Virologica Sinica 40 (2025) 374–387

Contents lists available at ScienceDirect

Virologica Sinica

journal homepage: www.keaipublishing.com/en/journals/virologica-sinica
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Research Article

Host factor RBM25 promotes HBV replication through Yin Yang 1-mediated cccDNA transcription

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ARTICLE INFO

Keywords:
Hepatitis B virus (HBV)
Covalently closed circular DNA (cccDNA)
cccDNA transcription
RNA binding motif protein 25 (RBM25)
Yin Yang 1 (YY1)
Acetylated histones

ABSTRACT

The persistence of covalently closed circular DNA (cccDNA) in hepatitis B virus (HBV)-infected hepatocytes remains a major obstacle to effective antiviral treatment. Understanding the molecular mechanisms regulating HBV cccDNA transcription is essential for developing novel therapeutic strategies. In this study, we investigated the role of RNA binding motif protein 25 (RBM25) in HBV replication, focusing on its interaction with cccDNA and its regulation of host transcription factors. The results demonstrated that RBM25 knockdown markedly inhibited HBV replication, reducing levels of HBV DNA, hepatitis B e antigen (HBeAg), hepatitis B surface antigen (HBsAg), HBV RNA, and L-HBs in HBV-replicating and infected cell models. Consistent results were observed in a mouse model hydrodynamically injected with 1.2 × HBV plasmid. Conversely, RBM25 overexpression significantly enhanced HBV replication. Mechanistically, RBM25 promoted HBV promoter activities by binding to cccDNA through its RE/RD and PWI domains. This effect was mediated by increased Yin Yang 1 (YY1) expression, which enhanced acetylation of cccDNA-bound histones, promoting HBV transcription. Furthermore, RBM25 expression was upregulated and translocated to the nucleus following core protein expression and accumulation, while overexpression of RBM25 promoted core protein degradation. In conclusion, this study demonstrates that RBM25 is a novel host factor that enhances HBV replication by upregulating YY1-dependent transcriptional activation of cccDNA. It also reveales a reciprocal regulatory mechanism between the HBV core protein and RBM25, which helps sustain HBV replication.

INTRODUCTION

Hepatitis B virus (HBV) infection remains a global health challenge, with an estimated 254 million people infected worldwide (Hsu et al., 2023; Wan et al., 2022). Despite the availability of vaccines and several approved therapies, approximately 820,000 deaths annually are attributed to end-stage liver disease in infected individuals (Sheena et al., 2022). This is partly due to the lack of therapies that directly target covalently closed circular DNA (cccDNA). Therefore, it is imperative to gain a comprehensive understanding of the host factors that interact with cccDNA and play a critical role in HBV replication.

HBV, a member of the *Hepadnaviridae* family, carries a 3.2 kb partially double-stranded relaxed circular DNA (rcDNA). Upon infection, the viral capsid releases rcDNA into the nucleus, where it is converted into

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https://doi.org/10.1016/j.virs.2025.05.004
Received 3 December 2024; Accepted 20 May 2025
Available online 22 May 2025

cccDNA. The cccDNA serves as a transcriptional template for pregenomic RNA (pgRNA) and subgenomic RNAs, which are essential for antigen production and viral replication within host cells (Wei et al., 2021). The pgRNA encodes both the HBV polymerase (Pol) and core protein, serving as a template for reverse transcription by Pol to generate rcDNA for progeny virus. The transcription of cccDNA is regulated by two enhancers (EnhI and EnhII) and four promoters (BCP/CP, SpI, SpII, and XP), which contain binding sites for both ubiquitous and liver-enriched host factors (Tsukuda et al., 2020). Numerous studies have shown that transcription factors, histone-modifying enzymes, and RNA-binding proteins regulate cccDNA transcription (Sun et al., 2017, 2022; Teng et al., 2021).

RNA binding motif protein 25 (RBM25), an RNA-binding protein, is capable of independently conducting pre-mRNA splicing. Additionally, RBM25 can interact with multiple splicing cofactors, such as SRm160/300, to form splicing complexes that regulate alternative splicing processes (Carlson et al., 2017; Zhang et al., 2021). While research on the role of RBM25 in disease progression remains limited, it has recently garnered attention for its potential regulatory role in viral infections (Wu et al., 2019). Studies suggest that certain RNA-binding proteins may interact with viral genomes or transcripts to modulate RNA metabolism and influence the stability of various viral and host RNAs (Makokha et al., 2019; Yao et al., 2019), thus representing a novel therapeutic target landscape. Intriguingly, RBM25 also binds DNA, and preliminary findings from our laboratory demonstrate its interaction with HBV rcDNA or cccDNA (Zheng et al., unpublished data). However, the specific role of RBM25 in HBV transcription and replication has not been well characterized.

In this study, we investigated the role of RBM25 in regulating HBV replication using both knockdown and overexpression approaches in HBV replicating cell and mouse models. The results demonstrate that RBM25 plays a crucial role in promoting HBV replication by enhancing HBV transcription through interactions with cccDNA. Furthermore, we provide evidence that RBM25's effects are mediated through Yin Yang 1 (YY1), a transcription factor known to modulate chromatin structure and implicated in HBV promoter activation (Shen et al., 2020; Wang et al., 2022). Notably, we also show that HBV core protein upregulates RBM25 expression, while RBM25 overexpression promotes core protein degradation. These findings provide a novel insight into the interplay between host factor and HBV replication.

