triethylene glycol spacer (TEG), giving rise to the rcDNA oligo bait (**Table 1**), which was further purified by HPLC. HepAD38 cells were cultured in T225 flasks with tet-free medium to induce HBV replication for 6 days with daily medium change. HepAD38 cells cultured in the presence of tet for 6 days were set as a negative control. The cells were collected, and the nuclear fractions were isolated using a Nuclear Extraction Kit (Cayman Chemical, 10009277). The obtained 300µl of nuclear fractions were then incubated with 1.25 nmol of biotinylated rcDNA oligo bait at 4°C for 16 h under constant gentle rotation. Next, 50 µl of streptavidin-coated MyOneC1 Dynabeads (ThermoFisher) were then added to the reaction and incubated for 4 h at 4°C under constant slow rotation. The paramagnetic beads were then pulled out of solution using a permanent neodymium magnet. The beads were washed in wash buffer containing 5 mM Tris-HCl (pH 7.5), 0.5 mM EDTA, and 1 M NaCl for 3 times. The beads were then spun down on a tabletop centrifuge and excess wash buffer was carefully

removed with a micropipette. The rcDNA-bound proteins were reduced, alkylated, and trypsin digested directly on beads, and the subsequent peptides were identified with liquid chromatography interfaced with a hybrid quadrupole-Orbitrap mass spectrometer (ThermoFisher Q Exactive HF Orbitrap LC-MS/MS system) at the Purdue University Proteomics Facility. Database search against Uniprot human database (August 2017) was conducted using MaxQuant to identify the probable source proteins of the peptides. A hit list was comprised comparing oligo pulldown samples of induced (tet-) versus uninduced (tet+) to differentiate between potential rcDNA-specific or -nonspecific bound proteins.

2.4 Chromatin Immunoprecipitation

HepAD38 cells were seeded and grown to confluency in T75 flasks. Once confluent the medium was switched to tet-free medium for 6 days to induce rcDNA synthesis. After 6 days of induction, the cells were fixed with 1% formaldehyde for 10 minutes under constant gentle rotation. The chromatin immunoprecipitation assay was conducted using the ChIP-IT Express Kit with protein G magnetic beads (Active Motif, 53008) according to manufacturer's manual with modifications. Cells were washed with ice cold 1x PBS for several seconds following 1% formaldehyde fixation. 1x glycine in PBS stop buffer was then added to the cells and put under constant gentle agitation for 5 minutes. The glycine stop buffer was poured off and the cells were washed again in ice cold 1x PBS. Ice cold scraping solution was then added to the plate and cells were gently scraped from the surface of the plate and carefully pipetted to a 15mL falcon tube. The scraped plate was then washed with additional remaining scraping solution to collect the remnant cells in the plate and pipetted into its corresponding 15mL falcon tube. The collected cells were centrifuged for 10 minutes at 800G's. The cell supernatant was discarded, and the cell pellet was

resuspended in lysis buffer and incubated on ice for 45 minutes. The cells should be swollen following the hypotonic lysis buffer incubation and was confirmed by observing cells under a microscope in trypan blue. Once the cells were confirmed to be swollen from the lysis buffer, the cell suspension was transferred to a Dounce homogenizer and stroked 100-200 times with pestle “A”. The cells were observed to be disrupted completely into a single cell solution under the microscope. The now singularized cell suspension was homogenized using pestle “B” to disrupt the cell membrane and release the cell nuclei. This was confirmed again by observing the cells under the microscope with trypan blue. The nuclei suspension was centrifuged for 10 minutes at 2,400G’s. The supernatant was discarded, and the nuclei pellet was resuspended in 350uL of shearing buffer. Briefly, chromatin was sheared using EpiShear ultrasound sonicator (Active Motif), with delivery of 50-250 Joules in dependence of cell line for obtaining chromatin fragments with DNA length around 500-1500 bp long. The sheared chromatin was precipitated with 5 µg of non-immune serum (NIS) IgG control (Sigma, I8765) or specific antibodies against DDB1 (Abcam, ab97522), DDB2 (Rockland, 100-401-A10), or HBc (DAKO, B0586). After the removal of formaldehyde crosslinks, immunoprecipitated chromatin was deproteinized with 10 µg/ml proteinase K. The input control and immunoprecipitated DNA fractions were purified with QIAquick PCR Purification Kit, and served as templates for qPCR amplification in LightCycler 96 System (Roche) using FastStart Essential DNA Probes Master kit (Roche) with HBV total DNA primers and probes as previously described (117). Occupancy of the specific protein on rcDNA was expressed in fold enrichment above NIS using $-\Delta\Delta Cq$ methods.

2.5 DDB2 Knockout Cell Line Establishment

DDB2 knockout cell lines were generated through CRISPR-mediated genome editing of DDB2 gene loci. The CRISPR RNA (crRNA) targeting human DDB2 gene were designed using <http://www.e-crisp.org/E-CRISP>. The CRISPR target should have a PAM site (NGG) adjacent to it. It is ideal to target a conserved region of the gene's exon to ensure the gene is critically damaged. The desired crRNA sequence should undergo a BLAST analysis to ensure there are minimal off targets for the crRNA to bind to. The synthetic scramble and DDB2 crRNA oligo pairs were annealed and cloned into BsmBI-digested lentiCRISPRv2 control vector (Addgene# 52961, gift from Dr. Feng Zhang). Ligated and expanded plasmids are sequenced to ensure proper ligation and plasmid isolation. Lentivirus preparation and titration were performed as previously published (117). Lentiviral transduction of HepAD38 or HepG2-NTCP cells and puromycin-selection were conducted to generate scramble control and DDB2 stable knockout cell lines. The DDB2 knockout phenotype was confirmed by western blot and indel sequencing assay. The corresponding coding sequence for epitope of DDB2 antibody has no overlap with the gene targeting sites of the designed crRNA. For indel sequencing analysis of DDB2 genes, total genomic DNA from the control and DDB2 knockout cells were extracted using DNeasy blood and tissue kit (Qiagen) according to the manufacturer's protocol. The genomic sequence region covering the crRNA target site was amplified by PCR using the indel detection primers (Table 1) and cloned into T vector pMD19 (Clontech) for Sanger sequencing. The DDB2 DNA sequences from control and knockout cells were aligned to determine the CRISPR-induced mutations.

