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Perspective

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Unfolding HBx for an epigenetic switch of HBV cccDNA minichromosome

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Hepatitis B virus (HBV) infection remains a worldwide health problem and is one of the major pathological factors for hepatitis, cirrhosis, hepatocellular carcinoma, and liver failure. Upon infection, HBV establishes a pool of covalently closed circular (ccc) DNA in the nucleus of infected hepatocytes, where it forms a minichromosome that exhibits resistance to antiviral therapies (Bock et al., 2001; Nassal, 2015). Notably, the cccDNA is typically silenced by host restriction factors and heterochromatin formation through an epigenetic restriction mechanism (Tsai and Cullen, 2020; Vivekanandan et al., 2010). The HBV-encoded protein X (HBx) can overcome this repression to initiate productive infections by interacting with multiple host proteins (Chuang et al., 2022; Van Damme et al., 2021). Intriguingly, the N-terminal motif of HBx (amino acids 2–21, the first Met residue is removed according to the N-terminal Met excision rule) (Giglione et al., 2004) is recognized as one of the most conserved regions among mammalian hepadnaviral genomes and demonstrates transrepressor activity by inhibiting the transactivation function of the C-terminal region ($HBx_{51–154}$) (Misra et al., 2004).

Emerging evidences suggest that epigenetic mechanisms are increasingly important strategies employed by both host cells and viruses to regulate their interplay. Recent studies have highlighted the involvement of host chromatin remodelers, chromatin-modifying enzymes, and transcription factors in modulating the epigenetic landscape of HBV cccDNA. For example, the recruitment of histone deacetylases (HDACs) to cccDNA-associated chromatin has been demonstrated to suppress HBV transcription and replication, whereas histone acetyltransferases (HATs) and methyltransferases have been implicated in enhancing cccDNA activity, thereby facilitating viral proliferation (Alarcon et al., 2016; Belloni et al., 2009). Additionally, it has been reported that heterochromatin protein 1 (HP1) and SET domain bifurcated histone lysine methyltransferase 1 (SETDB1)-mediated histone H3 lysine 9 trimethylation (H3K9me3) contribute to the silencing of HBV cccDNA transcription through modulation of chromatin structure, while HBx is capable of counteracting this repression, enabling the establishment of an active chromatin state (Rivière et al., 2015). Nonetheless, the precise molecular mechanisms underlying these processes remain to be fully elucidated.

Spindlin1 was initially identified as a highly expressed maternal protein in unfertilized mouse oocytes and two-cell stage embryos, where it plays a crucial regulatory role in oocyte maturation and early embryo development prior to zygotic genome activation (Oh et al., 1997, 1998; Staub et al., 2002). Recently, it is reported that the interaction between Spindlin1 and SPOC domain containing 1 (SPOCD1) is essential for spermatogenesis and piRNA-directed DNA methylation of young long interspersed nuclear element-1 (LINE1) elements (Dias Mirandela et al., 2024). Functional studies have demonstrated that Spindlin1 acts as an oncogene and is overexpressed in various cancer types, including gastric cancer, colorectal cancer, ovarian cancer, liposarcoma, and hepatocellular carcinoma (HCC) (Franz et al., 2015; Wang et al., 2012). Spindlin1 functions as a reader of multiple histone methylations via its tandem Tudor domains, of which the second Tudor domain specifically

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recognizes histone H3 lysine 4 trimethylation (H3K4me3) or histone H4 lysine 20 trimethylation (H4K20me3), while the first Tudor domain recognizes histone H3 arginine 8 asymmetric dimethylation (H3R8me2a) or H3K9me3 (Su et al., 2014; Wang et al., 2011, 2018; Yang et al., 2012; Zhao et al., 2020). Our previous research showed that the combinatorial readout of H3 "K4me3–R8me2a" or "K4me3-K9me3" methylation patterns by Spindlin1 significantly enhances binding affinity and promotes target gene expression with increased multivalency compared to single H3K4me3 mark readout (Ruthenburg et al., 2007; Su et al., 2014; Zhao et al., 2020). Recently, we and others reported that SPINDOC (a.k.a. C11orf84), an interactor of Spindlin1, modulates the transcriptional coactivator activity of Spindlin1 through direct interaction with its third Tudor domain (Du et al., 2021; Zhao et al., 2024). Notably, we discovered that HBx has evolved a conserved motif structurally similar to SPINDOC, which interacts directly with the third Tudor domain of Spindlin1 (Liu et al., 2023). Here we review how the histone methylation reader Spindlin1 is hijacked by HBV to facilitate its life cycle and contribute to liver pathogenesis in a manner dependent on HBx, involving damaged DNA binding protein 1 (DDB1), structural maintenance of chromosomes (SMC) 5/6 proteins, B cell lymphoma-2 (Bcl-2) protein, and H3 "K4me3–K9me3" readout. Understanding the intricate mechanisms underlying the epigenetic reprogramming of HBV cccDNA may provide potential new therapeutic avenues for the treatment of HBV-induced liver diseases.

The transactivation function of the C-terminal region of HBx

HBV is a partially double-stranded relaxed circular (rc) DNA virus that belongs to the Hepadnaviridae family. Upon infection, the rc-DNA is imported into the nucleus and converted into cccDNA, which acts as the template for the transcription of all viral RNAs, including the 3.5 kb pregenome (pg) RNA, preC mRNA, 2.4 kb preS mRNA, 2.1 kb S mRNA, and 0.7 kb HBx mRNA. The HBV genome comprises an overlapping region that encodes four canonical proteins named P, S, C, and X, encoding the DNA polymerase, HBsAg proteins, core and precore proteins, and the X protein (HBx), respectively.