RESULTS

Knockdown of endogenous RBM25 inhibits HBV replication

To investigate the role of RBM25 in regulating HBV replication, we designed three siRNAs specifically targeting the RBM25 gene and demonstrated that their combined use achieved the highest knockdown efficiency (Supplementary Fig. S1A). Based on these findings, we selected the combination of all three siRNAs for subsequent experiments and the results showed that these siRNAs significantly reduced the expression level of RBM25 mRNA in HepG2, HepAD38, and HepG2-NTCP cells. (Supplementary Fig. S1B, C). Next, we examined the levels of replication intermediates and viral proteins under conditions of RBM25 knockdown in HepG2 cells transiently transfected with prcccDNA/pCMV-Cre plasmids (prccc/cre), which produce recombinant cccDNA to initiate HBV replication (Qi et al., 2014). The results showed that RBM25 knockdown led to significant decreases in the levels of supernatant HBV DNA, hepatitis B e antigen (HBeAg), and hepatitis B surface antigen (HBsAg) (Fig. 1A–C). Additionally, Southern blot analysis confirmed the inhibitory effect of RBM25 knockdown on HBV replication (Fig. 1D). Consistent with these findings, L-HBs levels decreased following RBM25 knockdown, while core protein levels increased (Fig. 1E). Similar results were observed in HepAD38 cells, a commonly used HBV-inducible cell line (Fig. 1F–J).

To further corroborate these results, we analyzed HBV replication levels in RBM25-knockdown HepG2-NTCP cells infected with HBV virions (Fig. 1K). Seven days post-infection, RT-qPCR assays confirmed the effective knockdown of RBM25 (Supplementary Fig. S1C). Additionally, RBM25 knockdown significantly reduced the levels of HBV DNA, HBeAg, and HBsAg in the cell culture supernatants (Fig. 1L–N). Similarly, the intracellular core-associated HBV DNA levels were decreased following RBM25 knockdown (Fig. 1O). Interestingly, an increase in core protein levels was observed upon RBM25 knockdown (Fig. 1P). In summary, these findings demonstrated that RBM25 knockdown inhibited HBV replication in both HBV replicating and infected cell models.

Ectopic overexpression of RBM25 enhances HBV replication

To further validate the role of RBM25 in HBV replication, we established HepG2 cell lines with stable RBM25 overexpression via lentiviral transduction, followed by transfection with prcccDNA/pCMV-Cre plasmids. Overexpression of RBM25 significantly enhanced the levels of HBV DNA, HBeAg, and HBsAg in the supernatants, as well as core-associated HBV DNA and L-HBs within cells (Fig. 2A–E). Notably, RBM25 overexpression led to a downregulation of core protein levels (Fig. 2E). Similar results were observed in HepAD38 cells following RBM25 overexpression (Fig. 2F–J). Taken together, these findings suggested that RBM25 promoted HBV replication.

To further explore the relationship between RBM25 expression and HBV replication, we analyzed a published dataset of 69 chronic hepatitis B (CHB) patients in different phases (30 HBeAg-positive and 39 HBeAg-negative) (GEO: GSE230397) (Montanari et al., 2022). We observed a significant increase in RBM25 expression in liver tissues from HBeAg-positive patients compared to those from HBeAg-negative patients (Fig. 2K). Further stratification analysis revealed that RBM25 expression was significantly lower in the immune-active (IA), inactive carrier (IC), and HBeAg-negative (ENEG) phases than in the immunotolerant (IT) phase, which is characterized by the highest level of viral replication (Fig. 2L). Additionally, correlation analysis showed a positive correlation between HBV RNA load and hepatic RBM25 mRNA levels ($r = 0.5085$, $P = 0.0001$) (Fig. 2M). These findings suggested that RBM25 was a key host factor that facilitated HBV replication.

RBM25 increases HBV RNA expression by enhancing cccDNA transcriptional activity

RBM25 is known as an RNA-binding protein (Gong et al., 2013). To investigate whether RBM25 influenced HBV RNA expression, we measured the levels of 3.5 kb and total HBV RNA by RT-qPCR, as well as the levels of 3.5 kb and 2.4/2.1 kb HBV RNA using Northern blot in HBV replicating and infected cell models. The results showed that RBM25 knockdown significantly decreased intracellular 3.5 kb and total HBV RNA levels (Fig. 3A and E), without affecting cccDNA levels (Supplementary Fig. S1D) or cell proliferation (Supplementary Fig. S1G) in HepG2 cells transiently transfected with prcccDNA/pCMV-Cre plasmids, while RBM25 overexpression significantly increased intracellular HBV RNA levels (Fig. 3B and E), without affecting cccDNA levels (Supplementary Fig. S1E) or cell proliferation (Supplementary Fig. S1H). Consistent findings were observed in HepAD38 cells following RBM25 knockdown or overexpression (Fig. 3C, D and Supplementary Fig. S1D, E). Furthermore, 3.5 kb and total HBV RNA levels were reduced on day 7 post-infection following RBM25 knockdown in HBV-infected HepG2-NTCP cells (Fig. 3F), while cccDNA levels remained unchanged (Supplementary Fig. S1F).

