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## Silencing hepatitis B virus covalently closed circular DNA: The potential of an epigenetic therapy approach

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### Abstract

Global prophylactic vaccination programmes have helped to curb new hepatitis B virus (HBV) infections. However, it is estimated that nearly 300 million people are chronically infected and have a high risk of developing hepatocellular carcinoma. As such, HBV remains a serious health priority and the development of novel curative therapeutics is urgently needed. Chronic HBV infection has been attributed to the persistence of the covalently closed circular DNA (cccDNA) which establishes itself as a minichromosome in the nucleus of hepatocytes. As the viral transcription intermediate, the cccDNA is responsible for producing new virions and perpetuating infection. HBV is dependent on various host factors for cccDNA formation and the minichromosome is amenable to epigenetic modifications. Two HBV proteins, X (HBx) and core (HBc) promote viral replication by modulating the cccDNA epigenome and regulating host cell responses. This includes viral and host gene expression, chromatin remodeling, DNA methylation, the antiviral immune response, apoptosis, and ubiquitination. Elimination of the cccDNA minichromosome would result in a sterilizing cure; however, this may be difficult to achieve. Epigenetic therapies could permanently silence the cccDNA minichromosome and promote a functional cure. This review explores the cccDNA epigenome, how host and viral factors influence transcription, and the recent epigenetic therapies and epigenome engineering approaches that have been described.

**Key Words:** Chronic hepatitis B virus; Epigenetic gene silencing; Functional cure; Hepatocellular carcinoma; Hepatitis B surface antigen

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**Core Tip:** Epigenetic regulation of the hepatitis B virus (HBV) covalently closed circular DNA (cccDNA) minichromosome is important for establishing and maintaining infection. To do this HBV manipulates several cellular pathways, resulting in an intricate and complex interplay between the virus and the host. Epigenetic silencing of the cccDNA could permanently inhibit viral transcription. Therapies such as immune modulators, small molecules, and epigenome engineering tools could silence HBV DNA to promote a functional cure.

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## INTRODUCTION

Chronic HBV infection (CHB) remains largely incurable and is a major risk factor for the development of hepatocellular carcinoma (HCC)[1]. Regrettably, only a small percentage of chronically infected individuals are currently afforded therapy[2] and the annual rate of hepatitis-related deaths remains high. This underpins the importance of developing new therapeutics to treat the estimated 257-291 million people afflicted by this life-threatening disease[3]. Licensed direct-acting antivirals such as nucleoside/nucleotide analogues help mitigate infection by preventing viral replication but have little effect on established covalently closed circular DNA (cccDNA)[4]. Immunotherapy approaches using interferon alpha (IFN- $\alpha$ ) have demonstrated antiviral efficacy, particularly when using pegylated formulations, and have been paired with various nucleoside/nucleotide analogues for combination therapy[5]. However cost, serious side effects, and mixed results have limited the broad clinical feasibility of IFN-based therapies in their current form. Persistence of the cccDNA as a minichromosome-like structure in the nucleus of long-lived hepatocytes may account for the inability of these antivirals to achieve cure, even after long-term treatment[6]. A sterilizing cure can only be realized following complete elimination of intrahepatic cccDNA, while permanent loss of serum hepatitis B surface antigen (HBsAg) would achieve a functional cure. The cccDNA is thought to endure for years [7] and as the template for viral mRNA synthesis[8], it drives viral replication and remains the major obstacle in the development of curative antiviral therapies.

In recent years, treatments designed to directly silence or eliminate the cccDNA minichromosome have gained interest as they may provide a means for achieving cure [9]. Gene editing tools such as designer nucleases and nickases disrupt the viral DNA *via* site-directed cleavage. This stimulates either targeted mutagenesis or degradation of the cccDNA[10,11]. Zinc finger (ZF) nucleases, transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short palindromic repeats (CRISPR) RNA-guided nucleases have all demonstrated antiviral potential in preclinical models of infection. The simplicity of the CRISPR/Cas system has led to a surge in the field of anti-HBV gene editing, particularly as multiple studies have reported degradation of cccDNA following cleavage[12-15]. The CRISPR/Cas constructs developed by Seeger and Sohn[16] were shown to edit HBV DNA 15000 times more efficiently than IFN- $\alpha$ -induced APOBEC deamination. However, concerns regarding off-target cleavage, liver-specific delivery and expression, and Cas9 immunity will need to be addressed to ensure safety without compromising efficacy [17-20]. Viral vectors, including adeno-associated viruses (AAVs) and high-capacity adenoviral vectors (HCAdV) have recently been explored as hepatotropic delivery vehicles[13,21,22]. To overcome the packaging limitations of AAVs, the smaller *Staphylococcus aureus* endonucleases have been combined with HBV-specific guide RNAs to demonstrate cccDNA-targeting and antiviral efficacy in hNTCP-HepG2 cells [22] and transgenic mice[21]. On the other hand, the large packaging capacity of HCAdVs have been exploited to accommodate multiple HBV guide RNAs along with the larger *Streptococcus pyogenes* Cas9[13], which may improve the efficacy of this gene editing approach. Importantly, iterative modifications to the effector domains of traditional gene editing platforms have given rise to several new tools. CRISPR/Cas

base editors comprising an APOBEC1 deaminase, Cas9 nickase, and uracil glycosylase inhibitor introduced point mutations in cccDNA as well as integrated viral DNA[23]. A number of new epigenome engineering platforms have also been developed, which could be used to generate targeted epigenetic changes to control gene expression[24]. Such an approach may be well suited to treating CHB, as epigenetic modifications regulate cccDNA minichromosome organization and can either promote or repress viral transcription[25-27]. Host and/or virus-specific epigenetic therapies could promote silencing of HBV cccDNA and achieve a functional cure. This review will explore the formation and epigenetic regulation of the cccDNA minichromosome, how host and viral factors influence transcription, and whether epigenome editing could be used to silence HBV cccDNA permanently.

## CCC DNA FORMATION AND ORGANIZATION OF THE MINICHROMOSOME

cccDNA biogenesis is a multi-step process that relies on a number of host-cell DNA synthesis and repair factors[28,29]. Although the exact process is not fully understood it involves: (1) Removal of the covalently attached viral polymerase from the 5' end of the negative DNA strand; (2) Removal of the short RNA oligomer attached to the 5' end of the positive strand which is derived from the priming of positive-strand DNA synthesis; (3) Removal of one copy of the short terminal redundancy from the negative-strand; (4) Positive-strand elongation; and (5) Covalent ligation of the two strands[30-32]. Evidence from animal models has shown that between 1 and 50 copies of cccDNA may accumulate per infected cell[33-36]; however, more recent reports estimate up to 15 copies per cell[37,38]. Multiple rounds of infection are not necessary to establish the cccDNA pool as recycling of newly synthesized relaxed circular DNA (rcDNA) has been shown to occur, and contributes to the maintenance of cccDNA copy numbers[39-41]. Increasing evidence also indicates that the size of the pool is controlled by different host and viral mechanisms[42], but that initial *de novo* cccDNA formation is sufficient to maintain viral replication in the absence of rcDNA recycling [43].

Many different host factors have been implicated in cccDNA biogenesis, including host DNA polymerases[32,44], ligases[45], flap endonuclease I (FEN1)[46], topoisomerase I and II[47], SAMHD1[48], and tyrosyl-DNA phosphodiesterase 2[31]. Five essential components for cccDNA formation have recently been described by Wei and Ploss[49], who developed a cell-free biochemical system to mimic cccDNA biogenesis. The components are proliferating cell nuclear antigen (PCNA), replication factor C complex, DNA polymerase  $\delta$ , FEN1, and DNA ligase 1. Confirmation of these requirements in hepatocytes will be needed to validate the cell-free biochemical system[50]. Aphidicolin and CD437 were shown to block host DNA polymerases  $\alpha$ , which in turn inhibited intracellular amplification of cccDNA[44]. Also disubstituted sulfonamide compounds, CCC-0975 and CCC-0346, influenced conversion of rcDNA to cccDNA, most likely as a result of inhibiting deproteination[51]. Blocking the conversion of rcDNA to cccDNA with small molecule inhibitors may prevent accumulation of new replication-competent viral genomes in the nucleus of infected hepatocytes, but will not prevent *de novo* cccDNA biogenesis.