HBx is a multifunctional protein composed of 154 amino acids (aa), characterized by an N-terminal negative regulatory region (residues 1–50) and a C-terminal transactivation domain (residues 51–154) (Fig. 1A). It plays a pivotal role in diverse cellular processes, including gene transcription, signaling pathways, protein degradation, cell-cycle regulation, proliferation, and apoptosis (Slagle and Bouchard, 2016). Notably, the N-terminal and C-terminal regions of HBx exhibit distinct functional properties in various biological processes, with the transactivation domain being particularly critical for the enhancement of HBV transcription and replication through its interaction with multiple cellular proteins (Kumar et al., 1996) (Fig. 1A). The best characterized HBx-binding partner is DDB1, an adaptor protein for the cellular Cullin 4-RING E3 (CRL4 E3) ubiquitin ligase enzyme complex (Li et al., 2010). HBx binds to DDB1 through the H-box motif (residues 88–100), which redirects the DDB1-contaning E3 ubiquitin ligase to degrade the SMC5/6 proteins, thereby relieving the HBV transcription inhibition by SMC5/6 to allow productive HBV gene expression (Decorsière et al., 2016; Mitra and Guo, 2016). The HBx-DDB1 complex has also been shown to target WD repeat domain 77 protein, leading to the disability of methyltransferase activity of protein arginine methyltransferase 5 (PRMT5) and downregulation of histone H4 arginine 3 symmetric dimethylation (H4R3me2s) on the cccDNA minichromosome to promote HBV replication (Yuan et al., 2021). HBx interacts with the anti-apoptotic proteins Bcl-2 and Bcl-xL via a Bcl-2 homology region 3 (BH3)-like motif (residues 110–135) within its transactivation domain, leading to an increase in cytosolic calcium levels, which is essential for HBV DNA replication (Geng et al., 2012; Jiang et al., 2016; Zhang et al., 2019). Moreover, HBx (residues 58–119) modulates cAMP-response element binding protein (CREB) -mediated transcription through its interaction with /p300 (Cougot et al., 2007). HBx (residues 102–136) interacts with the tumor suppressor tumor protein p53 (p53), thereby inhibiting p53-mediated DNA binding and transcriptional activity *in vivo* (Truant et al., 1995; Wang et al., 1994). Additionally, HBx is well-established in transactivating various host biological processes by interacting with a multitude of transcription factors, including activator protein-1/2 (AP-1/2), activating transcription factor (ATF)/CREB, CCAAT/enhancer binding protein (C/EBP), E2 promoter binding factor (E2F), nuclear factor of activated T cells (NF-AT), p53, hypoxia inducible factor-1α (HIF-1α), hepatocyte nuclear factor 1 (HNF1), and SMAD family member 4 (SMAD4). Through these interactions, HBx can induce the transactivation of key cellular pathways, such as mitogen-activated protein kinase (MAPK), phosphoinositide 3-kinase/protein kinase B (PI3K/AKT), janus kinase/signal transducer and activator of transcription (JAK-STAT), SRC proto-oncogene, non-receptor tyrosine kinase (Src-kinase), Wnt/beta-catenin, nuclear factor kappa-B (NF-κB) signaling, calcium signaling, DNA repair, and apoptosis (Levrero and Zucman-Rossi, 2016). Recently, a study identified 189 proteins from HepG2 cells that specifically bind to the transactivation domain of HBx through glutathione S-transferase (GST) pull-down and mass spectrometry (MS) (Zhou et al., 2021). However, the detailed molecular mechanisms involving in the interplay between host factors and viral proteins remain to be fully elucidated.