RBM25 has potential effects on mRNA stability. To elucidate the mechanism by which RBM25 facilitates HBV RNA expression, we first examined its effect on the decay kinetics of HBV RNA. Although RBM25 could bind to HBV RNA, as detected by RNA immunoprecipitation (RIP) assays (Supplementary Fig. S2A), the degradation rates of HBV RNA were unchanged following RBM25 knockdown or overexpression (Supplementary Fig. S2B, C). Next, we performed luciferase reporter assays to

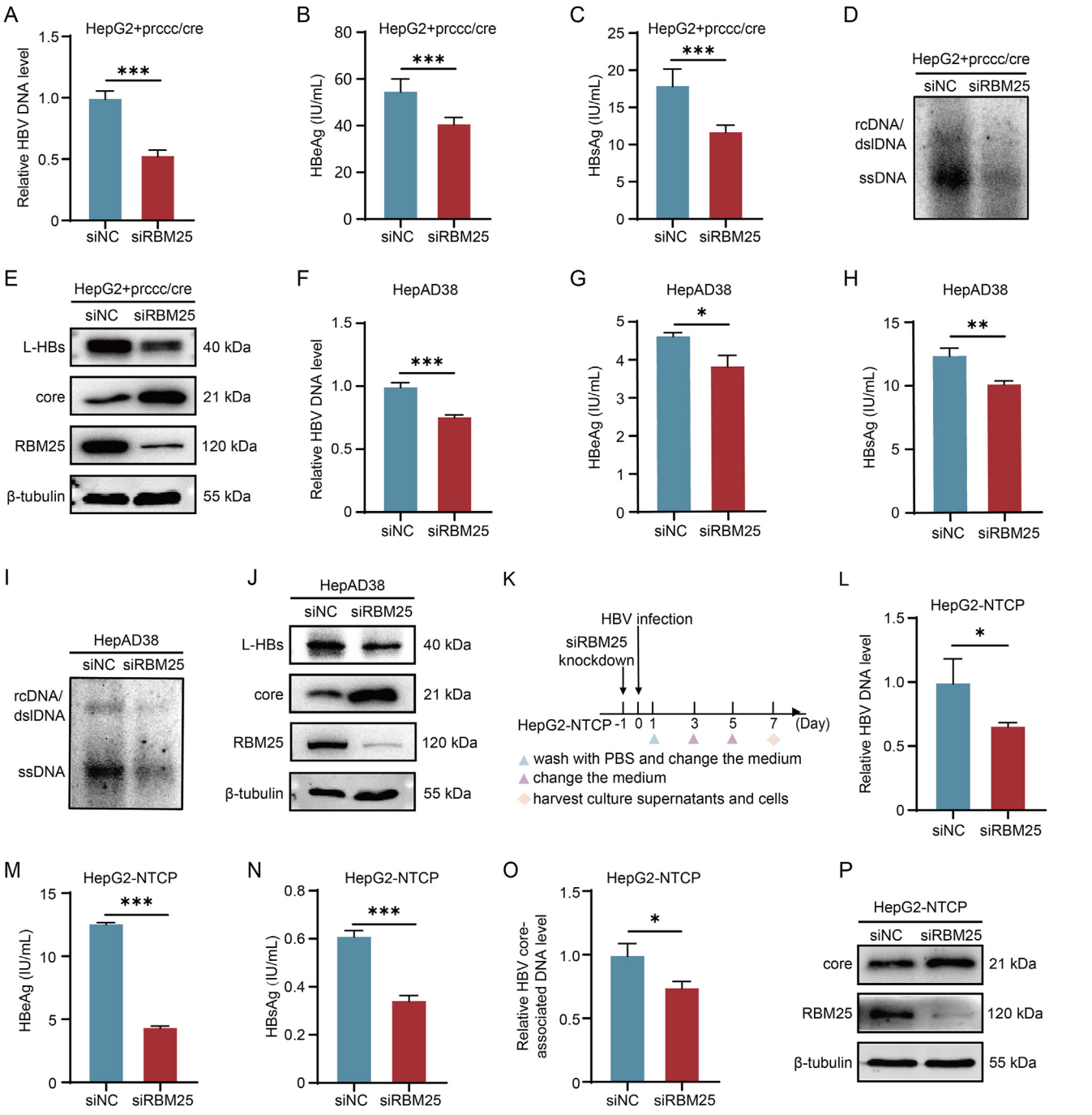

Fig. 1. Knockdown of endogenous RBM25 inhibits HBV replication. **A**–**E** HepG2 cells were transfected with siRBM25 or siNC, followed by the transfection of prcccDNA/pCMV-Cre plasmids. **A**–**C** Cells and culture supernatants were collected 48 h post-transfection. HBV DNA, HBeAg and HBsAg levels in the supernatants were measured by qPCR and CLIA. **D** HBV DNA replication intermediates levels were detected by Southern blot. **E** Intracellular protein levels were detected by Western blot. **F**–**J** HepAD38 cells were transfected with siRBM25 or siNC. The cells and culture supernatants were harvested 48 h post-transfection. Levels of HBV DNA, HBeAg, HBsAg, HBV DNA replication intermediates and intracellular protein were analyzed. **K** Schematic representation of the treatment process for HepG2-NTCP cells. **L**–**N** HBV DNA, HBeAg and HBsAg levels in the supernatants were measured by qPCR and CLIA. **O** Core-associated HBV DNA levels were analyzed by qPCR. **P** Intracellular proteins were detected by Western blot. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

detect the effects of RBM25 on the transcriptional activities of cccDNA. The results showed that RBM25 knockdown significantly suppressed the activities of the HBV BCP, CP, XP, and SpII, while RBM25 overexpression significantly enhanced these activities (Fig. 3G and H), suggesting that RBM25 increased HBV RNA expression through enhancing cccDNA transcription.