Once formed, cccDNA is organized into a nucleosome-decorated minichromosome [8,52]. It associates with histone and non-histone proteins, the latter of which are derived from both the host and the virus[29,33]. As a hepatotropic virus, the viral genome contains binding sites for ubiquitous and liver-specific transcription factors [53]. Thus, the recruitment, activity, and dynamic interplay of several host and viral factors are essential for efficient HBV gene expression[25-27]. Several histone proteins, transcription factors, chromatin modifying enzymes, as well as additional host enzymes and coactivators have been reported to bind to cccDNA (Table 1). Recruitment of these factors plays an essential role in regulating viral transcription which is amenable to epigenetic influences. Apart from host factors, viral elements such as HBx (HBV protein X) and HBc (HBV protein core) have been shown to influence gene transcription as well as reduce nucleosomal spacing, indicating a role in promoting viral replication and structural maintenance of the cccDNA minichromosome[8]. Thus, it is conceivable that post-translational modifications (PTMs) such as DNA methylation and histone modifications could be used to regulate cccDNA.

**Table 1 Host cell factors involved in the epigenetic maintenance of the covalently closed circular DNA minichromosome**

	Category	Epigenetic effect	Effect on HBV	Ref.
<b>Chromatin remodeling enzymes</b>				
Histone acetyltransferase p300/CBP	Writer	Increases H3K122a	Activation	Ananthanarayanan <i>et al</i> [99] and Tropberger <i>et al</i> [221]
P300/CBP-associated factor (PCAF)	Writer		Activation	Levrero <i>et al</i> [82] and Rivière <i>et al</i> [93]
Histone deacetylase 1 (HDAC1)	Eraser	Reduces acetylation of H3K9 and H3K27 with IFN	Inhibition	Pollicino <i>et al</i> [57] and Liu <i>et al</i> [98]
Sirtuin 1 and 3 (SIRT1/3)	Eraser	Reduces H3K9me3	Inhibition	Belloni <i>et al</i> [105] and Ren <i>et al</i> [114]
Enhancer of zeste homolog 2 (EZH2)	Writer	Increases H3K27ac and H3K27me3	Inhibition	Zhang <i>et al</i> [89]
Histone acetyltransferase 1 (HAT1)	Writer	Overexpression of HAT1 promotes acetylation of H3K27, H4K5 and H4K12	Activation	Yang <i>et al</i> [195]
Mixed lineage leukemia protein 3 (MLL3)	Writer	Increases H3K4me3	Activation	Tropberger <i>et al</i> [86] and Ananthanarayanan <i>et al</i> [99]
Protein arginine methyltransferase (PRMT)	Writer	PRMT5 interacts with HBc to increase H4R3me2s	Inhibition	Zhang <i>et al</i> [89]
Demethylases (KDMs)	Eraser	Increases H3K79me and function to transcriptional repression <i>via</i> SIRT1-mediated chromatin silencing	Inhibition	Kang <i>et al</i> [222]
Histone methyltransferase suppressor of variegation 3-9 homolog 1 (SUV39H1)	Writer	Increases H3K9me3	Inhibition	Peng and Karpen[223,224]
DNA methyltransferase (DNMTs)	Writer		Inhibition	Vivekanandan <i>et al</i> [81]
Methyl-CpG binding protein (MBPs)	Reader	Recruit chromatin remodeling and histone-modifying complexes to methylated sites resulting in histone methylation	Inhibition	Lopez-Serra and Esteller[94]
<b>Cellular transcription factors</b>				
Activating transcription factor 2 (ATF 2)		Inhibition viral transcription	Inhibition	Choi <i>et al</i> [225]
cAMP response element binding protein (CREB)		Enhances transcription	Activation	Kim <i>et al</i> [226] and Song <i>et al</i> [227]
Nuclear factor 1 (NF1)	Reader		Activation	Ori <i>et al</i> [228] and Shaul <i>et al</i> [229]
Transcription factor Yin Yang 1 (YY1)			Inhibition	Belloni <i>et al</i> [199] and Nakanishi-Matsui <i>et al</i> [230]
Specificity protein 1 (SP1)	Reader		Activation	Raney and McLachlan[231], Raney <i>et al</i> [232] and Li and Ou [233]
Nuclear Transcription Factor Y (NF-Y)		Recruit enzymes that both methylate and acetylate histone proteins	Activation	Lu and Yen[234], Maity and de Crombrughe[235] and Nardini <i>et al</i> [236]
Activator protein 1(AP-1)			Activation	Ren <i>et al</i> [111] and Choi <i>et al</i> [237]
TATA binding protein (TBP)	Reader		Activation	Bogomolski-Yahalom <i>et al</i> [238] and Chen <i>et al</i> [239]
Prospero-related homeobox protein (Prox1)			Inhibition	Qin <i>et al</i> [240]
Nuclear factor kappa-B (NF-κB)			Inhibition	Lin <i>et al</i> [241]
Histone-lysine N-methyltransferase SETDB1	Writer	In the absence of HBx, SETDB1 increases H3K9me2 and H3K9me3	Inhibition	Rivière <i>et al</i> [93]
<b>Hepatocyte factors</b>				
Retinoid X receptors (RXRα)		Increases acetylation of histones H4 and H3 by recruiting p300 to cccDNA minichromosome	Activation	Zhang <i>et al</i> [89] and Nkongolo <i>et al</i> [242]
Small heterodimer partner (SHP)			Inhibition	Oropeza <i>et al</i> [243]
CAAT enhancer-binding protein		C/EBP in low concentrations; C/EBP in high	Activation;	Raney and McLachlan[231] and

$\alpha$ and $\zeta$ (C/EBP)	concentrations	Inhibition	López-Cabrera <i>et al</i> [244]
Hepatocyte nuclear factor 1 $\alpha$ and $\beta$ (HNF1)	HNF1 and Oct 1 are essential co-activators of transcription. High HNF1 levels increase NF- $\kappa$ B expression and resulting in transcription inhibition	Activation and inhibition	Zhou and Yen[245], Zheng <i>et al</i> [246] and Lin <i>et al</i> [247]
Hepatocyte nuclear factor 3 $\alpha$ , $\beta$ , and $\gamma$ (HNF3)	Functions as a chromatin remodeler	Activation	Chen <i>et al</i> [248] and Li <i>et al</i> [249]
Hepatocyte nuclear factor 4 (HNF4)		Activation	Zheng <i>et al</i> [246], Cho <i>et al</i> [250] and Long <i>et al</i> [251]
Testicular orphan receptor 4 (TR4)		Inhibition	Lin <i>et al</i> [252]
Type I interferon (IFN- $\alpha$ )	Reduced acetylation of H3K9 and H3K27	Inhibition	Pollicino <i>et al</i> [57], Liu <i>et al</i> [98] and Yuan <i>et al</i> [253]

cccDNA: Covalently closed circular DNA; NF- $\kappa$ B: nuclear factor-kappa B.

## HOST FACTORS AND THE EPIGENETIC REGULATION OF HBV

Epigenetic modifications are reversible heritable phenotypic changes that alter chemical signatures on chromosomes without affecting the DNA sequence. This highly regulated process has a critical impact on gene expression, cell function as well as cell behavior[54]. Epigenetic marks can be added directly to DNA or placed on histone tails. Methylation of cytosines within CpG dinucleotides is the most common DNA epigenetic mark, and is critical for controlling transcription, genomic imprinting and cell type identity maintenance[55]. The wrapping of DNA around histones creates highly condensed chromatin structures with protruding N-terminal histone tails amenable to PTMs[56]. Histone acetylation and DNA methylation are required for cccDNA formation and viral replication[57], highlighting the importance of the epigenome in HBV infection.