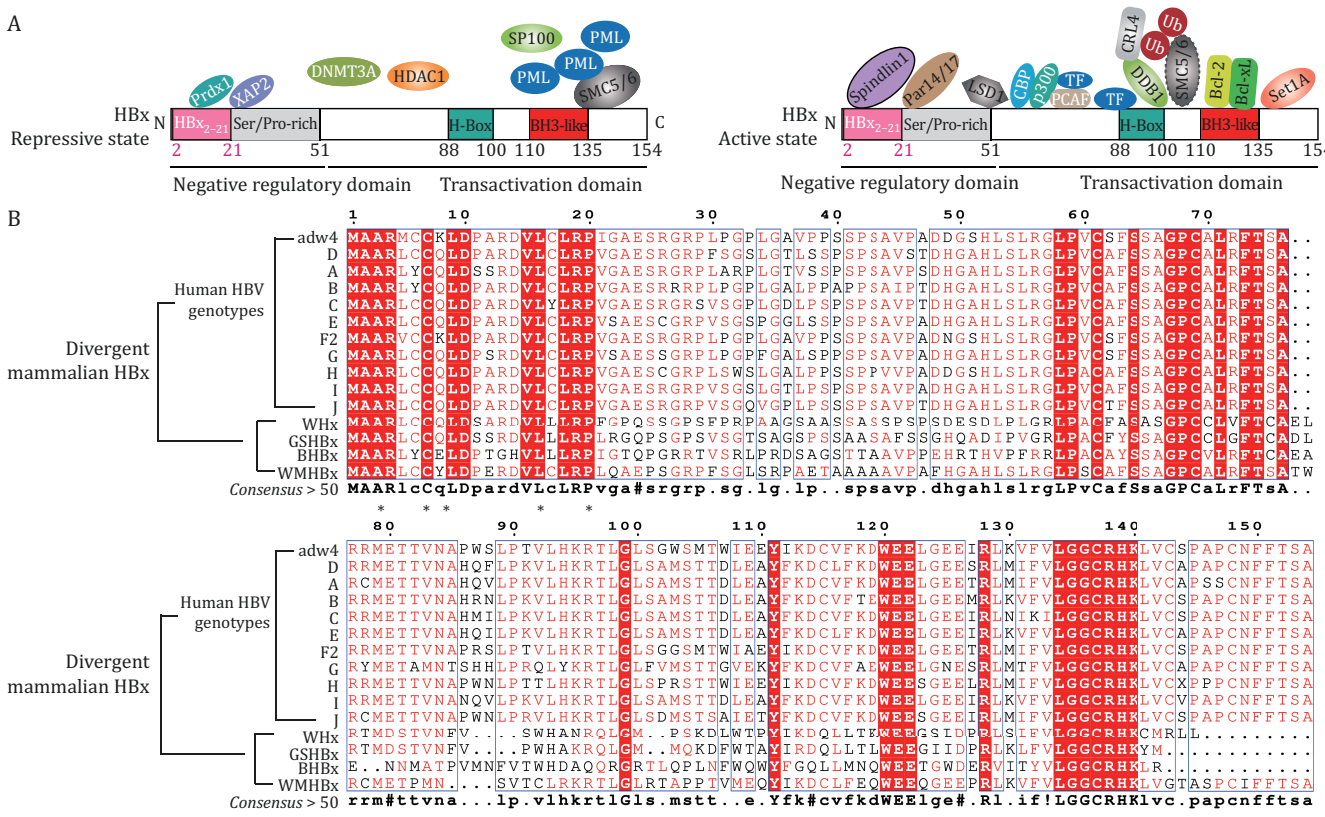

Figure 1. Binding partners and sequence alignment of the HBx protein. (A) Schematic model illustrating the binding partners of HBx in repressive state and active state. In the repressive state, HBx maintains HBV genome heterochromatin by recruiting co-repressors, such as HDAC1, DNMT3A, SMC5/6, Prdx1, and XAP2 (left). The binding of HBx to Spindlin1 can facilitate the transition of HBx to an active conformation. This shift promotes the recruitment of co-activators and transcriptional regulators, including DDB1, CBP/p300, PCAF, Bcl-2/Bcl-xL, LSD1, Set1A, and other HBV-specific transcription factors (right). (B) Sequence alignment of HBx protein across different human HBV subtypes (A–J), as well as HBx from woodchuck hepatitis virus (WHV), ground squirrel hepatitis virus (GSHV), bat hepatitis virus (BHV), and woolly monkey hepatitis virus (WMHV). *Key residues involved in Spindlin1 engagement.

The transrepression function of N-terminal motif of HBx

Characteristic motifs within HBx include an "A" region ($HBx_{2–21}$) (Kumar et al., 1996; Liu et al., 2023), a Ser/Pro-rich region (Tang et al., 2006), an H-box motif (Li et al., 2010), a BH3-like motif (Zhang et al., 2019), and a zinc finger motif formed by conserved Cys/His residues (C61, C69, C137, and H139) (Ramakrishnan et al., 2019) (Fig. 1A). Alignment of HBx proteins across various mammalian species, such as woodchuck hepatitis virus (WHV), ground squirrel hepatitis virus (GSHV), arctic squirrel hepatitis virus (ASHV), gorilla hepatitis virus (GOHHV), woolly monkey hepatitis virus (WMHV), chimpanzee hepatitis virus (CHV), gibbon hepatitis virus (GIHV), and different human HBV subtypes (A–J), reveals high sequence homology in the N-terminal motifs ($HBx_{2–21}$), suggesting a conserved biological function for this region (Liu et al., 2023) (Fig. 1B). Studies have demonstrated that the conserved N-terminal region exerts a transrepression function by specifically inhibiting the transactivation domain, without causing general inhibition or cytotoxicity (Murakami et al., 1994). Consistently, the N-terminal region interacts directly with the HBx protein, exerting a dominant-negative effect on HBx-mediated mitogenic pathway activation, endogenous gene expression, and apoptosis in 4pX-1 cells (Li et al., 2006). Furthermore, the N-terminal "A" domain (amino acids 1–20) of HBx has been shown a negative regulatory function, which is potentially through a transrepression mechanism involving protein-protein interactions that either squelch or inhibit a general transcription factor via the "A" region (Misra et al., 2004). Indeed, the host protein X-associated protein 2 (XAP2), which interacts with the residues 13–26 of HBx, has been demonstrated to inhibit the transactivation function of HBx by sequestering cellular proteins crucial for HBx transactivation (Kuzhandaivelu et al., 1996). Additionally, peroxiredoxin 1 (Prdx1), a novel HBx-binding protein through $HBx_{17–20}$, plays a role in negatively regulating HBV propagation by promoting HBV RNA degradation, further supporting the transrepressive role of HBx N-terminal region (Deng et al., 2019). Intriguingly, parvulin 14 and parvulin 17 proteins

have been reported to upregulate HBV transcription and replication from cccDNA chromatin through binding to the $^{19}R^{20}P$-$^{28}R^{29}P$ motifs of HBx (Saeed et al., 2019). Despite these insights, the precise biological significance of the $HBx_{2–21}$ region during HBV life cycle and the underlying mechanisms are largely unknown.