RBM25 enhances HBV transcription by interacting with cccDNA through its RE/RD and PWI domains

To determine whether RBM25 influenced promoter activity by binding to cccDNA, we next performed a DNA pull-down experiment. Biotinylated HBVcircle, a recombinant minicircle cccDNA model *in vitro*

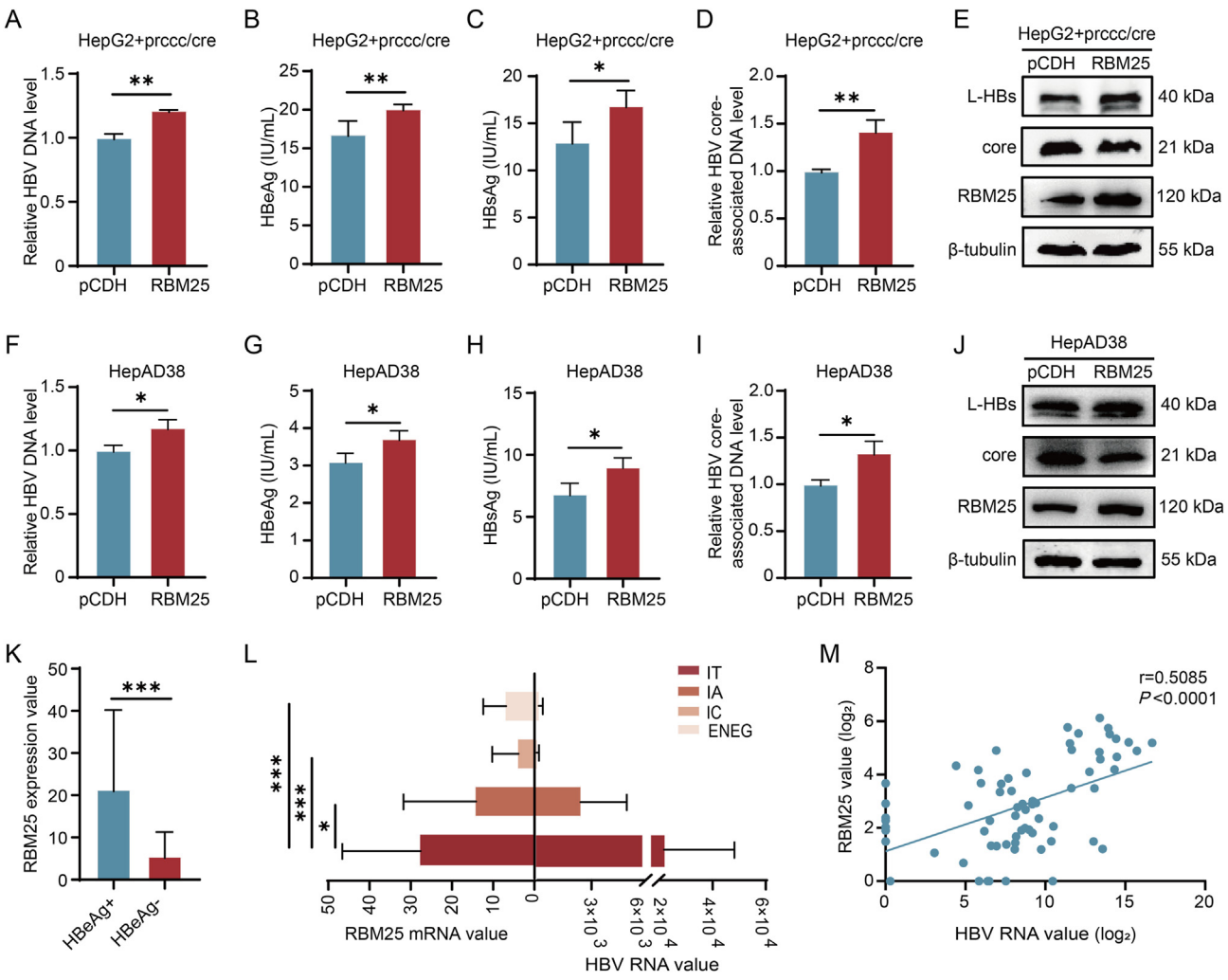

Fig. 2. Ectopic overexpression of RBM25 enhances HBV replication. **A**–**E** RBM25 was stably overexpressed in HepG2 cells, followed by the transfection of prcccDNA/pCMV-Cre plasmids. **A**–**C** HBV DNA, HBeAg and HBsAg levels in the supernatants were measured by qPCR and CLIA. **D** Core-associated HBV DNA levels were measured by qPCR. **E** Intracellular protein levels were detected by Western blot. **F**–**J** HepAD38 cells with stable RBM25 overexpression via lentiviral transduction were cultured for 48 h. Levels of HBV DNA, HBeAg, HBsAg, core-associated HBV DNA and intracellular protein were analyzed. **K** The hepatic RBM25 mRNA levels were analyzed between HBeAg-positive and HBeAg-negative patients using a published dataset (GEO: GSE230397). **L** RBM25 mRNA and HBV RNA levels were measured during IT, IA, IC, and ENEG phases. **M** Pearson's correlation analysis was performed to assess the relationship between RBM25 mRNA levels and HBV RNA load. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