2.6 HBV and HDV Infection in Vitro

The infectious HBV inoculum was prepared from the supernatant of HBV stable cell line HepDE19 as described previously (129). To prepare HDV inoculum, plasmids pSVL(D3) and pLMS were cotransfected into Huh7 cells and the virus particles were harvested from supernatant and virus titer was determined by qPCR according to a previous publication (204). HepG2-NTCP or PHH cells were seeded at a density of 1×10^6 cells/well of a 6-well plate (D₋₂). The next day (D₋₁) the cells were preincubated for 24 h with hepatocyte maintenance media (HMM) (Takara). After 24 h (D₀), the medium was replaced with an infection media prepared with HMM, 4% PEG-8000 (w/w), and HBV to have a multiplicity of infection (MOI) of 500 per well. After 16 h inoculation with virus (D₁), the medium was replaced with fresh HMM. From day 2 (D₂) onward until collection, the medium was changed daily with primary hepatocyte maintenance medium (PMM) (205). HDV infections were performed in an identical manner except that the HDV virus stock was used in place of HBV. When siRNA knockdown was included in the infection experiment, the siRNA transfection was conducted one day after cell seeding for the indicated time duration, followed by the same HBV infection workflow as described above.

2.7 Immunofluorescence

The HBV- or HDV-infected HepG2-NTCP cells were fixed with 4% paraformaldehyde for 20 min and then washed three times with cold PBS for 5 min each wash. The cells were then permeabilized with 0.5% Triton X-100 for 1 h at room temperature and washed again three times with cold PBS. The cells were then incubated with immunofluorescence assay (IFA) blocking buffer for 1 hour at room temperature. The cells were stained with rabbit anti-HBc (DAKO, B0586) or anti-HD δ Ag (a gift from Dr.

Severin Gudima) (206) for 16 h at 4°C under constant gentle agitation. The cells were then washed three times with cold PBS and blocked again with IFA for 30 min, followed by incubation with Alexa-Fluor 488 dye-conjugated goat anti-rabbit secondary antibody (ThermoFisher), and the nuclei were counterstained with DAPI for 60 min at room temperature. The cells were washed 3 more times and left in PBS. An EVOS M5000 microscope was used to take images of the stained cells under 20× magnification. ImageJ was used for counting cells on the images (207).

2.8 Enzyme-Linked Immunosorbent Assay (ELISA)

HBeAg in the supernatant of HBV-infected HepG2-NTCP cells was detected by an ELISA kit according to manufacturer's direction (CUSABIO, CSB-E13557h). Media was collected from induced or infected cells, following 24hr incubation at 37°C. 50µL of the collected media and was pipetted per well of the ELISA plate. It is recommended to have at least triplicates for each condition/timepoint. To each well 50µL of the HRP-conjugate was added and the plate was covered with an adhesive membrane. The plate was then incubated at 37°C for 30 minutes. The wells were gently aspirated and washed with 200µL of wash buffer. The wash buffer was allowed to rest for 20 seconds and this wash step was repeated 4 times. To ensure all excess wash buffer is removed, following the last wash the plate can be inverted and blotted against clean paper towels. 50µL of substrate A and substrate B both were added to each well following the wash step. The plate was covered with aluminum foil to protect it from light and incubated at 37°C for 15 minutes. Finally, 50µL of stop buffer was added to each well and the plate was tapped gently to ensure thorough mixing. The optical density of each well was observed through a microplate reader (Biotek Synergy HTX) set to 450nm.

2.9 Western Blot Assay

Laemmli buffer was used to collect whole cell lysates, which were then run on a 12% SDS-PAGE gel then transferred onto Immobilon PVDF-FL membranes (Millipore). The membrane was blocked with Western Breeze blocking buffer (ThermoFisher) and probed with antibodies against β -actin (Santa Cruz, sc-47778), DDB2 (Santa Cruz, sc-81246), DDB1 (Santa Cruz, sc-137142), Flag-tag (Sigma, F3165), HA-tag (Sigma, H3663), and T7-tag (Cell Signaling, 13246T).

2.10 HBV Nucleic Acids Analyses

HBV total RNA and core DNA were extracted and subjected to northern and Southern blotting as previously described (208). HBV cccDNA was extracted by a modified Hirt DNA extraction method (121). For cccDNA Southern blot assay, the Hirt DNA sample was heat denatured at 85°C for 5 min and then digested by EcoRI before gel loading, by which to denature the DP-rcDNA into ssDNA and subsequently linearize cccDNA into double stranded DNA for improved electrophoretic separation and hybridization (209). To detect CM-rcDNA, the Hirt DNA was treated with 3'→5' exonuclease ExoI/III and subjected to Southern blotting assay as previously described (133). A phosphorimager screen was used to record hybridization signals and was scanned using a Typhoon FLA-7000 (GE Healthcare). Hybridization signals were quantified with QuantityOne software (Bio-Rad). The quantitative PCR analysis of cccDNA was conducted with heat denatured and plasmid-safe ATP-dependent DNase (PSAD)-treated Hirt DNA sample and normalized to mitochondrial DNA according to our previous publication (117).

Table 1. Oligos used in this study

Name	Sequence (5'→3')
rcDNA oligo bait	Biotin-TEG- ACGCGGACTCCCCGTCTGTGCCTTCTCATCTGCCGGACCGTGTGCACTTCGC
HBV total DNA primers/probe	Forward: CCGTCTGTGCCTTCTCATCTG Reverse: AGTCCAAGAGTYCTTATGYAAGACCTT Probe: FAM- CCGTGTGCACTTCGCTTACCTCTGC-TAMRA
HBV cccDNA primers/probe	Forward: ATGGAGACCACCGTGAACGCCC Reverse: TCCCGATACAGAGCTGAGGCGG Probe: FAM-TTCAAGCCTCCAAGCTGTGCCTTGGGTGGC-TAMRA
Mitochondrial DNA primers	Forward: CCCTCTCGGCCCTCCTAATAACCT Reverse: GCCTTCTCGTATAACATCGCGTCA
DDB2 crRNA oligos*	Sense: <u>CACCGGGAGGGCTACATCCTTGGCG</u> Antisense: <u>AAACCGCCAAGGATGTAGCCCTCCC</u>
DDB2 indel PCR primers	Forward: TCATTTCTCTCTGTGGCAGGGG Reverse: GAACTGCTCACCCCTTTGATGA

Note: * the sticky ends for cloning are shown in italic and underlined.