Epigenetic reprogramming of cccDNA minichromosome during HBV transcription

HBV cccDNA is organized as a minichromosome in the nuclei of infected hepatocytes by histone and non-histone proteins. Increasing evidence suggests that HBV transcription can be controlled by epigenetic reprogramming of cccDNA, such as methylation, acetylation, and succinylation (Ren et al., 2024). HBV has been shown to induce methylation of both host and viral DNA *in vitro* through the induction of DNA methyltransferases (DNMT1, DNMT2, and DNMT3), leading to decreased viral gene expression (Vivekanandan et al., 2010; Zheng et al., 2009). Upon HBV infection, H3K9 methyltransferase SETDB1 and the heterochromatin protein HP1 can induce the silence of HBV cccDNA transcription through the modulation of chromatin structure (Rivière et al., 2015). Additionally, hypoacetylation status of the cccDNA-bound H3 and H4 and the recruitment of cellular HDAC1 onto cccDNA are correlated with low HBV transcription. HDAC11 is recruited to cccDNA and inhibits HBV transcription through decreasing the levels of cccDNA-bound histone H3 lysine 9 acetylation (H3K9ac) and histone H3 lysine 27 acetylation (H3K27ac) (Yuan et al., 2019). Moreover, various host factors involved in epigenetic modifications, including high mobility group box 1 (HMGB1), Sirtuin (SIRT) 1 and 3, histone methyltransferase SUV39H1, PRMT 1 and 5, enhancer of zeste homolog 2, histone methyltransferase SET domain containing 2 (SETD2), and activator of transcription 1 (STAT1) are associated with the silence of HBV cccDNA transcription (Belloni et al., 2009; Benhenda et al., 2013; Chen et al., 2017; Kim et al., 2022; Ren et al., 2018; Salerno et al., 2020; Zhang et al., 2017). In addition, $HBx_{50–100}$ was found to upregulate HBV m6A modification through interaction with METTL3/14, thereby lowing the expression of HBV proteins (Kim and Siddiqui, 2021).

To overcome the host restriction mechanisms, HBV has evolved various evasion strategies, of which HBx encoding is the most important. HBx is recruited to the cccDNA minichromosome and establishes an active chromatin state by direct or indirect recruitment of CBP /p300 acetyltransferase, CBP/p300-associated factor (PCAF), general control non-repressed protein 5 (GCN5), histone H3K9me1/2 demethylase lysine-specific demethylase 1 (LSD1) and H3K4me3 methyltransferase Set1A (Alarcon et al., 2016; Cougot et al., 2007). In addition, HBx antagonizes host inhibitory effects on cccDNA transcription induced by HMGB1, protein phosphatase 1(PP1)/HDAC1 complex, long intergenic non-coding RNA 01431 (LINC01431)-promoted H4R3me2a, SETDB1-medieated H3K9me3, HP1 recruitment and SIRT3 recruitment (Cougot et al., 2012; Kim et al., 2022; Ren et al., 2018; Rivière et al., 2015; Sun et al., 2022). HAT1 has been shown to active cccDNA transcription by modulating cccDNA minichromosome assembly through the acetylation of histone H4K5 and H4K12 (Yang et al., 2019). NQO1 (NAD(P)H:quinone oxidoreductase 1) was identified as a key player in promoting HBV transcription by stabilizing HBx and facilitating the establishment of an active chromatin structure on cccDNA through its interaction with the 20S proteasome (Cheng et al., 2021). Interferon-alpha (IFN-α) modulates the HBV cccDNA minichromosome by epigenetically regulating lysine acetyltransferase 2A (KAT2A, also termed GCN5)-mediated succinylation of histone H3K79 to clear HBV cccDNA (Yuan et al., 2020). These findings have greatly expanded the understanding of epigenetic reprogramming in cccDNA transcription. Nevertheless, the dynamic mechanisms controlling the establishment, maintenance, and remodeling of cccDNA modifications remain enigmatic.

Spindlin1-HBx interplay in epigenetic reprogramming of HBV genome

Spindlin1, a member of the Spin/Ssty family, has been characterized as a transcriptional coactivator through histone methylation readout, involving the first and second Tudor domains (Su et al., 2014; Wang et al., 2011, 2018; Yang et al., 2012; Zhao et al., 2020) (Fig. 2A). Both our research and others have revealed that $SPINDOC_{256–281}$ interacts with Spindlin1 through its third Tudor domain, and the combinatorial readout of H3 "K4me3–K9me3" by Spindlin1 in conjunction with SPINDOC can displace HP1 proteins from the poised rDNA loci, thereby facilitating rRNA expression (Du et al., 2021; Zhao et al., 2024). Recently, we have identified that the viral protein HBx has evolved a conserved N-terminal motif ($HBx_{2–21}$) that directly interacts with the third Tudor domain of Spindlin1 (Fig. 2B) in a way similar to SPINDOC (Liu et al., 2023). Interestingly, despite the proposed transrepression activity of $HBx_{2–21}$ on HBV transcription, our study highlighted a previously uncharacterized function of the N-terminal domain, where it could recruit Spindlin1 to derepress its repressive function, thereby promoting HBV transcription in a manner dependent on H3 "K4me3–K9me3" readout.

Upon entry into the target cells, many DNA viruses are recognized and loaded with histones marked with heterochromatin modifications, leading to the silence of viral gene expression. In the case of HBV, the SMC5/6 proteins (localized at pro-myelocytic leukemia nuclear bodies, PML-NBs, a.k.a. ND10), HDAC1, SETDB1, and HP1, act as restriction factors for HBV transcription by establishing a repressive cccDNA minichromosome state marked by