(Yan et al., 2017), was co-incubated with nuclear extracts from HepG2 cells. Unlabeled HBVcircle and biotinylated pcDNA3.1 plasmid DNA served as negative controls. Compared to the negative controls, the biotinylated HBVcircle successfully captured RBM25 (Fig. 4A). This interaction was further reversely validated through chromatin immunoprecipitation (ChIP) assays in HepAD38 cells and HepG2 cells transfected with prcccDNA/pCMV-Cre plasmids (Fig. 4B and C). To further investigate whether RBM25's binding to cccDNA depends on specific DNA sequence, HepG2-RBM25 cells were transfected with prcccDNA/pCMV-Cre plasmids to generate rcccDNA. We fragmented rcccDNA using micrococcal nuclease (MNase) and then incubated it with Flag antibodies, followed by qPCR with primers targeting different regions of the rcccDNA. The results revealed an extensive binding of RBM25 to different regions of rcccDNA (Supplementary Fig. S3A), suggesting that its binding was in an HBV DNA sequence-independent manner. Collectively, the results indicated that RBM25 promoted HBV promoter activity by interacting with cccDNA.

RBM25 contains three distinct domains, each essential for its functionality by interactions with nucleic acids and proteins (Szymczyna et al., 2003). A series of RBM25 truncated mutants, including pCDH-ΔRRM-Flag, pCDH-ΔRE/RD-Flag, and pCDH-ΔPWI-Flag, were generated to identify the domain(s) responsible for mediating the binding to cccDNA (Fig. 4D). Following confirmation of the successful expression of both wild-type RBM25 and these truncated mutants in HepAD38 and HepG2 cells (Supplementary Fig. S4A, B), ChIP assays demonstrated that deletion of either the RE/RD or PWI domains significantly reduced RBM25's binding to cccDNA (Fig. 4E and F). Further experiments revealed that deletion of the RE/RD or PWI domains significantly impaired RBM25's ability to upregulate 3.5 kb and total HBV RNA levels (Fig. 4G and H), as well as HBV DNA, HBeAg, and HBsAg levels in the supernatants of HepG2 cells transiently transfected with prcccDNA/pCMV-Cre plasmids (Fig. 4I–K). Similar results were observed in HepAD38 cells (Supplementary Fig. S5A-E). These findings suggested that the RE/RD and PWI domains of RBM25 were essential for promoting HBV transcription through binding to cccDNA.

RBM25 promotes YY1 expression and facilitates HBV transcription through YY1-mediated acetylation of cccDNA-associated histones

Our data demonstrated that RBM25 promoted HBV replication by increasing transcriptional activity of all tested HBV promoters (Fig. 3G