Numerous proteins are involved in the epigenetic regulation of genes and are generally categorized as writers, readers and erasers (Table 1)[54]. Writers are responsible for establishing epigenetic marks while readers recognize modified residues and recruit other protein complexes to regulate gene expression. Erasers are enzymes that remove epigenetic marks. Epigenetic therapies generally target one or more of these proteins, and studies using this approach for other chronic viral infections[58,59] suggest that this may be a promising treatment strategy for CHB.

### Methylation for transcriptional gene silencing

In mammalian cells, methylation is a common cellular defense mechanism known to silence invading foreign DNA[60]. It involves the addition of a methyl group from the methyl donor, S-adenosyl methionine, to the fifth carbon of cytosine in DNA (5mC). DNA methylation is most commonly detected within CpG islands and is associated with transcriptional gene silencing[61]. These inheritable 5mC marks can be 'written' (added) *de novo* in unmethylated regions which are either maintained or actively 'erased' (removed)[62]. In mammals, methyl-CpG binding proteins (MBPs) are responsible for the identification of methylation patterns[63] and a family of DNA methyltransferases (DNMTs) is responsible for establishment and maintenance of these patterns[64]. The DNMT family consists of DNMT1, DNMT2, DNMT3A, DNMT3B and DNMT3L (DNMT3-like). DNMT1 is primarily responsible for the maintenance of the methylation patterns during cell division and shows a preference for hemimethylated DNA[65,66]. *De novo* methylation patterns are established by the two active DNMT3A and DNMT3B enzymes and their activity is enhanced by interaction with DNMT3L which lacks catalytic activity[67,68]. In addition, DNMT2 plays an important role in methylation of structural RNA[69]. More than 20 years ago, it was discovered that integrated HBV DNA could be methylated and this was frequently observed in CHB patients with HCC[70]. Since then methylation of non-integrated HBV DNA as well as cccDNA has been observed in patients' liver samples [71,72]. Several studies have reported that HBV cccDNA can be methylated to various extents, resulting in repression of transcription, decreased viremia and loss of hepatitis B e antigen (HBeAg)[71,73,74]. Thus, the methylation pattern of cccDNA is a crucial component of viral replication and may affect pathogenesis.

HBV cccDNA has three predicted CpG islands which are strategically located in the regulatory elements of the viral genome[75]. The first CpG island (CpG I) overlaps the ATG start site of the sequence encoding the small HBsAg, the second (CpG II) overlaps

the enhancer I and II, the core promoter, and HBx promoter sequence, and the third (CpG III) spans the SpI promoter and ATG start codon of the polymerase gene[72,76]. Among the 10 distinct HBV genotypes (A-J), CpG I is present in five genotypes (A, B, D, E, and I) whereas CpG II and III have been shown to exist in all genotypes[76]. Varying degrees of methylation have been reported to occur at the three CpG islands. In patients with CHB, methylation frequencies of 14, 0.6 and 3.7% within CpG I, II and III, respectively, were observed[77]. However, a separate computational study revealed that 50% of HBV sequences lacked CpG I, whereas CpG II and II were conserved across genotypes[76]. This suggests that methylation patterns are likely to differ between the genotypes. Vivekanandan *et al*[72] demonstrated that increased methylation of CpG I and II correlated with reduced viral protein production when analyzing HBV DNA samples isolated from CHB liver biopsies. In addition, the authors showed that patients with occult HBV infection had increased methylation at CpG II when compared to non-occult CHB individuals. A subsequent study compared the methylation status of CpG II in liver tissues from HBeAg+ and HBeAg- individuals [71]. Methylation was higher in HBeAg- samples (48%) compared to HBeAg+ samples (14%), indicating that an increase in methylation may reduce viral protein production. Similarly, a link between methylation of CpG III and lower HBsAg levels has been reported[74]. A cohort of cirrhosis patients failed to show an association between cccDNA methylation and HBsAg expression, but did indicate that higher methylation density was associated with lower viral load and virion productivity[73]. These results show the impact methylation can have on viral gene expression in CHB[78]. A recent comprehensive meta-analysis revealed significant hypermethylation of certain host genes depending on the geographical location of the population[79]. This included six genes in HBV-positive carcinoma tissues (*p16*, *RASSF1A*, *GSTP1*, *APC*, *p15* and *SFRP1*), two genes in HBV-positive carcinoma sera (*p16* and *APC*) and one gene in HBV-positive adjacent tissues (*GSTP1*). The study also indicated that DNA methylation could lead to the development of HBV-related HCC, an important consideration for epigenetic therapy.

The gene silencing effects of methylation have also been validated in different cell culture models of HBV replication. Transfection of HepG2 cells with methylated HBV DNA led to a reduction in viral mRNA levels, decreased HBV core antigen (HBcAg) and HBsAg expression, and reduced secretion of viral proteins[80]. *In vitro* infection experiments showed an increase in DNMT expression in response to HBV, which led to hypermethylation of the viral DNA, a reduction in viral mRNAs and proteins, and decreased HBV replication[81]. Furthermore, co-transfection of HBV DNA and DNMT3a was associated with decreased production of HBeAg and HBsAg[81].

### **Histone modifications and the cccDNA epigenome**

HBV cccDNA is associated with host histones, whose “bead-on-a-string” organization serves to compact the viral genome and provide a means for regulating gene expression[52]. As such transcription can be controlled by epigenetic modifications to the cccDNA-bound histones, thereby regulating HBV replication[82]. Nucleosome-associated histones undergo numerous types of PTMs that are generally reversible and mainly localized at the amino-terminal histone tails. These direct modifications of the N-terminal tails most commonly include acetylation, methylation, ubiquitination, phosphorylation, SUMOylation, ADP-ribosylation, and deamination[83]. Histone modifications can also indirectly regulate chromatin structure by serving as binding sites for the recruitment of other regulatory proteins[84]. Current studies have shown that PTMs significantly impact HBV replication, maturation and infection[85,86]. Several enzymes such as histone acetyltransferases (HATs) and deacetylases (HDACs), lysine and protein arginine methyltransferases (KMTs and PRMTs), demethylases (HDMTs), kinases, phosphatases, ubiquitin ligase, and deubiquitinases, modify cccDNA-associated histones[83,87]. The epigenome is further influenced by HBx and HBc, adding to the intricate and complex interplay between host and viral proteins on the cccDNA minichromosome (discussed later). Regulation of histone modifications has been proposed as a likely method to reduce cccDNA[8,52,57]. HATs facilitate the acetylation of lysine residues, making the histone-associated DNA more accessible to transcription factors, effectors and RNA polymerase II, to promote gene expression[88, 89]. Conversely, HDACs catalyze the removal of acetyl groups from lysine residues, leading to heterochromatin formation and gene repression[90,91]. Although less characterized, MBPs are reported to recruit HMTs and HDACs to promote histone methylation, which in turn recruits heterochromatin protein 1 factors (HP1) to promote DNA methylation[92-94].

HBV transcription is regulated by the acetylation status of cccDNA bound histones 3 and 4 (H3 and H4) both in cell culture models of viral replication[57,95] and in CHB patients[57]. Recruitment of HDAC1 and hypoacetylation of H3 and H4 is associated with low HBV replication and viremia *in vitro* and *in vivo*[57]. In this study, Pollicino *et al*[57] demonstrated that acetylation of cccDNA-bound H4 correlated with high levels of replication, an effect that was maintained when using HDAC inhibitors. Furthermore, the recruitment of the HATs, CREB-binding protein (CBP) and p300 as well as HDAC1 was associated with low HBV replication in an HBx-dependent manner. NIREF, an E3 ubiquitination ligase, has been shown to reduce acetylation of cccDNA-bound H3 as well as promote proteasomal degradation of HBc[96]. Gong and colleagues previously reported similar results for acetylation of cccDNA-bound H3, and identified roles for histone methylation and phosphorylation in controlling viral replication[85]. Surprisingly, HDAC inhibitors suppressed duck hepatitis B virus (DHBV) cccDNA transcription and reduced viral replication in a dose-dependent manner[97,98], suggesting cell and virus-specific epigenetic factors differ between the avian and human models[98].