hypoacetylation and H3K9me3 modifications (Belloni et al., 2009; Rivière et al., 2015; Tsai and Cullen, 2020). HBx can counteract this heterochromatin barrier and promote viral transcription through interactions with multiple cellular factors (Belloni et al., 2009; Cougot et al., 2007; Levrero and Zucman-Rossi, 2016; Lucifora et al., 2011). We discovered that Spindlin1-HBx engagement promotes HBV gene expression depending on the epigenetic conversion of cccDNA from an H3K9me3-enriched repressive state to an H3K4me3-marked active state. Remarkably, we observed an exceptionally strong binding affinity (K_d = 3.8 nmol/L) between the Spindlin1-$HBx_{2–21}$ complex and the H3 "K4me3–K9me3" peptide, representing the most potent histone modification recognition event reported to date (Fig. 2C). Chromatin imunoprecipitation-quantitative polymerase chain reaction (ChIP-qPCR) analysis further demonstrated an elevated Spindlin1 signal at the HBV cccDNA minichromosome in WT HBV-infected HepG2-NTCP (sodium taurocholate cotransporting polypeptide) cells compared to HBx-deficient HBV-infected cells (Liu et al., 2023). Structural comparison of the Spindlin1-$HBx_{2–21}$ complex with previously reported structure of the Spindlin1-H3 "K4me3–K9me3" complex revealed that the engagement of Tudor 3 with the HBx N-terminal region and histone binding by Tudors 2 and 1 occur simultaneously without any discernible structural conflicts (Fig. 2C). Collectively, these findings suggest a role for $HBx_{2–21}$ in stabilizing Spindlin1 at the poised cccDNA minichromosome marked with H3 "K4me3–K9me3" and highlight Spindlin1's role in facilitating HBx chromatin targeting, as HBx lacks any known chromatin-binding domains.

The bivalent chromatin domain, marked by the co-occurrence of H3K4me3 and H3K27me3 on the opposite H3 tails of a single nucleosome, was initially identified in embryonic stem cells and is associated with pluripotency and rapid gene activation upon onset of differentiation programs (Bernstein et al., 2006; Voigt et al., 2012). Here, we present evidence for the functional importance of another type of bivalent modification, where H3K4me3 and H3K9me3 co-occur on the same histone H3 tail, recognized by the Spindlin1-HBx complex to promote HBV transcription. We propose that the HBV cccDNA minichromosome exists in three chromatin states: a repressive state enriched with H3K9me3, a poised state marked by bivalent H3 "K4me3–K9me3" methylation, and an active state marked by H3K4me3 (Fig. 2D). It has been reported that the mixed lineage leukemia protein-1 (MLL1) core complex can methylate H3K4 in H3K9me3-modified regions, thereby enzymatically generating a bivalent H3 "K4me3–K9me3" methylation pattern (Patel et al., 2014). Then HBx could recruit Spindlin1 to effectively target the bivalent methylation pattern and induce an epigenetic

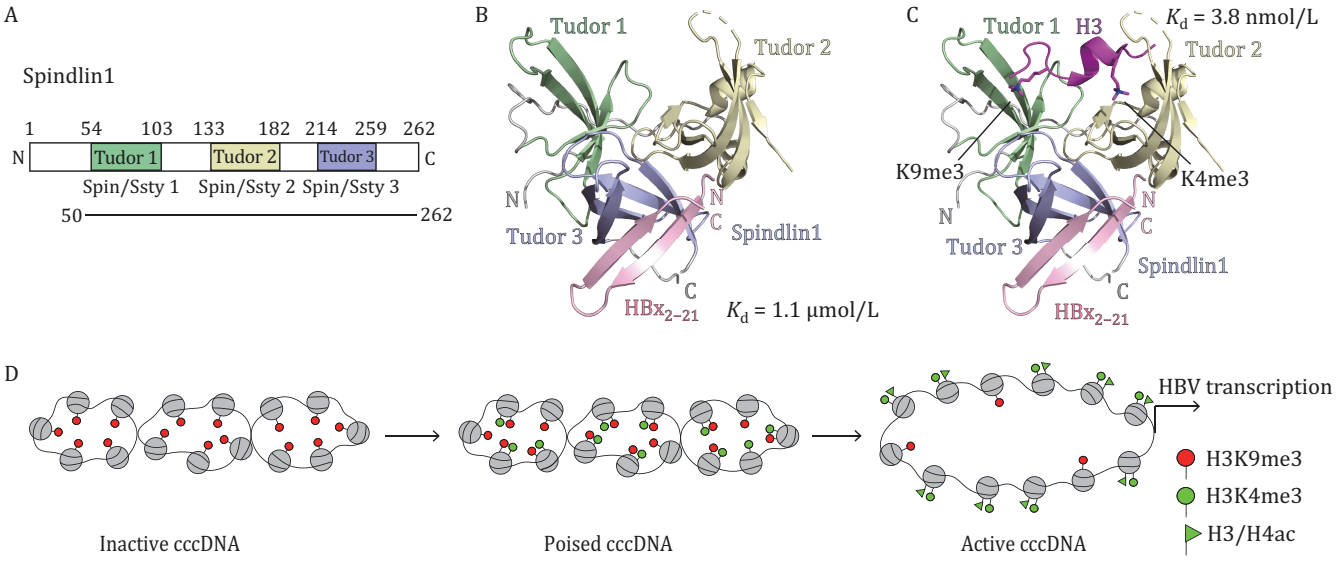

Figure 2. Epigenetic mechanism underlying Spindlin1-HBx engagement in promoting HBV transcription from the cccDNA minichromosome. (A) Domain architecture of Spindlin1. (B) Overall structure of $Spindlin1_{50–262}$-$HBx_{2–21}$ complex in ribbon view (PDB ID 8GTX). The dissociation constant (K_d) between Spindlin1 and $HBx_{2–21}$ peptide is 1.1 μmol/L. (C) Cartoon representation of structural comparison of $Spindlin1_{50–262}$ bound to $HBx_{2–21}$ (PDB ID 8GTX) with $Spindlin1_{50–262}$ bound to H3 "K4me3–K9me3" (PDB ID 7BQZ). The K_d between Spindlin1-$HBx_{2–21}$ complex and H3 "K4me3–K9me3" peptide is 3.8 nmol/L. (D) Schematic diagram illustrating Spindlin1-HBx-mediated transcriptional derepression of cccDNA minichromosome. HBV genome is typically silenced by host restriction factors and forms a repressive chromatin state marked by H3K9me3 (left). To overcome the heterochromatin barrier, histone methyltransferases (such as H3K4MT) can target H3K9me3-enriched cccDNA to create bivalent H3 "K4me3–K9me3" modifications (middle). Spindlin1-HBx complex then binds to H3 "K4me3–K9me3" marked poised state chromatin and cooperatively establish an active cccDNA chromatin state in concert with other host transcription factors and co-activators to promote HBV transcription (right).

switch from a poised-state cccDNA minichromosome to an H3K4me3-marked active chromatin, thereby promoting HBV transcription. Likewise, SPINDOC has been reported to promote rRNA transcription through the recruitment of Spindlin1 and subsequent readout of the H3 "K4me3–K9me3" methylation pattern in a poised rDNA chromatin state (Du et al., 2021), suggesting a common mechanism underlying both HBV and rRNA transcription.