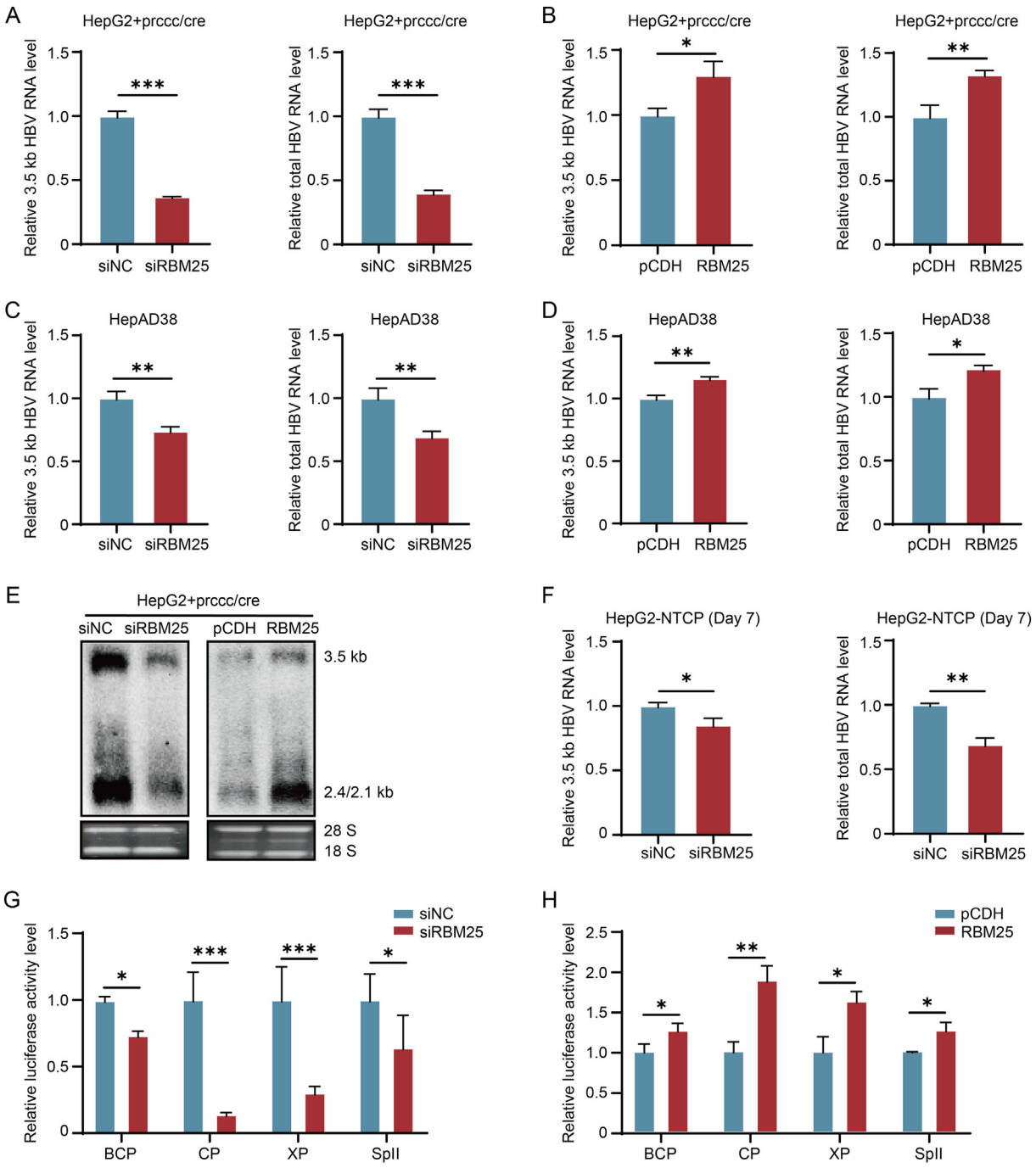

Fig. 3. RBM25 increases HBV RNA expression by enhancing cccDNA transcriptional activity. **A, B, E** HepG2 cells with RBM25 knockdown or overexpression were transfected with prcccDNA/pCMV-Cre plasmids. The cells were harvested 48 h post-transfection. Intracellular HBV RNA levels were measured by RT-qPCR and Northern blot. **C, D** HepAD38 cells with RBM25 knockdown or overexpression were cultured for 48 h. Intracellular HBV RNA levels were measured by RT-qPCR. **F** The intracellular 3.5 kb and total HBV RNA of HepG2-NTCP cells on day 7 post-infection were quantified by RT-qPCR. **G, H** HepG2 cells with RBM25 knockdown or overexpression were transfected with HBV promoter-luciferase plasmids. Promoter activity was measured by dual-luciferase assay. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

and H). Hepatitis B virus X protein (HBx) is a well-known transcriptional co-regulatory factor that regulates HBV transcription (Belloni et al., 2009). Given that RBM25 knockdown resulted in global transcription suppression, or vice versa, a prcccDNA/pCMV-Cre recombinant plasmid system lacking HBx was utilized to assess whether the effects of RBM25 were mediated through HBx. As expected, HBV replication was generally lower in the HBx-deficient group compared to the wild-type, confirming the system's efficacy. Nonetheless, even in the absence of HBx, RBM25 overexpression still significantly increased 3.5 kb and total HBV RNA levels (Supplementary Fig. S6A), while RBM25 knockdown decreased them (Supplementary Fig. S6D). Similar results were observed in the HBV DNA (Supplementary Fig. S6B, E) and HBeAg levels (Supplementary Fig. S6C, F) in the supernatants. These findings demonstrated that RBM25 facilitated HBV transcription by broadly upregulating promoter activity, independently of HBx.

RBM25 has been reported to mediate YY1 function in chromatin binding, DNA looping, and transcription (Xiao et al., 2019), and YY1 has been shown to enhance HBV replication (Zhou et al., 2024). To further