CHAPTER THREE

RESULTS

3.1 Co-immunoprecipitation Coupled with Mass Spectrometry (CoIP-MS) Identified Novel Interactions Between Host Proteins and Nuclear HBV rcDNA

To accelerate the search for the DNA repair factors involved in cccDNA formation, we developed a new screen to uncover novel interactions between the host DNA repair machinery (**Fig.6**). A biotinylated DNA oligo complimentary to the single stranded region of HBV rcDNA was synthesized and used as bait to pull down nuclear rcDNA together with the binding proteins. To this end, HBV replication was induced in HepAD38 for 6 days in tet-free medium, a time point that nuclear DP-rcDNA has emerged without detectable cccDNA by Southern blotting (8), which is ideal to capture nuclear rcDNA early to mid-transition to cccDNA. The uninduced HepAD38 cells would not produce rcDNA and would therefore serve as a control for non-specific or integrated HBV DNA binding of the biotinylated oligo or streptavidin coated magnetic beads. The nuclear extract was prepared from induced and uninduced cells separately and incubated with the biotinylated oligo “bait”, then oligo-rcDNA-protein complexes were subsequently precipitated with streptavidin-covered paramagnetic beads. It is noted that the nuclear extraction kit used in this assay does not extract chromatin DNA, hereby avoiding the potential coprecipitation of HBV transgene by oligo bait. On-bead trypsin digestion was then performed, and the digested peptides were analyzed and identified through liquid chromatography paired with mass spectrometry (LC/MS). The Uniprot database of human proteins were used to match the peptide fingerprints with their probable protein sources. When comparing the coIP-MS lists from noninduced and induced HepAD38 cells, while many of the proteins were

nonspecific to the rcDNA-positive cells as revealed by the noninduced control, or are not the known DNA repair factors, a handful of proteins of interest (POIs) caught our attention (**Fig.6**). Our goal was qualitative and not quantitative in nature, because of this we focused on the peptide hits unique to induced samples. Consistent with previous reports, several known proteins or components of pathways involved in cccDNA formation were re-identified in our assay, including topoisomerases, Pol δ , RFC subunits, and ATR pathway related proteins (108, 136, 145). The potential novel nuclear rcDNA-binding DNA repair factors identified from the assay are the Fancaconi Anemia Group Proteins and DNA damage-binding protein 1 (DDB1). DDB1 is the large subunit of the UV-DDB complex, a heterodimer comprised of DDB1 and a small DDB2 subunit (210), and DDB1 had surfaced as a hit of cccDNA formation participant from a previous shRNA screen (117). Therefore, we prioritized DDB1 for further investigation in this study.

Figure 6. Nuclear HBV rcDNA CoIP-MS workflow. HepAD38 cells were cultured in tet-free medium for 6 days to induce HBV pgRNA transcription and DNA replication. Some of the rcDNA including DP-rcDNA is transported into the nucleus, where it undergoes a rate-limiting DNA repair to form cccDNA. The uninduced HepAD38 cells served as control. The nuclear fractions are isolated and incubated with the biotinylated oligos (rcDNA bait), complimentary to the open (-) strand of rcDNA. Streptavidin coated magnetic beads were then used to precipitate the oligo-bound rcDNA and proteins associated with the complex. An on-bead trypsin digestion was performed, and the resulting peptides were analyzed through liquid chromatography-mass spectrometry (LC/MS). See Materials and Methods for technical details. The representative proteins of interest (POIs) with known DNA repair functions and their subunits are listed.

3.2 UV-DDB Complex Binds to Nuclear rcDNA

To validate the binding of DDB1 to nuclear rcDNA, we performed chromatin immunoprecipitation (ChIP) assay in HepAD38 cells induced in tet-free medium for 6 days, which gave ample time for rcDNA to be accumulated in the nucleus but before cccDNA became unambiguously detectable. The primers used in this assay do not discriminate between rcDNA and cccDNA, but the 6 days post induction time point allow for a temporal separation between rcDNA and cccDNA populations. Uninduced HepAD38 cells were set as a negative control. The cells were collected, and the ChIP coupled with qPCR (ChIP-qPCR) assay targeting each of the two components of the UV-DDB complex, DDB1 and DDB2, was performed (**Fig.7**). Non-immune serum IgG and anti-HBc served as background and positive control, respectively. The ChIP-qPCR results showed specific precipitation of rcDNA along with the DDB1, DDB2, and HBc, thus, suggesting that the DDB complex does in fact bind to nuclear rcDNA. This also confirmed the validity of the novel rcDNA coIP-MS assay in its potential ability to identify novel host-HBV DNA interactions. The strong ChIP signal by anti-HBc indicated a tight association of HBc with nuclear rcDNA, which may predominantly exist in nucleocapsid format after nuclear import, followed by a slow rcDNA uncoating and cccDNA formation.

3.3 DDB2 Knockout in HepAD38 Cells Reduces HBV cccDNA Formation

CRISPR RNAs (crRNAs) targeting DDB1 and DDB2 were designed and created. We proceeded to transduce HepAD38 cells with the lentiCRISPR virus encoding DDB1 or DDB2 crRNA. However, DDB1 crRNA failed to produce clones, and we noted that cells treated with the DDB1-crRNA lentivirus suffered extensive cell death prior to puromycin selection. This could suggest that the complete loss of DDB1 may be a lethal mutation to

Figure 7 The association of UV-DDB with nuclear rcDNA. HepAD38 cells were induced (tet-) for 6 days to induce nuclear rcDNA accumulation. ChIP assay was then conducted with non-immune serum IgG or antibodies against, DDB1, DDB2, and HBV core protein (HBc), respectively, followed by qPCR of total HBV DNA. The same rcDNA ChIP-qPCR assay performed on uninduced HepAD38 cells (tet+) served as cell background control for the integrated HBV transgene. The ChIP-qPCR results were expressed as fold enrichment over the IgG controls (mean $\pm$ SD, n=3).

cells, given the importance of DDB1 in host genome stability and cell cycle progression (210, 211). Alternatively, the DDB2 crRNA targeting the third exon in its ORF successfully knocked out the gene in HepAD38 cells (**Fig.8A**). The successful knockout of DDB2 was confirmed through sequencing and western blotting (**Fig.8A-B**). DDB2 is the DNA binding subunit of the UV-DDB complex, and by knocking it out, the UV-DDB's DNA binding and downstream DNA repair signaling pathway would be completely disrupted (210, 212). The HepAD38 DDB2 K/O cells were induced for 14 days in tet-free medium to maximize time allotted to cccDNA formation. In comparison to control K/O cells, HBV core DNA replicative intermediates, including rcDNA and DP-rcDNA (the precursors for cccDNA), were slightly decreased in the DDB2 K/O cells, accompanying

with a more profound reduction of cccDNA, as revealed by Southern blotting. Furthermore, a cccDNA-specific qPCR normalized to total HBV Hirt DNA and cellular mitochondrial DNA confirmed a similar reduction in cccDNA corresponding to the Southern blot results. These results suggest a substantial role is played by DDB2 in the formation of HBV cccDNA through the rcDNA recycling pathway.