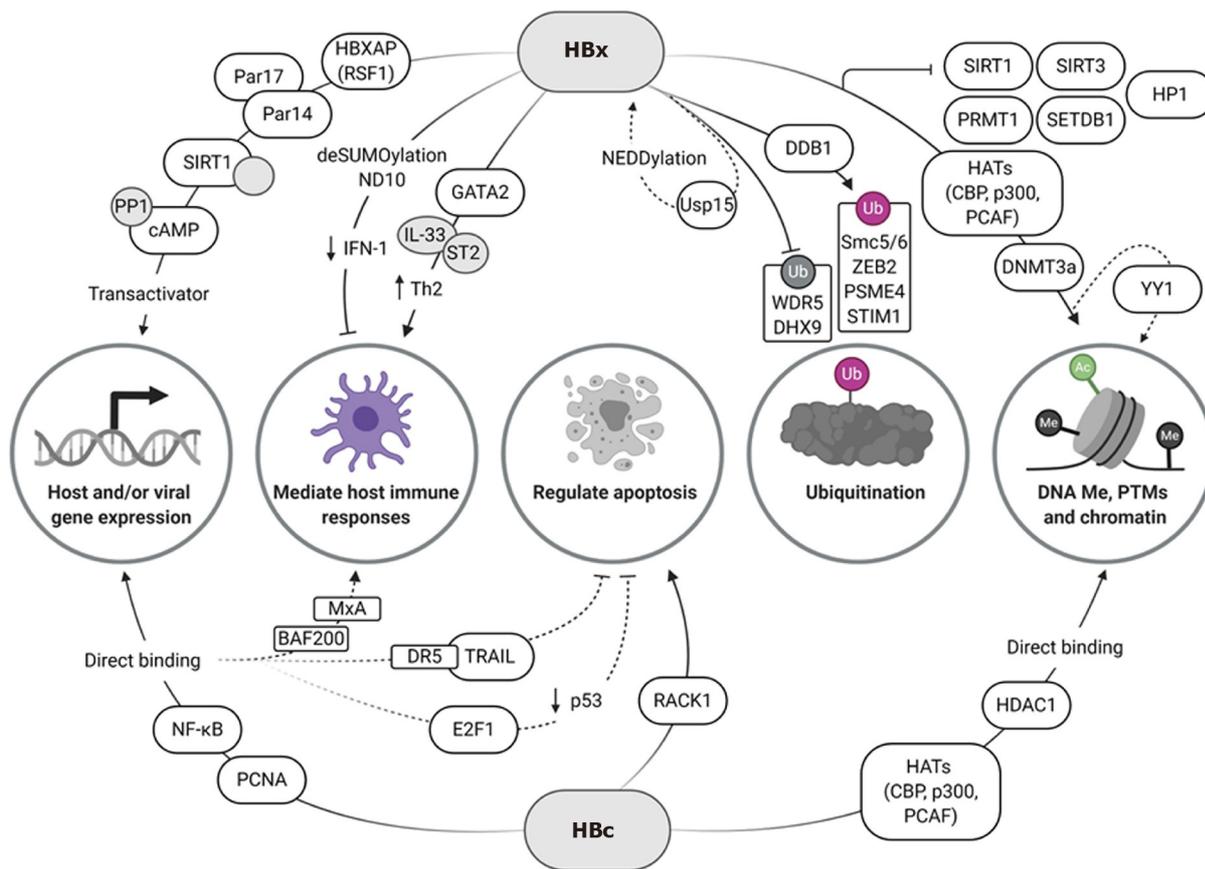
Genome wide studies of PTMs on the cccDNA minichromosome revealed high levels of trimethylation at lysine 4 of histone H3 (H3K4me3) and acetylation of lysine residues of histone H3 (H3K27ac and H3K122ac) at specific loci, which lead to gene activation or repression[86]. H3K4me3 modifications are crucial for active transcription of the sodium-taurocholate cotransporting polypeptide (NTCP) gene in HepG2 cells[99]. MLL3, a component of the ASCOM complex, is responsible for this modification and may indirectly facilitate HBV infection, given that NTCP is the hepatotropic receptor[97,99]. Knockdown of another methyltransferase, the enhancer of zeste homolog 2 (EZH2), resulted in upregulation of HBsAg and HBeAg production indicating that EZH2 represses HBV gene expression[89]. A recent study by Zhang *et al*[89] showed that PRMT5 induced symmetric dimethylation of arginine 3 on histone H4 (H4R3me2s) on cccDNA when associated with the hSWI/SNF chromatin remodeling complex and HBc. This epigenetic modification repressed viral transcription by inhibiting binding of RNA polymerase II. Interestingly, encapsidation of pregenomic RNA was also restricted in an epigenetically-independent manner, suggesting multiple antiviral roles for PRMT5[89].

## VIRAL FACTORS AS EPIGENETIC REGULATORS

Two viral proteins, HBx and HBc, have been identified as important modulators of HBV transcription which influence the cccDNA epigenome (Figure 1). HBx is a small 17 kDa oncogenic protein that has been shown to interact with multiple host factors to manipulate both host and viral gene expression, mediate host immune responses, and control apoptosis[100]. Although HBx lacks DNA-binding motifs and does not directly bind to the cccDNA minichromosome, it is able to act as a trans-activator of viral replication by recruiting other factors to the minichromosome[101]. HBc is a 21 kDa structural polypeptide that forms the viral nucleocapsid and is essential for reverse transcription of the pgRNA[102]. As such, HBc is important for the packaging and secretion of new virions during HBV replication. Interestingly, it also forms part of the cccDNA minichromosome and is involved in its epigenetic regulation[8,57]. Unlike HBx, the HBc C-terminal domain (CTD) is capable of binding directly to cccDNA[103] as well as host gene promoter regions[104]. As such, HBc binding affects minichromosome organization as it can adjust the number and spacing of cccDNA-associated nucleosomes[57,105,106].

### ***HBx acts as a ‘master regulator’***

HBx has been shown to recruit a variety of host transcription factors and co-activators to regulate cccDNA transcription (Figure 1). These include ATF/CREB, ATF3, c/EBP, NF-κB, IL-6, Ets, Egr, SMAD4, Oct1, RXR receptor, and p53[82]. HBx-associated protein HBXAP (RSF1) has been shown to bind to HBx *via* its plant homology domain, and acts as a co-activator of viral transcription[107]. Recently, Saeed *et al*[108] demonstrated that HBx can directly bind to parvulin 14 (Par14) and 17 (Par17), which increased the stability of HBx and mediated its translocation to the nucleus and mitochondria. In addition, binding of Par14 and Par17 to the cccDNA at enhancer and promoter regions resulted in upregulation of viral transcription, an effect that was abrogated in the absence of HBx[108]. HBx has also been shown to recruit, block and promote degradation of host antiviral proteins that would normally prevent HBV replication and cccDNA persistence, as well as activate host genes to enhance viral