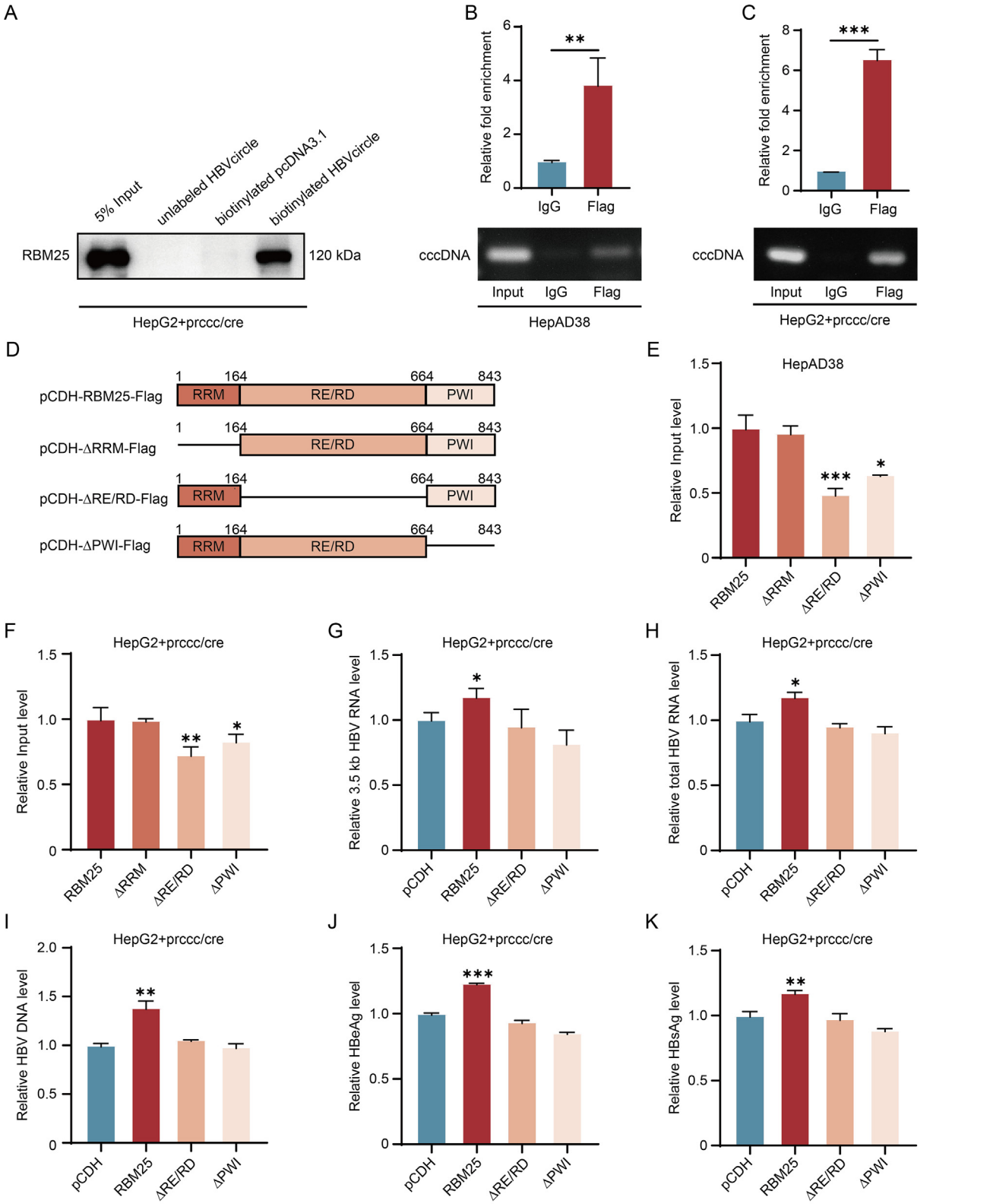

Fig. 4. RBM25 enhances HBV transcription by interacting with cccDNA through its RE/RD and PWI domains. **A** DNA pull-down assays were conducted using unlabeled HBVcircle, biotinylated pcDNA3.1 plasmid DNA, and biotinylated HBVcircle incubated with HepG2 nuclear extracts. RBM25 binding was detected by Western blot. **B, C** cccDNA ChIP assays were performed in HepAD38 and HepG2-rcccDNA cells overexpressing RBM25, and the results were analyzed by qPCR and PCR. **D** Schematic representation of RBM25 truncated mutants. **E–K** HepAD38 and HepG2 cells were transduced with lentiviral vectors expressing either wild-type RBM25 or its truncated mutants to establish stable cell lines, followed by the transfection of prcccDNA/pCMV-Cre plasmids into HepG2 cells. **E, F** cccDNA ChIP assays were performed using Flag antibodies, with results analyzed by qPCR. **G, H** Levels of 3.5 kb and total HBV RNA were quantified by RT-qPCR. **I–K** HBV DNA, HBeAg and HBsAg levels in the supernatants were measured by qPCR and CLIA. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

assess whether the effect of RBM25 on HBV transcription depends on YY1-mediated promoter activation, we designed three siRNAs specifically targeting the *YY1* gene and demonstrated that their combined use achieved the highest knockdown efficiency (Supplementary Fig. S7A). Then YY1 was knocked down in HepG2 cells (Fig. 5A) prior to RBM25 overexpression. YY1 knockdown significantly reduced 3.5 kb and total HBV RNA levels, and RBM25 overexpression failed to upregulate these levels in the absence of YY1 (Fig. 5B and C). This finding was further validated by the levels of HBV DNA, HBeAg, and HBsAg in the culture supernatants (Fig. 5D–F). Additionally, we first knocked down RBM25 and then overexpressed exogenous YY1 in HepG2 cells transfected with the prcccDNA/pCMV-Cre plasmid. The results showed that RBM25 knockdown significantly decreased intracellular total HBV RNA levels, as well as HBeAg and HBsAg in the culture supernatant. Notably, in RBM25 knockdown HepG2 cells, YY1 overexpression failed to upregulate these levels (Fig. 5G–I). In summary, these results suggested that RBM25 and YY1 synergistically interacted to enhance HBV replication.

Next, we explored the relationship between RBM25 and YY1. In HepAD38, HepG2-rcccDNA, and HepG2-NTCP cells, RBM25 knockdown significantly reduced YY1 expression at both mRNA and protein levels (Fig. 5J and K). Conversely, stable overexpression of RBM25 upregulated YY1 expression in HepAD38 and HepG2-rcccDNA cells (Fig. 5L and M). Furthermore, analysis of RNA-seq datasets from liver biopsy tissues of 69 CHB patients (GEO: GSE230397) revealed a strong positive correlation between RBM25 and YY1 expression ($r = 0.6545$, $P < 0.0001$) (Fig. 5N). However, knockdown or overexpression of YY1 had no effect on RBM25 mRNA and protein levels (Supplementary Fig. S7B–D). These results demonstrated that RBM25 promoted YY1 expression by enhancing its transcription.