In addition, our previous study revealed that Spindlin1 can also recognize histone lysine-arginine methylation patterns, such as H3 "K4me3–R8me2a", to promote Wnt signaling and cell cycle (Su et al., 2014). This suggests that Spindlin1 may activate chromatin in a stepwise manner by recognizing distinct methylation patterns or types to fine-tune chromatin dynamics and gene expression. Future studies are needed to clarify the role of Spindlin1-HBx in distinct methylation pattern recognition during HBV transcription and its contribution to viral replication and host cell regulation.

Linking HBx conformation switch to epigenetic reprogramming

It has been demonstrated that the conserved N-terminal region of HBx exerts a transrepression function by specifically inhibiting the transactivation function of the C-terminal region (Murakami et al., 1994). Here, our study revealed a transcriptional activation role of $HBx_{2–21}$ by recruiting Spindlin1. This seemingly contradictory roles of $HBx_{2–21}$ in HBV transcription may be reconciled by the conformational switch of HBx state triggered by Spindlin1 binding.

Structural studies have revealed that residues 88–100 of the HBx H-box binds DDB1 through an α-helical motif (Li et al., 2010), and residues 110–135 and 113–135 of the HBx BH3-like motif adopts an amphipathic α-helix when binding to Bcl-2 and Bcl-xL (Fig. 3A) (Jiang et al., 2016; Zhang et al., 2019). Interestingly, according to the predicted structure of HBx by AlphaFold (Jumper et al., 2021), the N-terminal motif of HBx ($HBx_{2–21}$), the DDB1-binding motif ($HBx_{88–100}$) and the Bcl-2-binding motif ($HBx_{110–135}$) cluster together and are buried into a hydrophobic core when folded (Fig. 3A). However, upon engagement with Spindlin1, HBx unfolds, exposing the N-terminal motif, and concurrently, the two C-terminal motifs for DDB1 and Bcl-2 recruitment. This suggests that HBx has two functional states: a folded, repressive state that maintains HBV genome heterochromatin by recruiting co-repressors, such as HDAC1, DNMT3A, SETDB1, HP1, and XAP2, and an unfolded state that loses co-repressor binding while recruiting Spindlin1, Bcl-2/Bcl-xL, DDB1, CBP/p300, PCAF, LSD1, and Set1A to establish euchromatin and promote transcription (Fig. 3B). Conceivably, the N-terminal motif may inhibit the exposure of the C-terminal motifs needed for transactivation by hydrophobic core formation. Meanwhile, Spindlin1, DDB1, Bcl-2, and other factors likely cooperate to unfold HBx. Indeed, our HBx pull-down results show reduced DDB1 binding in Spindlin1 knockdown HepG2-NTCP cells (Liu et al., 2023) and decreased HDAC1 but increased p300 binding in Spindlin1 overexpressing HEK293T cells (data unpublished). Additionally, SMC6 degradation assays reveal that decreased SMC6 levels by HBx are partly restored in Spindlin1 knockdown cells (Liu et al., 2023).

These findings support a positive correlation between Spindlin1-HBx, DDB1-HBx, and p300-HBx, as well as DDB1-HBx-mediated SMC5/6 degradation, but a negative correlation between Spindlin1-HBx and HDAC1-HBx. Our study underscores the conformational switch of HBx and its modulation by Spindlin1, offering insight into the dual roles (repression vs. activation) of HBx in HBV transcriptional regulation. The dynamic shift between HBx conformation, as well as the chromatin state of cccDNA, is reminiscent of the lytic and lysogenic cycles in bacteriophages, where two opposing mechanisms compete for dominance through distinct pathways. Future research should focus on evaluating the role of various cellular factors, both known and unknown, in inducing the conformational switch of HBx, which is crucial for understanding the molecular mechanisms underlying HBV transcriptional regulation and developing novel therapeutic strategies against HBV infection. Additionally, it has been reported that conserved HBx residues, including Ser25, Ser41, and Thr81, which are exposed in the predicted three-dimensional (3D) structure of HBx, are potential phosphorylation sites that may regulate HBx function (Prieto et al., 2021). Recombinant HBx has been shown to be phosphorylated *in vitro* by both protein kinase C and MAPK, specifically, Ser41 are phosphorylated by extracellular signal-regulated kinases 1 and 2 (ERK1/2) (Hernández et al., 2012; Noh et al., 2004). Given that the N-terminal motif and C-terminal transactivation domain of HBx are clustered to form a hydrophobic core under repressive conditions, it would be intriguing to explore whether the post-translational modifications, particularly phosphorylation, play a role in modulating the conformational switch of HBx to release its N-terminal and C-terminal motifs to interact with multiple cellular factors.