**Figure 1** Hepatitis B virus proteins X and core act on multiple host cell pathways to promote viral persistence. An intricate epigenetic regulatory network is established during Hepatitis B virus infection. Hepatitis B virus proteins X and core contribute to this by either directly or indirectly controlling gene expression, modifying chromatin structure or location, mediating host immune responses, regulating apoptosis, and promoting or preventing ubiquitination. Pathways discussed in 'Viral factors as epigenetic regulators' are depicted here. Created with BioRender.com. HBx: Hepatitis B virus X protein; HBc: Hepatitis B virus core protein; IFN: Interferon; IL: Interleukin; NF- $\kappa$ B: nuclear factor-kappa B; PTM: Post-translational modifications; SIRT1: Sirtuin 1; YY1: Yin-Yang 1; HATs: Histone acetyltransferases; HDAC: Histone deacetylase; PCNA: Proliferating cell nuclear antigen; HBXAP: HBx-associated protein; RSF1: Remodeling and spacing factor 1; Par14: Parvulin 14; Par17: Parvulin 17; PP1: Protein phosphatase 1; cAMP: Cyclic adenosine monophosphate; ND10: Nuclear domain 10; GATA2: GATA binding protein 2; ST2: Interleukin 1 receptor-like 1; Th2: T helper cell type 2; Usp15: Ubiquitin-specific peptidase 15; DDB1: Damaged DNA-binding protein 1; Smc5/6: Structural maintenance of chromosomes 5/6; ZEB2: Zinc finger E-box-binding homeobox 2; PSME4: Proteasome activator subunit 4; STIM1: Stromal interaction molecule 1; WDR5: tryptophan-aspartic acid (WD) repeat domain 5 protein; DHX9: DEXH-box RNA helicase 9; Ub: Ubiquitination; SIRT3: Sirtuin 3; PRMT1: Protein arginine methyltransferase 1; SETDB1: SET domain bifurcated histone lysine methyltransferase 1; HP1: Heterochromatin protein 1 factors; CBP: CREB-binding protein; PCAF: p300/CBP-associated factor; DNMT3a: DNA methyltransferase 3a; MxA: Myxovirus resistance gene A; DR5: Death receptor 5; TRAIL: Tumor necrosis factor-related apoptosis-inducing ligand; E2F1: E2F transcription factor 1; RACK1: Receptor for activated protein kinase C 1.

gene expression.

Initiation of viral replication and stabilization of the cccDNA minichromosome is thought to occur through HBx-mediated PTMs of histones associated with hypomethylated CpG islands (mainly GpG I and III)[74,109]. Recruitment of HATs such as CBP, p300, and the p300/CBP-associated factor (PCAF) to the cccDNA minichromosome allows for upregulation of viral transcription[105]. In the presence of an HBx mutant, Belloni *et al*[105] showed increased recruitment of HDAC1 and sirtuin 1 (SIRT1) to the cccDNA, which correlated with a reduction in acetylation of cccDNA-bound H4 and viral transcription. These results suggest a role for HBx in promoting cccDNA histone acetylation to enhance viral transcription. However, the involvement of SIRT1 in the promotion or suppression of HBV replication appears to vary. Other studies found that SIRT1 positively regulated HBV replication by deacetylating PGC-1 $\alpha$  and FXR $\alpha$ [110], and promoting binding of activator protein 1 to the core promoter [111]. However, these studies did not investigate the role of SIRT1 in cccDNA-dependent models of viral replication. More recently Deng *et al*[112] showed that in HepG2-NTCP cells, SIRT1 complexes with and stabilizes HBx, and promoted the recruitment of HBx and other co-activating factors to cccDNA. This increased viral transcription in a deacetylase-independent manner. These results suggest a dual role for SIRT1 in the regulation of HBV replication, which may be dependent on the presence or absence of HBx.

Binding of HBx to PRMT1 inhibited the methyltransferase activity of this enzyme, ultimately enhancing viral replication[113]. Recently, HBx was found to inhibit SIRT3 expression by downregulating its expression and possibly inhibiting its recruitment to the cccDNA[114]. SIRT3 promotes cccDNA-histone methylation (H3K9me3) by directly removing H3K9ac marks and recruiting histone methyltransferase SUV39H1 and SETD1A to the cccDNA[114]. However, in the presence of HBx, H3K9me3 marks were reduced and H3K4me3 and H3K9ac modifications increased to promote HBV transcription. This effect has also been observed for other transcriptional repressors. HBx reduced the chromatin structure alteration-mediated repression of histone-lysine N-methyltransferase SET domain, bifurcate 1 (SETDB1) and HP1[93]; however, the exact molecular mechanisms of this rescue remain to be elucidated. Upregulation of DNMTs by HBx and direct interaction of HBx with DNMT3A has been shown to either upregulate transcription of host genes, or block activity by facilitating host promoter methylation[115]. For example, SOCS-1, SUFU and TIRAP are downregulated, and this could be important in mediating an immune response in CHB, albeit the exact mechanisms still need to be elucidated[81,116]. HBx-mediated downregulation of tumor suppressors may be a factor leading to the development of HCC[115,117-119]. HBx-mediated deSUMOylation removes transcription factors from nuclear domain 10 (ND10), which in turn downregulates IFN-I-response pathway elements and host epigenetic modifications by p300/HDAC1, resulting in viral persistence[120]. Host gene promoters and enhancers can also be stimulated by HBx to regulate HBV replication[121-123]. Upregulation of host *ST2* expression by HBx-GATA2 binding to its promoter and subsequent induction of the IL-33/*ST2* axis stimulated a Th2 immune response, instead of the desired antiviral Th1 response[124], which is likely to play a role in the chronicity of infection[125].

As stated before, CREB is recruited by HBx to the cccDNA minichromosome; however, HBx also increases this activator's longevity[126]. By binding to protein phosphatase 1, the activating phosphorylation induced by cAMP has a longer half-life, resulting in increased viral transcription[126]. Interestingly, not only does HBx mediate recruitment of factors directly to the cccDNA, but also promotes spatial localization of the minichromosome. Shen *et al*[127] showed that HBx and the transcription factor Yin-Yang 1 (YY1) aligned the cccDNA minichromosome with a region of the human genome rich in a highly active enhancer element, resulting in increased HBV replication.

Negative regulation by HBx has also been shown through an interaction with peroxiredoxin 1 (Prdx1), a cellular hydrogen peroxide scavenger, which recruits exosome component 5 (Exosc5) to degrade HBV RNA. Although the ability of Prdx1 to bind to HBV RNA was independent of HBx, HBx was required for degradation of the RNA[128].

### **HBx-mediated ubiquitination**

Indirect epigenetic regulation can also occur through HBx-mediated proteasomal degradation of host factors, or by preventing degradation or deactivation of host factors. Damaged DNA-binding protein 1 (DDB1), an adapter protein for the cullin-RING ligase 4 (CRL4) E3 Ligase complex, is responsible for recruitment of DDB1-Cul4-associated factors (DCAFs), which allow for ubiquitination and subsequent degradation of proteins[129]. Interactions between HBx and DDB1 were implicated in early studies[130]. The interaction is mediated by a motif in HBx that mimics the DCAF proteins' binding motif, allowing HBV to hijack the host protein ubiquitination system[131]. Years later, Decorsière *et al*[132] and Murphy *et al*[133] identified the structural maintenance of chromosomes 5/6 (Smc5/6) complex as a target for HBx-mediated proteasomal degradation. The Smc5/6 complex is a host antiviral restriction factor that enables transcriptional repression of the cccDNA minichromosome, by localizing to the ND10 without inducing an innate immune response[134]. However, the epigenetic effect is transient as it does not silence HBx mRNA expression, ultimately leading to Smc5/6 degradation and initiation of viral transcription[135]. Like Smc5/6, other HBV restriction factors are degraded by HBx-mediated polyubiquitination, to increase viral transcription and HBV replication[136]. This includes ZEB2, which inhibits HBV replication by interaction with the core promoter[137], proteasome activator subunit 4 (PSME4), which induces degradation of acetylated histones involved in upregulation of HBV[138,139], and stromal interaction molecule 1 (STIM1), known to regulate cytoplasmic calcium levels[140].

On the other hand, HBx is also able to inhibit proteasomal degradation[141]. This has recently been shown for the WD repeat domain 5 protein (WDR5), a core subunit of H3K4 methyltransferase complexes[142]. HBx stabilized WDR5 by preventing DDB1/cullin-4-induced degradation and in turn, promoted viral gene expression

[142]. Shen *et al*[143] demonstrated how HBx could also inhibit MDM2-mediated degradation of DExH-box RNA helicase 9 (DHX9) to enhance viral DNA synthesis.