Next, we examined the effect of DDB2 K/O on production of CM-rcDNA, a potential intermediate during rcDNA-to-cccDNA conversion. As shown in **Fig.9**, after normalizing the input amount of HBV core DNA to an equal amount of rcDNA between control and DDB2 K/O HepAD38 cells, the proportional ExoI/III-treated Hirt DNA samples exhibited a significant reduction of both CM-rcDNA and cccDNA in DDB2 K/O cells. The results indicate that the nuclear rcDNA-binding DDB2 may embark on cccDNA formation at a step prior to CM-rcDNA formation.

3.4 DDB2 siRNA K/D in vitro HBV infection systems reduces *de novo* HBV cccDNA formation

Having demonstrated the role of DDB2 in cccDNA formation *via* rcDNA recycling pathway in HBV stably transfected HepAD38 cells, we set out to investigate its role in *de novo* HBV cccDNA formation following initial infection. To this end we used HepG2-NTCP cells as well as primary human hepatocytes (PHH), both of which are permissive to HBV infection and first round cccDNA formation (129, 213, 214). As shown in **Fig.10**, DDB2 siRNA efficiently knocked down DDB2 expression in HepG2-NTCP and PHH cells. HBV cccDNA was observed to be largely reduced through Southern blotting and/or qPCR analysis, when compared to control siRNA knockdown. DP-rcDNA was essentially undetected in either the control or DDB2 K/D cells, which is consistent with previous

Figure 8 DDB2 K/O in HepAD38 cells reduced cccDNA production. (A) A crRNA (underlined sequence) was designed to target the 3rd exon of the DDB2 gene. The knockout of DDB2 gene in HepAD38 DDB2 K/O cells was confirmed by indel-PCR sequencing, which revealed an ORF-disrupting deletion around the PAM site. The HepAD38 control K/O cell line made with scramble crRNA contains wild type DDB2 sequence. (B) The HepAD38 scramble K/O and DDB2 K/O cells were induced for 12 days. The expression levels of DDB2 protein were analyzed by western blot with β -actin serving as loading control. The cytoplasmic HBV core DNA and total HBV Hirt DNA were analyzed by Southern blot. The Hirt DNA samples were heated at 85°C for 5 min and subsequently digested by EcoRI before gel loading. Relative levels of HBV rcDNA and cccDNA hybridization signal in each sample are expressed as the percentage of control (% of ctrl) and indicated underneath the blots. The Southern blots represent one of two experiment trials. HBV cccDNA was also quantified by using a cccDNA-specific qPCR assay, the cccDNA copy numbers were normalized by mitochondrial DNA and plotted as relative levels of control (fold change of 1) (mean $\pm$ SD, n=3).

reports that the rcDNA recycling pathway in the HepG2-NTCP-based HBV infection system is much less efficient than that of HBV stable cell lines (107, 124, 125, 129). Though the underlying mechanism remains unknown, this phenotype makes HepG2-NTCP cells a suitable system for specifically studying the first round of cccDNA formation from the incoming virus. Thus, the reduction of cccDNA mediated by DDB2 transient knockdown in HepG2-NTCP and PHH cells suggests that DDB2 plays an important role in *de novo* HBV infection and cccDNA formation.

Figure 9 DDB2 K/O reduced CM-rcDNA production in HepAD38 Cells. The HepAD38 scramble K/O and DDB2 K/O cells were induced for 14 days. HBV core DNA was analyzed by Southern blot (top panel). Hirt DNA was extracted and treated with ExoI/III, and the remaining cccDNA and the minus strand circular DNA moiety of CM-rcDNA were detected by Southern blot (bottom panel). The results are representative of three separate trials.

Figure 10 DDB2 K/D reduced cccDNA formation in HBV-infected cells. (A) HepG2-NTCP cells were transfected with control siRNA or DDB2-specific siRNA for 32h, followed by HBV infection with a MOI of 500 for 6 days. The DDB2 K/D was assessed by western blot (top panels). HBV cccDNA was analyzed by Southern blot after heat denaturation and EcoRI linearization (middle panel), the relative level of cccDNA hybridization signal in each sample is expressed as the percentage of control (% of ctrl). The cccDNA copy numbers were quantified by qPCR and normalized to mitochondrial DNA and expressed as relative levels of control (mean $\pm$ SD, n=3) (bottom panel). (B) PHH cells were transfected with control siRNA or DDB2-specific siRNA for 48h, followed by HBV infection with a MOI of 500 for 4 days. The levels of DDB2 protein and HBV cccDNA were analyzed by western blot and qPCR, respectively, in the same way as panel (A).

3.5 DDB2 K/O in HepG2-NTCP cells reduces cccDNA and its downstream indicators upon HBV infection

To further investigate the dependence on DDB2 by *de novo* HBV cccDNA formation, we decided to knock out DDB2 expression in HepG2-NTCP cells. The same crRNA designed and used to knockout DDB2 in HepAD38 cells was used in HepG2-NTCP cells. Successful knockout clone was selected through sequencing and western blotting (**Fig.11A-B**). Comparing to HBV infection in the scramble control K/O cells, the infection of DDB2 K/O cells exhibited markedly reduced levels of cccDNA and multiple downstream indicators of cccDNA such as intracellular HBV RNA and HBc (**Fig.11B-D**). To ensure DDB2 was specifically playing a role in *de novo* cccDNA formation but not the upstream step(s) in viral entry, Hepatitis D virus (HDV) infections were carried out in HepG2-NTCP DDB2 K/O cells. HDV, a satellite RNA virus to HBV, can only propagate in HBV-infected cells as it coats itself with the HBV envelop proteins and therefore invades hepatocytes *via* NTCP identically to HBV but possesses a distinct post-entry replication cycle (37, 215). Therefore, if DDB2 was involved in HBV entry but not cccDNA formation, it would also inhibit HDV infection. However, HDV-infected DDB2 K/O cells had similar level of HD δ Ag immunofluorescence signals compared to HDV-infected scramble K/O cells (**Fig.11D**), indicating that DDB2 does not play a role in HBV/HDV entry but is rather a critical factor in *de novo* HBV cccDNA formation.

It is worth noting that DDB2 K/O did not completely blocked cccDNA formation in HBV stable cell lines or infection model (**Fig.8-11**), indicating that a redundancy mechanism(s) exists to maintain partial capacity of cccDNA formation or compensate for the loss of DDB2.