As the anti-apoptotic proteins, Bcl-2 and Bcl-xL have been reported to interacts with HBx through its BH3-like motif, and that this protein interaction is crucial for HBx-induced cytosolic calcium elevation, cell death, and viral DNA replication (Geng et al., 2012). Consistently, our study suggests that the interaction between HBx and Bcl-2 also facilitates the Spindlin1-mediated conformational switch of HBx, thereby enhancing HBV transcription and replication. These results highlight a synergistic mechanism by which HBx leverages its interaction with Bcl-2 to modulate both apoptotic pathways and viral replication. This dual role of the HBx-Bcl-2 interaction underscores the complexity of HBV-host interactions and provides new insights into the molecular mechanisms underlying HBV

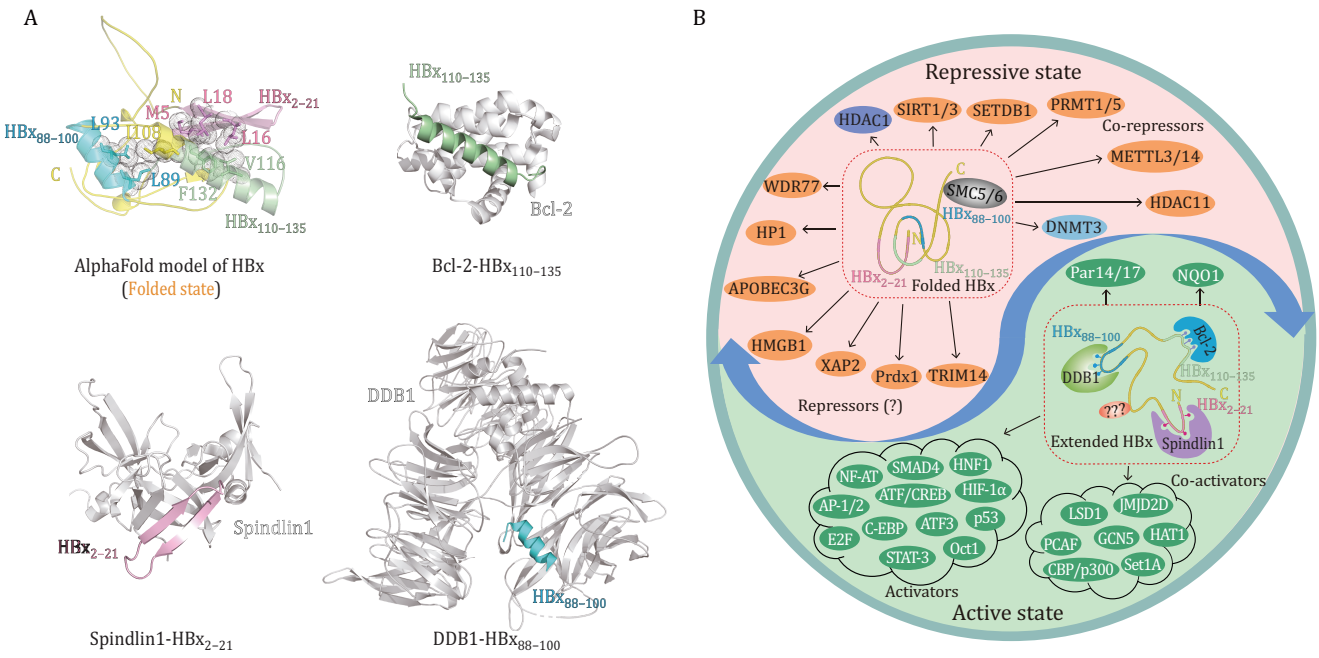

Figure 3. A proposed model of Spindlin1-mediated HBx conformational switch during HBV cccDNA transcription. (A) AlphaFold model of free HBx and structures of $HBx_{2–21}$, $HBx_{88–110}$ and $HBx_{110–135}$ peptides in complex with Spindlin1 (PDB ID 8GTX), Bcl-2 (PDB ID 5FCG), and DDB1 (PDB ID 3I7H). In the folded state, key residues of HBx involved in Spindlin1, DDB1, and Bcl-2 binding are buried within the hydrophobic core. (B) A proposed model of HBx transitioning from the repressive state to an active one by binding to Spindlin1. In the folded HBx state, HBV cccDNA exists in a repressed chromatin state characterized by methylation, hypoacetylation, and histone H3K9 methylation through direct or indirect recruitment of DNMT3A, HDAC1, SIRT1, PRMT1/5, H3K9 methyltransferase SETDB1, and the heterochromatin protein HP1. Whereas, the binding of HBx to Spindlin1 can unlock the transrepression activity of the N-terminal motif, and enables a functional switch of HBx from organizing a repressive chromatin state to an open one by blocking the host restriction factors and recruiting activation-related factors such as DDB1, CBP/p300, PCAF, Bcl-2, or other HBV-specific co-activators.

persistence and pathogenesis. Further exploration of this interplay may reveal novel therapeutic targets for disrupting HBV replication and associated apoptotic signaling.

Conclusion and perspectives

Here we demonstrate that the N-terminal negative regulatory motif of HBx can physically recruit Spindlin1 to unlock the motif's repressive function. This process is associated with the blocking of co-repressors and recruiting multiple co-activators, thereby modulating the epigenetic state of cccDNA minichromosome. Our analysis suggests a dual-state conformational switch mechanism of HBx in driving epigenetic reprogramming of the viral genome, providing novel insights into HBV transcription and new treatment strategies. Nevertheless, several questions related to this process remain to be addressed.