The epigenetic regulation of viral transcription is further enhanced as a result of HBx-mediated *trans*-activation of its own promoter[144]. Along with this, post-translational modifications of HBx through interactions with the deubiquitinating ubiquitin-specific peptidase 15 (Usp15) enzyme, increase the half-life of the viral protein[145]. This may also be a result of the close interaction of HBx with components of the proteasome machinery, such as the DDB1-CRL4 complex, which may inadvertently result in ubiquitination of HBx itself. HDM2-mediated NEDDylation of HBx was also found to occur, probably because of its close proximity to Cul4, resulting in increased stability and chromatin localization[101,146]. NEDDylation-dependent reduction of ubiquitination and subsequent degradation of HBx by E3 Ligases, such as Siah-1, may account for the increased stability of this viral protein. MLN4924 (pevonedistat), a NEDD8-activating enzyme inhibitor, has been shown to impede HBV replication by reducing cullin[147] and HBx NEDDylation[146]. In addition, MLN4924 promotes upregulation of phosphorylated extracellular signal-regulated kinases (ERKs) resulting in reduced HNF1 $\alpha$ , HNF4 $\alpha$  and C/EBP $\alpha$  transcription factor levels[147], which are known activators of HBV transcription. Overall, the maintenance of persistent HBV infection by HBx contributes to HCC, by inducing host epigenetic modifications implicated in cancer.

### **HBc associates with cccDNA and host genes**

Direct interaction of the HBc CTD[103] with CpG islands across the cccDNA, especially CpG II, has been shown to induce hypomethylation and subsequent upregulation of viral transcription and regulation[74,148]. This hypomethylation causes increased CBP binding and resulting histone acetylation, further increasing viral gene expression[57,148] albeit at lower levels than with HBx[149,150]. The association of HBc with HDAC1 was also shown[148], as well as P300 and PCAF/CBP [151]. Increased NF- $\kappa$ B binding upstream of *ENII* induced by HBc is thought to increase pre-C promoter activity[152]. Host DNA polymerase coordinator PCNA, which is known to maintain genetic and epigenetic integrity[153], is recruited to cccDNA by HBc which upregulates viral gene expression and plays a role in the development of HCC. However, the exact mechanism of this process is still unclear [154].

HBc can additionally mediate host gene expression through the binding of HBc to endogenous promoter regions. Guo *et al*[104] identified nearly 3100 host promoter regions with potential HBc binding sites. Examples of these include binding to the MxA promoter to evade a host IFN-induced immune response[155] and competitive binding to BAF200, resulting in the downregulation of interferon-induced transmembrane protein 1 (IFITM1) mRNA[156]. Binding of HBc to the death receptor 5 (DR5) promoter reduced DR5 expression, thereby reducing cellular apoptosis through TRAIL which is thought to support CHB infection[157].

Transcription factor binding to mediate host expression has also been shown. The binding of HBc to E2F1 prevented its natural association with the p53 promoter, and hence decreased p53 levels[158]. This could reduce p53-related apoptosis[159] and along with DR5 downregulation further support the development of chronic infection. However, competitive binding of HBc to the receptor for activated protein kinase C 1 (RACK1) prevented phosphorylation of mitogen-activated protein kinase 7 (MKK7) which sensitized cells to apoptosis[160]. This suggests a dual role for HBc in the control of apoptosis and requires further study to determine the impact of these pathways on HBV infection. The subcellular location of HBc is dependent on cell cycle phase, with *in vitro* studies showing that predominantly nuclear localization occurs during G1 phase[161]. Since E2F transcription factors and p53 both have effects on cell cycling[162-164] and are differentially regulated during HBV infection, further investigation of this phenomenon and the possibility of epigenetically controlling the cell cycle is needed. HBc has also been shown to recruit APOBEC3A/B to cccDNA to promote deamination[165]. Since so many HBc binding sites are predicted to occur in endogenous promoter regions, there is the possibility that APOBEC3A/B is recruited to host genes to influence their expression, and may play a role in the development of HCC[166].

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## **REGULATORY NONCODING RNAs**

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Noncoding RNAs (ncRNAs) consist of small ncRNAs (< 200 nt) and long ncRNA

(lncRNA > 200 nt)[167]. Small ncRNAs, including miRNAs, are involved in post-transcriptional gene silencing[167], while lncRNA are implicated in a plethora of host functions including post-transcriptional and chromatin modifications[168]. By altering the host's epigenetic signature, HBV dysregulates the ncRNA landscape to control viral replication and influence hepatocarcinogenesis.

### **Epigenetic influences of miRNAs**

miRNAs have been widely studied in HBV infection, and have been shown to facilitate or inhibit viral replication[169]. An increase in replication can occur by targeting host factors that normally restrict viral replication, for example miR-15b downregulates HNF-1 $\alpha$ [170], or by indirectly upregulating factors to promote replication, such as miR-1 upregulation of farnesoid X receptor  $\alpha$  (FRX $\alpha$ ) expression[171]. Interestingly, miR-1-mediated downregulation of E2F5 and HDAC4 was implicated in G1 cell cycle arrest and upregulation of hepatocyte differentiation factors, to increase HBV replication[171]. This may be related to other viral epigenetic factors such as HBc, which showed similar associations. Indirect FRX $\alpha$  upregulation by miR-449a targeting of CREB5[172], HDAC4 downregulation by miR-548ah[173] and G1 arrest by miR-125b-5p-mediated inhibition of retinoblastoma protein phosphorylation[174] implicate redundant epigenetic pathways in HBV regulation.

A recent study by Moon *et al*[175] has suggested that miR-20a can act in an epigenetic manner and promote methylation of cccDNA. They found that AGO2-miR-20a binding to the cccDNA may recruit DNMT3 to silence gene expression; however, further studies are needed to confirm the exact mechanism. Interestingly miR-146a could play a role in cccDNA formation, through a positive feedback loop with FEN1 [176], and in silencing ZEB2 expression[177].

Induction of autophagy has been associated with many miRNAs including miR-146a-5p[178], the miR-99 family[179] and miR-192-3p, which is downregulated by HBx [180]. miR-155 has also been implicated in autophagy; however, this miRNA is downregulated following HBV infection[181]. HBV upregulates expression of miR-192-5p and miR-215-5p, which results in downregulation of apoptosis[182]. Contrasting effects were observed during HBV-mediated upregulation of miR-194-5p, which resulted in the downregulation of anti-apoptotic proteins SODD and cFLIP and sensitization of liver cells to apoptosis[182]. Once again, the role of HBV in promoting and preventing apoptosis needs further clarification.

Certain cancer-related miRNAs, namely miR-15a/miR-16-1, the miR-17-92 cluster and miR-224, are also associated with decreased viral replication through modification of cccDNA promoters and histones[183]. Interestingly, miR-122, which targets the HBV polymerase ORF and *core* 3'UTR[184], has also been shown to inhibit HBV by targeting cyclin G1 to increase p53-mediated inhibition of replication[185]. However, HBx abrogates these antiviral effects by downregulating miR-122[183]. Host miRNAs can also indirectly inhibit viral replication by regulating various host factors[169] HBx-mediated downregulation of miR-122 alters regulation of its natural host targets, including heme oxygenase-1[186], CCNG1 and NDRG3[187]. Similarly, miR-141 was implicated in targeting both PPARA[188] and SIRT1[189]. Reduced SIRT1 inhibited autophagy, and miR-130a targets liver pyruvate kinase which is thought to reduce energy supply and hence HBV replication[190]. Induction of immune suppression may occur through the increase of miR-199a-5p, miR-221-3p and Let-7a-3p, as shown in immune tolerant HBV-infected patients[191]. HBV-miR-3, a miRNA produced by the virus, reduces replication by directly targeting viral transcripts[192] or upregulating the anti-HBV IFN immune response[193].