Figure 11. DDB2 K/O reduced cccDNA and downstream indicators in HBV-infected HepG2-NTCP cells. A) The DDB2 crRNA-mediated mutation of DDB2 gene in HepG2-NTCP DDB2 K/O cells was detected by indel-PCR sequencing and aligned with the wild type sequence from HepG2-NTCP scramble control K/O cells. (B) The control and DDB2 K/O cells were infected by HBV (MOI: 500) for 6 days. The expression levels of DDB2 protein were analyzed by western blot, β -actin served as loading control; the intracellular total HBV RNA were detected by northern blot, ribosomal RNA (28S and 18S) served as loading control. (C) cccDNA copy numbers were quantified by qPCR and normalized to mitochondrial DNA and expressed as relative levels of control (mean $\pm$ SD, n=3). (D) HepG2-NTCP scramble K/O and DD2 K/O cells were infected by HBV or HDV at MOI of 500, respectively, for 6 days. The intracellular HBc (top grids) and HD δ Ag (bottom grids) were detected by immunofluorescence. Cell nuclei were counterstained with DAPI. Each image is a representative of five different microscopic fields, the percentage of antigen-positive cells was indicated.

3.6 Ectopic DDB2 expression rescues cccDNA formation in HepG2-NTCP DDB2 K/O cells

After demonstrating a loss of DDB2 led to a reduction in cccDNA, we wanted to strategically confirm the cccDNA reduction was specifically due to DDB2 depletion. We transfected HepG2-NTCP DDB2 K/O cells with a wild type (WT) DDB2 expression plasmid to rescue DDB2 expression, followed by HBV infection. As shown in **Fig.12**, western blotting confirmed that DDB2 expression was restored in DDB2 K/O cells transfected by WT DDB2, while the expression of endogenous DDB1 was unchanged in the absence or presence of DDB2 (**Fig.12A, top panels**); the levels of cccDNA and HBeAg were restored in the DDB2 K/O cells upon DDB2 transcomplementation (**Fig. 12A, bottom panels**). HBc immunofluorescence also showed a rebound of HBc expression in the HepG2-NTCP DDB2 K/O cells upon restoration of DDB2 expression (**Fig.12B**). Collectively, the above results further demonstrated a critical role of DDB2 in cccDNA formation.

3.7 The DNA- and DDB1-binding activity and ubiquitination of DDB2 play a role in establishing HBV infection

To investigate what functional domains of DDB2 may be involved in the interaction between UV-DDB and HBV cccDNA formation, we employed several DDB2 mutant forms (**Fig.13A**), including 1) two naturally occurring mutations K244E and R273H involved in the rare genetic disorder xeroderma pigmentosum (type E) (216, 217), which result in structural instability within the protein leading to their inability to tightly bind damaged DNA (218-220); 2) WD motif (aa238-278) and C-terminal deleted DDB2

Figure 12 Ectopic DDB2 expression rescued HBV infection in HepG2-NTCP DDB2 K/O cells. HepG2-NTCP scramble K/O cells and DDB2 K/O cells were transfected with control vector or plasmid Flag-DDB2 as indicated for 32 h, followed by HBV infection with an MOI of 500 for 6 days. (A) DDB1 and DDB2 protein expression were detected by western blot using antibodies against endogenous DDB1 and DDB2, respectively. The intracellular HBV cccDNA and supernatant HBcAg were analyzed by qPCR and ELISA, respectively, and expressed as relative levels of control (mean $\pm$ SD, n=3). (B) Intracellular HBc was detected by immunofluorescence. Cell nuclei were counterstained with DAPI. Each image is a representative of five different microscopic fields, the percentage of HBc-positive cells was indicated.

mutants (WDA Δ , 1-320, and 1-380) that have lost their DDB1-binding ability, leaving the DDB2 unable to form the DDB heterodimeric complex (199, 200); 3) the N7KR ubiquitination mutant had its seven N-terminal lysine residues substituted with arginine and the Ndel mutant has an N-terminal deletion removing the entire region containing the seven N-terminal lysines (201). The ubiquitination of DDB2 by DDB1-CUL4A E3 ligase is essential for it to hand off the damaged DNA to its downstream effectors for the initiation

of the nucleotide excision repair (NER) pathway (220-222). We transfected the HepG2-NTCP DDB2 K/O cells with the above mutant DDB2 expression plasmids and confirmed their expression through western blotting (**Fig.13B**). The HepG2-NTCP scramble control K/O cells, and DDB2 K/O cells transfected with WT and mutant DDB2 were infected with HBV for 6 days, and the infection was assessed by HBc immunofluorescence. As observed before, WT DDB2 rescued widespread HBc expression in the DDB2 K/O cells, however, the DDB2 mutants all failed to rescue HBV infection in DDB2 K/O cells (**Fig.13C**). Therefore, the results indicate that, for an optimal cccDNA formation, DDB2 must be part of the UV-DDB complex that binds directly to a precursor to cccDNA and signals to its downstream effector(s) *via* its N-terminal ubiquitination.

Figure 13 DDB2 loss-of-function mutants were unable to rescue HBV infection in HepG2-NTCP DDB2 K/O cells. (A) List of DDB2 mutant plasmid names, the brief description of mutations, along with their corresponding wild type (WT) DDB2 expression plasmids. (B) Each of the plasmids listed in (A) was transfected into HepG2-NTCP DDB2 K/O cells for 32 h, followed by HBV infection (MOI: 500) for 6 days. The expression of WT and mutant DDB2 proteins was assessed by western blot using antibodies against T7-tag (left blot) and HA-tag (right blot), respectively. β -actin served as loading control. (C) Intracellular HBc was detected by immunofluorescence. DAPI was used to stain nuclei. HepG2-NTCP scramble K/O cells transfected with control vector, and HepG2-NTCP DDB2 K/O cells transfected with control vector and Flag-DDB2 (F-DDB2), respectively, were infected with HBV as above described and served as reference for rescue experiment (top grids). Each image is a representative of five different microscopic fields, the percentage of HBc-positive cells was indicated. Scale bar: 200 μ m.

CHAPTER FOUR

DISCUSSION AND FUTURE DIRECTIONS

The mechanism of cccDNA formation remains a major knowledge gap in our understanding of the HBV life cycle. It is generally acknowledged that HBV rcDNA hijacks host DNA repair machinery to repair it into cccDNA. To date, most if not all the known host factors involved in cccDNA formation were discovered by hypothesis testing and/or phenotypic screening (223), but targeted identification of rcDNA-interacting host DNA repair factors has not been reported. For this reason, we sought out an unbiased exploratory assay to discover novel interactions between host factors and cccDNA formation (**Fig.6**). With our nuclear rcDNA coIP-MS assay we were able to confirm previously reported interactions, such as ATR pathway proteins, RFC subunits, multiple host polymerase subunits, and topoisomerases, which validated the assay's feasibility and practicality.