As the key regulatory protein of HBV genome, HBx is crucial for regulating HBV transcription from cccDNA templates by interacting with various host factors. All solved complex structures to date, including DDB1-$HBx_{88–100}$ (Li et al., 2010), Bcl-2-$HBx_{110–135}$ (Jiang et al., 2016), Bcl-xL-$HBx_{113–135}$ (Zhang et al., 2019), and Spindlin1-$HBx_{2–21}$ (Liu et al., 2023), are related to an unfolded HBx state with a transcriptional activation outcome. While AI has predicted a three-dimensional structure of folded HBx with moderate confidence, an experimental structure of HBx remains unavailable, partly due to its flexible nature, as well as the challenges of obtaining sufficient well-behaved protein samples. It is anticipated that the co-repressors of HBx, such as HDAC1 and DNMT3A, likely interact with HBx in its folded state via protein surfaces distinct from its extended state. This hypothesis awaits exploration in future structural and biochemical studies.

HBV infection triggers cellular mechanisms that silence cccDNA transcription through heterochromatin formation, involving Smc5/6 in ND10 bodies, SETDB1-mediated H3K9me3, and HP1 (Rivière et al., 2015). We demonstrated that HBx can overcome this epigenetic repression by hijacking the host co-activators such as Spindlin1. However, the mechanisms by which HBx is initially expressed from the heterochromatic HBV genome remain elusive. Notably, studies have reported that HBx is the earliest-expressed viral gene after infection of primary human hepatocytes, and can be detected very early, prior to the depletion of SMC6 (Niu et al., 2017). Similarly, a recent study demonstrated that early HBx gene transcription is linked to the chromatinization of

cccDNA, which may be mediated by the preinitiation complex (PIC)-Mediator complex, a mega-complex that can facilitate transcription through a chromatinized promoter (Prescott et al., 2024). Based on these findings, we propose that HBx transcription likely occurs at a low basal level, with the protein primarily existing in a folded, inactive state to nucleate cccDNA heterochromatin formation. These HBx transcripts may arise from leaky heterochromatin or indicate a specific transcription regulatory process that remains to be fully explored, akin to the RNA-based heterochromatin assembly pathway (Grewal, 2023; Saksouk et al., 2015). During HBV infection, HBx employs a delicate dual-state switch mechanism, transitioning between repressive and active conformations in response to the cellular environment to sustain the virus's life cycle. Understanding this complex relationship will provide valuable insights into viral behavior and host interactions, highlighting the sophisticated strategies employed by viruses to ensure their survival and propagation.

Here we highlight the pivotal role of the HBV-encoded protein HBx in enhancing HBV transcription by epigenetically reprogramming chromatin through its interaction with Spindlin1. This mechanism is not unique to HBV, viral proteins often recruit host proteins to alter chromatin states, thus promoting viral gene expression. For example, during herpes simplex virus-1 (HSV-1) infection in fibroblasts, host defenses can epigenetically silence HSV-1 genome through the H3K9me3 modification (Cohen et al., 2018; Tsai and Cullen, 2020). However, the viral protein VP16 counters this suppression by recruiting host factors to remove the repressive H3K9me3 mark and deposit the activating H3K4me3 mark on viral chromatin (Liang et al., 2009, 2013; Narayanan et al., 2007). Notably, Spindlin1 also plays a crucial role in transcriptional regulation of HSV-1 (Ducroux et al., 2014). Exploring whether Spindlin1 has a conserved role in regulating other DNA viruses via similar epigenetic mechanisms would be intriguing. Additionally, DNA methylation can act as a host defense strategy against viral genomes (Barlow, 1993). HBV infection can induce methylation of both host and viral DNA to suppress viral gene expression (Vivekanandan et al., 2010). How viral DNA methylation functions along with H3K9me3 to regulate epigenetic reprogramming, and the roles of Spindlin1 and HBx in this process, remain areas for further investigation.

HBV infection is a major risk factor for HCC, with HBx being the predominant expressed HBV protein in malignant HCC cells and playing a crucial role in hepatocarcinogenesis (Levrero and Zucman-Rossi, 2016). Although the exact mechanisms remain unclear, HBx is believed to facilitate HCC progression through interaction with a variety of host proteins. Notably, Spindlin1 is often overexpressed and contributes to transcriptional programs in several cancers, including ovarian, colorectal, breast, liposarcoma, and HCC (Franz et al., 2015; Wang et al., 2012). Given that HBx can promote chromatin targeting of Spindlin1 by boosting its H3 "K4me3–K9me3" readout activity (Liu et al., 2023), the Spindlin1-HBx complex likely plays an oncogenic role by driving host cell gene expression in cancer pathways. Furthermore, HBx is thought to adopt two distinct conformations, facilitating either corepressor or coactivator recruitment. Genomic analyses of HCC patient samples with HBV infection have identified frequent mutations in HBx that enhance its carcinogenic potential (Zhou et al., 2022). While these mutations do not alter the Spindlin1-binding motif, they affect either the residues forming the hydrophobic core or those responsible for co-repressor binding, such as DNMT3A (Zheng et al., 2009). These changes may either destabilize the hydrophobic core, maintaining HBx in an unfolded state to effectively interact with Spindlin1, or disrupt its association with co-repressors, potentially promoting HCC progression. Understanding the complex interplay between Spindlin1 and HBx, along with the impact of HBx mutations, will be crucial for developing more effective strategies for HCC prevention, treatment, and prognosis prediction.

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Conflict of interest

The authors declare no competing interests.

Ethics approval

This perspective does not contain any studies with human or animal subjects performed by any of the authors.

Consent to participate

All authors agree to participate.

Consent for publication

All authors agree to publish this manuscript.

Data availability

All data generated or analyzed during this study are included in this published article.

Code availability

The PDB codes of the previously determined structures used in this manuscript are: 8GTX (Spindlin1$_{50–262}$-HBx$_{2–21}$), 7BQZ (Spindlin1-H3 "K4me3–K9me3"), 3I7H (DDB1-HBx$_{88–100}$), 5FCG (Bcl-2-HBx$_{110–135}$).

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