### **Newly discovered lncRNAs implicated in the control of HBV**

lncRNAs have been identified as important polyfunctional epigenetic regulators and are associated with disease progression, particularly carcinogenesis[194]. Novel lncRNAs related to development of HBV-associated HCC are continuously being identified; however, a few have also been implicated in epigenetic regulation of viral transcription. lncRNAs highly up-regulated in liver cancer (HULC), DLEU2, and lncRNA 32 have been identified as epigenetic regulators of HBV. HAT1, which is co-activated by HBx-Sp1 binding, is transported to the cccDNA by HULC in an HBc-dependent manner[195]. HULC also mediates upregulation of HBx and the subsequent HBx-STAT upregulation of miR-539, which targets APOBEC3B. This results in reduced cccDNA degradation, with resultant increased transcription of viral genes, viral persistence, and HCC progression[196]. Interestingly, the promotion of HBV transcription by the lncRNA HULC is dependent on both HBx and HBc, which forms a positive feedback loop[195]. In the case of lncRNA DLEU, HBx both upregulates its expression and is co-recruited with it to the cccDNA, where the

complex may bind to the histone methyltransferase EZH2[197]. Computational analysis of this interaction suggests that binding alleviates EZH2 transcriptional repression[197]. LncRNAs have also been implicated in the host antiviral response. To increase IFN-stimulated gene (*ISG*) expression, lncRNA 32 binds to activating transcription factor 2 (ATF 2) and promotes gene expression. However, during HBV infection, the expression of this lncRNA is reduced[198], ultimately dampening the immune response to the virus.

## ANTI-HBV EPIGENETIC THERAPY

A number of epigenetic strategies has been identified as having therapeutic potential for CHB[27]. Some promote the host's natural antiviral defense mechanisms while others aim to target specific pathways involved in the epigenetic control of cccDNA.

### *IFN- $\alpha$ therapy*

The antiviral and epigenetic properties of IFN- $\alpha$  therapy are well established[199], and have been adopted as a strategy to treat CHB[5]. Type 1 IFNs interact with the IFN- $\alpha/\beta$  receptor complex to modulate transcription of ISGs, ultimately to evoke strong innate immune responses against viral infections[200]. Administration of IFN- $\alpha$  to HBV-infected cells and HBV-infected chimeric uPA/SCID mice resulted in the inhibition of viral replication through epigenetic regulation of cccDNA[105]. Hypoacetylation of cccDNA-bound histones was achieved following the active recruitment of histone deacetylases hSIRT1 and HDAC1, methyltransferase EZH2, and the transcriptional repressor YY1[199]. Binding of transcription factors STAT1 and STAT2 to the IFN- $\alpha$  sensitive response element on active cccDNA was shown to be reduced after IFN- $\alpha$  treatment[199]. More recently, the structural maintenance of chromosomes flexible hinge domain containing 1 (SMCHD1) and promyelocytic leukemia protein have, along with STAT1, been implicated in the stimulation of cccDNA-associated histone PTMs in response to IFN- $\alpha$ [201]. Furthermore, IFN- $\alpha$ -based treatment may regulate the SMC5/6 complex to promote transcriptional repression of cccDNA[202].

When comparing IFN- $\alpha$  treatment to the effects of C646 (a small molecule epigenetic modifying agent that inhibits p300/CBP), Tropberger *et al*[86] demonstrated that C646 reduces HBV transcription in primary human hepatocytes in a dose-dependent manner. Like IFN- $\alpha$ , C646 treatment reduced the levels of active PTMs but without activating the innate immune response. However, neither treatment reduced cccDNA levels[86]. When combined with lymphotoxin- $\beta$  receptor activation, IFN- $\alpha$  treatment did eliminate cccDNA in an APOBEC-dependent manner[165]. Yet the clinical utility of currently licensed IFN- $\alpha$ -based treatments has proved tricky because some patients respond to therapy, but others show no benefit. High costs and adverse side effects have also limited the use of IFN- $\alpha$ . Alternatives are being investigated and an iterative approach has led to the identification of new IFNs with increased potency, such as the recently described IFN- $\alpha$  14[203].

### *Small molecule inhibitors and epigenetic drugs*

Epigenetic therapies and immunomodulators have shown promise as anti-HCC agents [204], of which HDAC inhibitors such as 5-Azacytidine (5-Aza) and the EZH2 inhibitor 3-Deazaneplanocin A (DZNep) have recently been explored as combination therapies [205]. Such therapies have also been proposed for the treatment of CHB to silence the cccDNA minichromosome. Two small molecule inhibitors, AGK2 and GS-5801, are currently being investigated as anti-HBV epigenetic drugs[206,207]. AGK2 is an inhibitor of the SIRT2 deacetylase, while GS-5801 prevents erasing of epigenetic marks by lysine demethylase 5 (KDM5). Both have been shown to repress viral replication; however, preliminary results from a single dose of GS-5801 suggest that H3K4me3 may not be permanently associated with cccDNA[207]. Recently Dicoumarol, an inhibitor of NAD(P)H:quinone oxidoreductase 1 (NQO1), has been shown to promote the silencing of cccDNA[208] indirectly. The epigenetic effect of Dicoumarol is thought to arise from the destabilization of the HBx/NQO1 interaction, as the upregulation of NQO1 was shown to increase the half-life of HBx[208]. Although promising, the feasibility of using epigenetic modulators to silence HBV is complex, especially as the virus employs many normal host factors to regulate gene expression. The influence on development of HBV-related HCC and viral integration are additional important considerations for advancing epigenetic therapy. For instance, treating HCC with HDAC inhibitors is likely to promote viral transcription[57], while epigenetic silencing

cccDNA gene expression by increasing DNA methylation may promote hepatocarcinogenesis[209].

### **Targeted epigenetic gene silencing**

Epigenome engineering has become a popular targeted gene therapy approach to promote locus-specific epigenetic changes to treat genetic diseases[24]. Effector domains based on a variety of transcriptional activators, repressors, and chromatin remodeling enzymes including the typical writers, readers and erasers (Table 1) have been fused to DNA binding domains (DBD) to promote site-specific modifications. The targeting capabilities of epigenome engineering tools could overcome the non-specific effects epigenetic drugs may have on host gene expression. As such, cccDNA epigenome editors are currently being developed as a novel antiviral gene therapy for CHB (Figure 2).

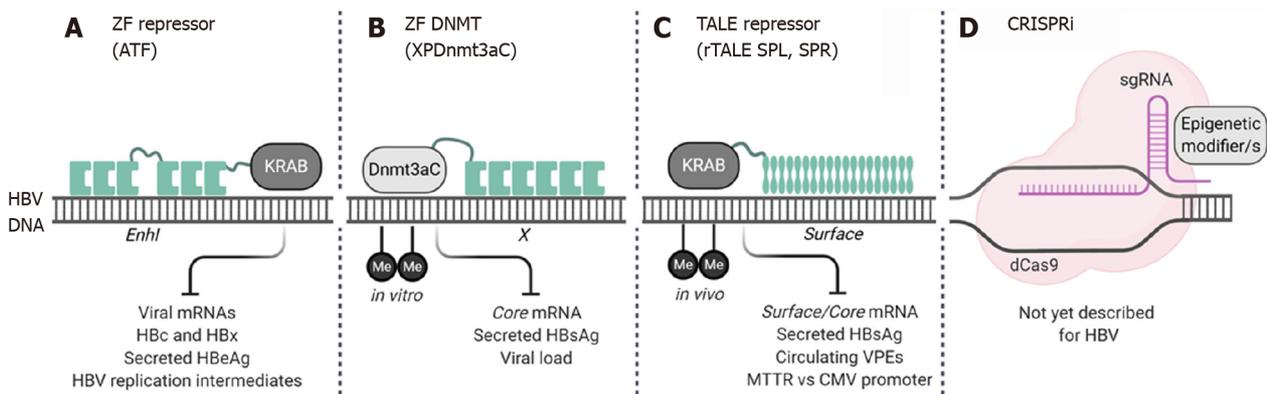
ZF and transcription activator-like effector (TALE) DBDs have been used to direct different epigenetic effectors, primarily transcriptional repressors, to the HBV genome [210-213]. In 2013, Zhao *et al*[210] described the first ATF which was designed to target an 18 bp region of the HBV X or enhancer I (EnhI) sequence. The ATF was generated by joining six ZF DBDs to the Krüppel-associated box (KRAB) repression domain. Although not tested in a typical HBV replication model, the ATF achieved repression of the HBx promoter in a Hep3B reporter cell line, although accompanying cellular growth arrest was also observed[210]. Building on this study, Luo and colleagues demonstrated the antiviral activity of their EnhI-specific ATF in HepG2.2.15 cells and transgenic mice[211]. Viral replication was inhibited, in this case without affecting cellular growth, despite both models harboring integrated HBV sequences. Interestingly, the EnhI ZF-array alone achieved transcriptional repression, an effect that has previously been reported when using anti-HBV TALENs[214]. The antiviral effect of the ATF was further confirmed in the HBV transgenic mouse model where reductions in viral DNA, HBcAg and HBeAg were observed[211]. By replacing the repressor domain with a methyltransferase, ZF arrays have also been used to achieve targeted methylation of viral DNA. Xirong *et al*[213] fused the C-terminal region of the DNMT3A to a six-finger ZF (XPDnmt3aC) and tested the epigenome editor in HepG2 cells and HBV transgenic mice. The XPDnmt3aC was designed to bind to the HBx promoter region and achieved *de novo* methylation of the HBV DNA in HepG2 cells which was accompanied by a reduction in markers of viral replication. However, in transgenic mice the antiviral effect lasted for about two weeks[213], suggesting that if epigenetic modification was achieved it was not permanent.