A limitation of our coIP-MS assay is the limited region of rcDNA sequence for designing biotinylated DNA oligo to target and pull down nuclear rcDNA complexes. The oligo must target the single stranded region of rcDNA, where an approximately several hundred nucleotides long gap is left between the variable 3' end of plus strand DNA and the DR2 region (**Fig.4**). Therefore, we designed a 52 nt oligo with immediate upstream sequence of DR2 (nt 1540-1591) to maximize the coverage of nuclear rcDNA molecules (**Fig.6**). However, we have previously found that a portion of cytoplasmic DP-rcDNA population contained 3' end of plus strand beyond nt 1540 or even into the DR2 motif (224), which, if existed in the nucleus, would not be efficiently annealed to the oligo bait. To a lesser extent, the oligo could possibly competitively bind to these rcDNA species with complete or almost complete but unligated plus strands. However, the hypothetical rcDNA

intermediates with a complete and ligated plus strand (225), if any, the cccDNA, and the integrated HBV transgene would not be able to be pulled down by the rcDNA oligo bait. On the other hand, the nuclear HBV double stranded linear DNA (dsl-DNA) or deproteinated dslDNA, a minor HBV genome by-product resulting from the failed 2nd template switch during rcDNA synthesis (24, 223), could be precipitated by the oligo bait granted that the plus sense strand is incomplete and the minus strand complementary to the oligo is readily available for binding. Though dslDNA can be a precursor to cccDNA, the dslDNA-derived cccDNA is commonly observed with extensive indels (34, 114). Studies investigating dsl-DNA-derived cccDNA have discovered accumulating evidence of the non-homologous end joining (NHEJ) DNA repair mechanism is responsible for the conversion of dslDNA to cccDNA, including the involvement of NHEJ sensor Ku70/Ku80 complex and the NHEJ-specific DNA ligase LIG4 (116, 117). However, Ku70/80 and LIG4 were not detected in the coIP-MS assay, perhaps due to the low abundance of nuclear dslDNA input and/or the sensitivity of the assay. Nonetheless, the small population of incomplete nuclear dslDNA, if any, is not expected to create serious complications in our results. Another potential concern is that there is a possibility the induced HBV transgene could contain an exposed single strand DNA region for oligo binding, but DNA repair factor(s) may not be associated with the integrated HBV DNA if no DNA damage is generated. Furthermore, the nuclear extraction kit used in this study does not extract chromosome DNA. On the other hand, there may a small number of cccDNA present at the 6 days post induction time point in this assay, and the cccDNA being actively transcribed may also be targeted by the oligo bait, however, if cccDNA is precipitated it would likely be in insignificant amounts, and it also does not interfere with the principle

of the assay as host DNA repair factors could possibly still be bound to the newly formed cccDNA. With all these in mind, the designed oligo bait should be able to precipitate a majority of nonencapsidated rcDNA populations within the nucleus including DP-rcDNA and CM-rcDNA, which contain incomplete and un-ligated plus strands (8, 34, 133, 224). If we accept these populations to be intermediates to cccDNA formation, as it has been implied, then our assay should be able to observe host factors involved in cccDNA formation at least after rcDNA nuclear uncoating and before plus strand completion and ligation.

The rcDNA CoIP-MS assay delivered a long list of proteins, we specifically focused on the DNA repair proteins that were unique to induced HepAD38 cells. A new nuclear rcDNA-interacting hit that stood out to us, was the DDB1 protein, a subunit in the UV-DDB complex and major factor in the GG-NER pathway (210). Interestingly, DDB1 was also a hit in our previous shRNA screen for host DNA repair genes involved in cccDNA formation (117). UV-DDB is comprised of DDB1 and DDB2, with DDB2 being the DNA scanning and binding subunit of the complex (210). In line with this, the ChIP-qPCR assay showed both subunits of the UV-DDB complex being associated with the nuclear HBV rcDNA (**Fig.7**). However, DDB2 was not present in the rcDNA coIP-MS assay, the reason for this was unclear but could be perhaps due to the lack of suitable DDB2-specific proteolytic peptides for mass spec detection. To further investigate the role of UV-DDB in cccDNA formation, we attempted a knockout of DDB1 and DDB2 in HepAD38 cells. The DDB2 knockout was successful and confirmed with sequencing and western blot (**Fig.8**). However, we failed to establish viable DDB1 knockout cells likely due to DDB1 being essential to cell viability. In line with this, previous studies have

demonstrated that the loss of DDB1 leads to cell apoptosis (226-228). Furthermore, in the rare genetic disorder xeroderma pigmentosum (XP), which leaves carriers with extreme sensitivity to ultraviolet light-mediated DNA damage, human DDB2 has several natural mutations which lead to this genetic disorder (219), while, to the best of our knowledge, there are no DDB1 mutations described leading to XP, thus leading us to believe that further effort to knock out DDB1 would be futile. DDB2 is the DNA binding and sensing subunit of the UV-DDB complex (212), therefore, we continued with our focus on assessing the effect of DDB2 on cccDNA formation. The HepAD38 DDB2 K/O cells showed a marked decrease in HBV cccDNA formation exclusively mediated by the intracellular rcDNA recycling pathway (**Fig.8**).

Furthermore, DDB2 K/O resulted in a concurrent reduction of CM-rcDNA (**Fig.9**), a putative intermediate precursor for cccDNA formation (133), indicating that DDB2 plays a role in cccDNA formation prior to the covalent circularization of minus strand of nuclear rcDNA. It is plausible that the binding of DDB2 (or the UV-DDB complex) to nuclear rcDNA recruits downstream factors to repair the minus strand of rcDNA first. It has been reported that topoisomerase 1 and ATR-Chk1 DNA damage response (DDR) pathway are involved in CM-rcDNA formation (136, 145), thus, there may be a potential interplay among UV-DDB and these factors in CM-rcDNA synthesis. In line with this notion, DDB2 has been shown to recruit ATR kinase to the UV DNA damage sites for initiating DDR (229). Furthermore, as mentioned above, whether the nuclear HBV dsDNA can be sensed by UV-DDB or ATR-Chk1 awaits further investigation.