Anti-HBV repressor TALEs (rTALEs) are engineered proteins with DBDs derived from the *Xanthomonas* bacteria which have been fused to the KRAB effector domain [212]. Two rTALEs, SPL and SPR, which were designed to bind to the *surface* ORF were tested in both *in vitro* and *in vivo* models of viral replication. Both the SPL and SPR rTALEs inhibited HBV replication, and significant reductions in HBsAg, viral mRNAs, and circulating viral particles were observed[212]. Importantly, a comparison between the liver specific mouse transthyretin (MTTR) or ubiquitously active CMV promoters was performed. The MTTR conferred liver-specific expression which may improve the safety of this epigenome engineering approach. Quantitative DNA methylation analysis using EpiTYPER™ Technology[215] was used to show that increased targeted methylation of intrahepatic HBV DNA was achieved at CpG II [212]. This confirmed the principle of this epigenome engineering approach; however, further validation of these rTALEs on cccDNA is warranted.

Interestingly, despite widespread studies using CRISPR/Cas gene editing technologies to disrupt cccDNA, the alternative epigenome editing platforms have yet to be investigated. CRISPRi and CRISPRa are RNA-guided epigenome modifiers that can repress or promote gene expression, and are generated by fusing single or multiple effector domains to a dead (nuclease deficient) Cas[216]. This means that site-directed epigenetic modifications can be achieved by designing guide RNAs to bind to the cccDNA. Although not yet reported, the field of CRISPR epigenome engineering is constantly expanding and may present a novel way of silencing cccDNA.

## **CONCLUSION**

The epigenetic regulation of the cccDNA minichromosome to either promote or repress viral transcription involves an intricate association between host cell factors and viral proteins that is not yet fully understood. While there is currently debate around whether HBV should be considered a stealth virus[217], it is clear that it can



**Figure 2 Novel epigenome engineering tools that target hepatitis B virus DNA.** Three different types of epigenetic editors have been designed to silence viral gene expression. The Zinc finger (ZF) repressor-based artificial transcription factors (ATF) comprise two three-finger ZF DNA binding domains (DBD) which are fused, with a C-terminal linker peptide, to the Krüppel-associated box (KRAB) repressor domain. ATFs were designed to target the *EnhI* region (A). A ZF-based methyltransferase (DNMT) designed to target the *X* region (XPDnmt3aC). The XPDnmt3aC comprises a six-finger ZF DBD fused to the catalytic domain of Dnmt3a (Dnmt3aC). Methylation of the viral DNA was achieved *in vitro* (B). To generate transcription activator-like effector (TALE) repressors, TALE DBDs targeting two regions of *surface* were fused to the KRAB repressor domain (N-terminal). *In vivo*, methylation of intrahepatic viral DNA was shown (C). Although not yet described for HBV, the CRISPRi epigenome editing tools provide a novel means of silencing covalently closed circular DNA. RNA guides (sgRNA) targeting the viral genome could be used to recruit a dead Cas (dCas9) with one or more epigenetic modifying domains (D). Created with BioRender.com. HBV: Hepatitis B virus; ZF: Zinc finger; ATF: Artificial transcription factor; HBx: Hepatitis B virus X protein; HBc: Hepatitis B virus core protein; HBeAg: Hepatitis B e antigen; HBsAg: Hepatitis B surface antigen; TALE: Transcription activator-like effector; MTTR: Mouse transthyretin; CMV: Cytomegalovirus; VPE: Viral particle equivalent.

manipulate the host cells replication, transcription and translation machinery for its own benefit. HBx appears to have a key role in viral persistence, not only as a regulator of cccDNA but also as a modulator of RNAs, ubiquitination, apoptosis, and antiviral responses. Although these intricate host-viral pathways require further clarification, the research to date has helped to identify possible feedback loops which could act as new targets for anti-HBV therapy.

There are conflicting views regarding the involvement of certain proteins, for example the epigenetic and deacetylase-independent roles of SIRT1. While some factors may have dual roles, the model systems used to evaluate epigenetic regulation of HBV need to be considered carefully. Although CHB is associated with HCC, epigenetic therapies designed to achieve a functional cure are likely to be given prior to the development of cancer. Using different liver cancer cell lines may skew the HBV epigenetic landscape as regulation of the host cells' epigenome and gene expression is irregular. Establishing an ideal model system is however, easier said than done. Infectious primary human hepatocyte-based models require specialized facilities, as for example is required for the generation of humanized mouse models[218]. Similarly, studies on primary human hepatocytes and three-dimensional liver cultures are costly. Viruses infecting small animals, like the DHBV and woodchuck hepatitis virus, which are commonly used to test novel therapies, may also pose problems for analysis of the cccDNA epigenome. The different effects that HDAC inhibitors had on HBV and DHBV infection suggest differences between the hosts' endogenous epigenetic pathways. Standardizing cccDNA quantification using droplet digital polymerase chain reaction and the liver biopsy-adapted chromatin immunoprecipitation (ChIP)-quantitative polymerase chain reaction technique (micro-ChIP) may help detect low levels of cccDNA as well as measure epigenetic marks[219].

Epigenetic drugs, improved interferon therapies, and epigenome editing tools are at an interesting stage of development, as new ways to silence cccDNA transcription. Accomplishing targeted epigenetic modifications and long-term viral suppression will be important, and consideration of the HCC epigenetic profile will need careful evaluation. Integrated viral sequences may also be amenable to cccDNA-specific epigenome editing, and undesirable off-target effects on the host genome need to be carefully assessed. Next-generation sequencing platforms like RNA-Seq would help to establish transcriptome profiles and identify potential off-target sites. To date only four targeted epigenome editors have been investigated, and although action on HBV DNA has been shown, a direct effect on the cccDNA minichromosome is yet to be established. Furthermore, as with designer nucleases, liver-specific delivery[19] and immune responses to the foreign TALE and Cas proteins will need to be addressed [220]. Delivering epigenome editors as *in vitro* transcribed mRNA transcripts would avoid the packaging constraints of viral vectors[10]. Despite the current challenges

associated with epigenetic therapies, there is potential for such an approach to achieve cccDNA gene silencing and perhaps cure. As this relatively new field of research continues to grow, overcoming the hurdles could lead to a promising class of new antivirals.

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