According to previous studies and this study, the relative proportions of nuclear DP-rcDNA (or PF-rcDNA), CM-rcDNA and cccDNA are approximately 10 (or more):1:1

in HepAD38 and other inducible HBV stable cell lines (Fig.4-5) (8, 34, 133, 139, 209). Serving as a putative precursor for cccDNA, DP-rcDNA greatly outnumbers CM-rcDNA and cccDNA, but the reason for such an inefficient DP-rcDNA to CM-rcDNA/cccDNA conversion *via* rcDNA recycling pathway is largely unclear. The rate limiting step(s) is possibly at nuclear rcDNA uncoating and/or DNA repair, leaving a significant fraction of DP-rcDNA behind as laggards or even dead ends. As shown in Fig.3, a large amount of nuclear rcDNA remain associated with HBc, indicating an inefficient rcDNA uncoating in nucleus. Furthermore, although the cytoplasmic DP-rcDNA exhibit almost homogenous characteristics in terms of DNA terminal sequences and modifications (224), but it remains possible that a big portion of DP-rcDNA are further modified upon delivery into nucleus, making them incapable of cccDNA formation. Thus, further characterizing the nuclear DP-rcDNA molecules in detail would help to address these issues. On the other hand, although the cccDNA formation efficiency appears to be low in *in vitro* HBV infection systems despite the high virus inoculum used, there is a much less accumulation of DP-rcDNA compared to that in HBV stable cell lines. In this regard, growing evidence has indicated that certain different regulations of DP-rcDNA and cccDNA formation exist between the intracellular rcDNA recycling and *de novo* HBV infection (107, 139, 223, 230).

We continued to explore the role of DDB2 in the HBV infection model cell line, HepG2-NTCP, by knocking down DDB2 with siRNA. This resulted in a greater than 50% reduction in cccDNA formation (**Fig.10A**). To further validate the physiological relevance of these findings, we wanted to confirm these results in PHH. The DDB2 K/D in PHH cells revealed a similar impact on cccDNA formation (**Fig.10B**). CRISPR-based DDB2 K/O in HepG2-NTCP cells similarly resulted in a roughly 50% reduction in cccDNA levels

following HBV infection (**Fig.11**). Furthermore, the reduced cccDNA phenotype in DDB2 K/O cells could then be rescued by DDB2 transcomplementation (**Fig.12**), suggesting a direct role of DDB2 in cccDNA formation during *de novo* HBV infection.

Though DDB2 is involved in cccDNA formation through both *de novo* infection and rcDNA recycling route, a complete loss of cccDNA was not achieved under DDB2 K/O condition, indicating that a compensatory mechanism(s) is in place for cccDNA biosynthesis in the absence of DDB2. In line with this notion, the redundant effects of host DNA repair factors on cccDNA formation have been reported in previous studies, including topoisomerases (145), DNA polymerases (107, 139), and ligases (117). These phenomena indicate that HBV has evolved to take advantage of the host redundant DNA repair systems for maintaining the viral replication cycle.

It appears that the manipulation of DDB2 expression level posed a slightly higher impact on viral transcription and antigen expression than cccDNA formation in HBV infection setting (**Fig.11-12**), which indicated that DDB2 might possess an additional effect on cccDNA transcription. In this regard, a well-studied function of DDB1, the binding partner of DDB2 in UV-DDB complex, in HBV life cycle is to interact with HBx protein to recruit the CUL4 E3 ubiquitin ligase to ubiquitinate SMC5/6 complex for proteasomal degradation, a process to protect cccDNA minichromosome from SMC5/6-mediated epigenetic silencing (231-233). However, an HBx-independent stimulation of HBV transcription by DDB1 has also been reported (234). Furthermore, it has been reported that DDB2 induces nuclear accumulation of HBx independently of binding to DDB1 (235). Considering that HBx is not required for cccDNA formation but indispensable for cccDNA transcription (56), and the loss of DDB2 does not affect the steady state level of DDB1

(**Fig.12**), the potential effect of DDB2 on cccDNA transcription in the context of UV-DDB complex and in the presence or absence of HBx awaits investigation in future studies.

The DDB1 and DDB2 duet uses DDB2 as the DNA damage binding subunit, and DDB1 forms a ubiquitin E3 ligase complex with CUL4A, which in turn ubiquitinates DDB2 and xeroderma pigmentosum complementation group C protein (XPC) to kick off the GG-NER pathway (236, 237). In this scenario, DDB2 also serves as a DDB1 and CUL4-associated factors (DCAFs) like HBx. Indeed, the DDB1-CUL4 complex pairs with dozens of known host and viral DCAFs to ubiquitinate specific protein substrates to regulate a variety of cellular functions (238, 239). In order to determine the domains/functions of DDB2 necessary for its role in HBV cccDNA formation, we assessed the mutant forms of DDB2 for their ability to rescue the DDB2 K/O phenotype in HBV infection (**Fig.13**). The well-characterized DDB2 mutants contained aberrations inhibiting the complexing with DDB1, DNA damage binding, and ubiquitination for downstream signaling/handoff to XPC (**Fig.13A**) (199-201, 216-220). All the mutant DDB2 constructs failed to rescue HBV infection in DDB2 K/O cells (**Fig.13C**), indicating that DDB2 must be in complex with DDB1 to scan nuclear HBV rcDNA, sense it as damaged, and be ubiquitinated to hand off the rcDNA to the next factor(s) in the cccDNA formation pathway. These observations demonstrate a significant and intimate role played by the UV-DDB complex, or more specifically DDB2, in the crucial lifecycle step of HBV cccDNA formation. DDB2 likely plays an early role in sensing the DP-rcDNA or other unidentified rcDNA species upon nuclear entry and signaling for host DNA repair factors to repair rcDNA to cccDNA.

While we have stated DDB2 as an early effector in cccDNA formation, this is mostly concluded by DDB2's function as a DNA damage detecting factor, and the early nuclear rcDNA selective bias of our assay. In this regard, there is, thus far, a lack of direct evidence for establishing a timeline/chronological order of host factors binding to nuclear rcDNA (110, 223). In this study, one lingering question remaining to be answered is the specific binding site of DDB2 along the nuclear rcDNA. Mechanistically, UV-DDB complex employs DDB2 to continually scan the genome for DNA damage and establishes high-affinity DNA interactions once damage is detected, such as UV-induced cyclobutane pyrimidine dimers (CPDs) and 6-4 photoproducts (240). DDB2 also exhibits a remarkable substrate diversity to recognize a variety of helix distorting "blocking" DNA lesions (241-243). Thus, we hypothesize that DDB2 within the UV-DDB complex randomly finds HBV rcDNA upon nuclear import and repeatedly loosely binds to it before finding a specific structure along rcDNA, likely the terminal redundancy of minus strand and its resulting flap formation or the gap/nick on plus strand DNA, where it will tightly bind to and recruit presumably the GG-NER pathway or another unidentified pathway to begin forming CM-rcDNA and then cccDNA (**Fig.14**). While this updated model of cccDNA formation awaits further development, it is worthy of note that some late factors in the NER pathway like PCNA, RFC, polymerases (Pol δ and Pol κ), and ligases (LIG1 and LIG3) have also been previously implicated in cccDNA formation (107, 108, 117, 139, 142, 237, 244). Therefore, it is of interest to further assess the potential involvement of other GG-NER midstream components in cccDNA formation, such as XPC, transcription factor II H (TFIIH), XPG, etc. Moreover, it will also be interesting to investigate the prevalence and pathophysiology of HBV infection in XP patients.

Figure 14. Model of UV-DDB-mediated cccDNA formation. The cytoplasmic HBV rcDNA undergoes deproteination to generate DP-rcDNA, the latter is transported into nucleus and sensed as "damaged" DNA by UV-DDB and/or ATR-CHK1 complexes. While there are potential single strand break (ssb) sites on DP-rcDNA for ATR to bind to, it is uncertain what structure triggers DDB2 to tightly bind to rcDNA. It is possible that DDB2 and ATR could detect the nick and/or terminal redundancy on minus strand DNA individually or cooperatively, as both are involved in CM-rcDNA formation. DDB2, in conjunction with DDB1, is ubiquitinated by the CUL4A ubiquitin ligase, facilitating its handoff to a yet to be defined DNA damage response pathway(s). The pathway then assembles DNA repair factors to facilitate the repair of the remaining terminal aberrations of rcDNA into cccDNA. Interestingly, seven of the previously reported proteins implicated in cccDNA formation are involved in the late stages of the GG-NER pathway, which is commonly activated by the UV-DDB complex upon substrate anchoring.

Taken together, by making use of a newly developed HBV rcDNA coIP-MS assay, this study has identified UV-DDB as a novel nuclear rcDNA binding factor and established adequate evidence to implicate DDB2, as part of the UV-DDB complex, in the formation of HBV cccDNA, providing new insights into the molecular mechanism of cccDNA formation and potential antiviral targets for treatment of hepatitis B.

The mystery of cccDNA formation is no longer as ambiguous as previously thought. Based on the findings, an updated proposed model of cccDNA formation would be: The mature rcDNA is deproteinated in the cytoplasm and transported into the nucleus, followed by nuclear uncoating of DP-rcDNA. The UV-DDB binds to the nuclear DP-rcDNA and senses it as damaged which in turn potentially activates the ATR-Chk1 DNA damage response (8, 9, 34, 100, 123, 136). One copy of the (-) strand terminal redundant

repeats is cleaved from the 5' terminus by FEN1 or other nuclease(s) (133, 142), followed by DNA resynthesis catalyzed by polymerase α and/or δ (108, 139). The (-) strand is then ligated shut to form CM-rcDNA (133). Host DNA polymerases (α , δ , κ , η , etc.) then complete the (+) strand DNA for ligation (107, 108, 139). Host DNA ligases (LIG1/3) and topoisomerases are involved in the ligation steps, while topoisomerases may have other functions beyond ligation (117, 145) (**Fig.4A**). Although this cccDNA formation pathway seems possible, it is important to note that the participating host factors and the order of DNA repair reactions are likely to be appended and redefined as the research proceeds. It remains possible that these identified DNA intermediates (DP-rcDNA, CM-rcDNA) are minor or even byproducts during cccDNA formation, major or additional intermediate(s) may exist and awaits further exploration. In addition, no single host factor, including UV-DDB, completely inhibited cccDNA formation when the host factors of interest were either knocked out or inhibited. This casts some doubt whether these are the essential factors for cccDNA formation and could suggest there are alternative host factors and/or pathways that serve redundant roles at each step of the cccDNA formation pathway. In this regard, there is a plausible scenario of “all roads lead to Rome” during the conversion of rcDNA to cccDNA. In contrast, the involvement of NHEJ pathway in dsDNA-based cccDNA formation and integration seems more straightforward and well-documented (**Fig.5B**).

Taken together, by making use of a newly developed HBV rcDNA CoIP-MS assay, we were able to identify UV-DDB as a novel nuclear rcDNA binding factor and established adequate evidence to implicate DDB2, as a part of the UV-DDB complex, in the formation of HBV cccDNA, providing new insights into the molecular mechanism of cccDNA formation and potential antiviral targets for treatment of hepatitis B.

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Education

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Publications

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- Marchetti AL, Guo H. New Insights on Molecular Mechanism of Hepatitis B Virus Covalently Closed Circular DNA Formation. *Cells*. 2020 Nov 6;9(11):2430. doi: 10.3390/cells9112430. PMID: 33172220; PMCID: PMC7694973.
- Cai D, Yan R, Xu JZ, Zhang H, Shen S, Mitra B, Marchetti A, Kim ES, Guo H. Characterization of the Termini of Cytoplasmic Hepatitis B Virus Deproteinized Relaxed Circular DNA. *J Virol*. 2020 Dec 9;95(1):e00922-20. doi: 10.1128/JVI.00922-20. PMID: 33055252; PMCID: PMC7737736.

- Long Q, Yan R, Hu J, Cai D, Mitra B, Kim ES, Marchetti A, Zhang H, Wang S, Liu Y, Huang A, Guo H. The role of host DNA ligases in hepadnavirus covalently closed circular DNA formation. PLoS Pathog. 2017 Dec 29;13(12):e1006784. doi: 10.1371/journal.ppat.1006784. PMID: 29287110; PMCID: PMC5747486.

Abstracts

- Marchetti, A., Long, Q., Kim, E., Zhang, H., Mitra, B., Wang, M., Guo, H. Host DNA damage binding protein 2 is involved in HBV cccDNA formation. Viral entry, cccDNA biogenesis, and homeostasis. The 2019 International HBV meeting, October 1-5th, 2019, Melbourne, Australia.

Conferences and Presentations

- 2019 International HBV meeting. Host DNA damage binding protein 2 is involved in HBV cccDNA formation. Melbourne, Australia.

Awards

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- 2019 International HBV Meeting Travel grant

Mentorship Experience

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Professional Experience

- Cancer cell line culture

- Primary human cell culture
- Western immunoblotting
- Vector designing and cloning
- Southern Blot DNA hybridization
- Northern Blot RNA hybridization
- Chromatin immunoprecipitation (ChIP)
- Co-Immunoprecipitation of proteins and DNA (CoIP)
- Polymerase Chain Reactions (RT, quantitative, etc.)
- ELISA spectrophotometry
- Bacterial transformation
- DNA/siRNA transfection
- Gene cloning
- Viral Transduction and Infection Assays
- CRISPR RNA (crRNA) sequence design
- Generation of stable CRISPR knockout cell lines
- Confocal Microscopy and Imaging
- Flow Cytometry
- Immunofluorescence Microscopy and Imaging
- HALO linked live cell imaging
- Hirt DNA extraction
- Agarose and Polyacrylamide Gel Electrophoresis