YY1 is known to recruit histone acetylases or deacetylases to promoter regions, thereby modulating promoter activity (Wang et al., 2022). We found that RBM25 knockdown significantly reduced the binding of YY1 to cccDNA (Fig. 5O). Importantly, the ChIP assay showed that, although RBM25 knockdown did not affect trimethylation of histone H3 lysine 4 (H3K4me3) binding to cccDNA (Supplementary Fig. S8A), it significantly reduced the binding of acetylated H3 (ac-H3), acetylated H4 (ac-H4), and histone H3 lysine 27 acetylation (H3K27ac) with cccDNA (Fig. 5P–R). Furthermore, the knockdown of RBM25 had no significant effect on the protein levels of ac-H3, ac-H4 and H3K27ac (Supplementary Fig. S8B–D). These findings suggested that RBM25 promoted YY1 expression and enhanced its binding ability to cccDNA. YY1 further increased histone acetylation of cccDNA, thereby activating cccDNA transcription and promoting viral replication.

Silencing of RBM25 inhibits HBV replication in HBV-replicating mouse model

To evaluate the effect of RBM25 on HBV replication *in vivo*, CRISPR-sgRbm25-1 and CRISPR-sgRbm25-2 plasmids were constructed by inserting murine Rbm25 gene gDNA into the PX458 vector. Both plasmids significantly reduced Rbm25 protein levels in Hepa1-6 cells, with co-transfection resulting in a more pronounced decrease (Fig. 6A). Subsequently, the pBB4.5-HBV1.2 plasmid, in combination with CRISPR-sgRbm25-1 and CRISPR-sgRbm25-2 plasmids, was hydrodynamically injected into C57BL/6 mice. Blood and liver samples were collected at the specified time points (Fig. 6B). Mice were euthanized on day 7 post-injection for immunohistochemistry and on day 14 for mRNA and protein quantification. The results showed that Rbm25 knockdown significantly decreased serum HBeAg and HBsAg levels (Fig. 6C and D). Immunohistochemical analysis further demonstrated that Rbm25 knockdown significantly reduced *in-situ* HBsAg protein expression, while hepatitis B core antigen (HBcAg) levels increased (Fig. 6E and F). In the liver, CRISPR-sgRbm25 significantly reduced Rbm25 expression and suppressed Yy1 expression at both mRNA and protein levels compared to the control group (Fig. 6G and H), resulting in decreased 3.5 kb and total HBV RNA levels (Fig. 6I), consistent with our findings in the cell model. These findings suggested that silencing RBM25 inhibited HBV replication by downregulating YY1 expression in *in vivo* models of HBV replication.

HBV core protein upregulates RBM25 expression

According to our results above, although RBM25 overexpression upregulated HBV RNA levels and promoted HBV replication, it suppressed core protein levels. We speculated that the observed alterations in core protein levels following RBM25 modulation may be due to changes in protein stability. The results of protein turnover assays showed that RBM25 overexpression significantly shortened the half-life of core protein compared to the control cells (Fig. 7A and B), suggesting that RBM25 promoted the degradation of HBV core protein.

HBV has been reported to reshape the infection environment to facilitate virus replication, including upregulating host factors that promote replication and downregulating those that inhibit it (Turton et al., 2020). To test whether RBM25 expression was regulated by active HBV replication, we assessed RBM25 expression changes in HBV replicating and infection cells. The results showed that RBM25 expression was significantly elevated at both mRNA and protein levels in HepG2-rcccDNA, HepAD38 and HBV infected HepG2-NTCP cells compared with the control cells (Fig. 7C and D). This increase was localized to the nucleus rather than the cytoplasm (Fig. 7E), indicating that active HBV replication specifically enhanced RBM25 expression and nuclear translocation. To figure out how HBV regulated RBM25 expression, HBV proteins (Core, Pol, HBx, and HBs) were overexpressed in HepG2 cells. Among them, core protein rather than other viral proteins significantly upregulated RBM25 expression at both the mRNA and protein levels (Fig. 7F and G). To better describe the correlation between core protein and RBM25, we examined the expression levels of RBM25 and core protein in HepAD38 cells at 0, 2, 4, 6, and 8 days following tetracycline (Tet) withdrawal. The results indicated that as the expression level of the core protein increased, the expression of RBM25 was also upregulated (Fig. 7H). Additionally, to better mimic the natural infection process of HBV, HepG2-NTCP cells were infected with concentrated HBV virions. Cells were collected at 0, 3, 6, and 9 days post-infection to analyze RBM25 and core protein expression. The results were consistent with those observed in HepAD38 cells (Fig. 7I). These findings further supported the hypothesis that the core protein initially upregulated RBM25 expression during HBV infection.

Taken together, these findings indicated that RBM25 overexpression downregulated core protein expression at the post-transcriptional level by reducing its stability. RBM25 expression was upregulated and translocated to the nucleus following core protein expression and accumulation, revealing a reciprocal regulatory mechanism between HBV replication and host factor RBM25.

DISCUSSION

In this study, we demonstrated that RBM25 significantly increased the production of HBV progeny viruses in both HBV-replicating cell and mouse models. Mechanistically, RBM25 enhanced YY1 expression and recruited YY1 to cccDNA, facilitating the recruitment of ac-H3, ac-H4, and H3K27ac to activate cccDNA transcription. Interestingly, changes in core protein levels due to RBM25 knockdown or overexpression did not align with trends observed in other virological indicators. Further analysis revealed that RBM25 promoted core protein degradation, leading to a significant reduction in core protein levels. On the flip side, we also observed that HBV replication upregulated RBM25 expression, with this upregulation being dependent on core protein levels. Therefore, the reciprocal regulation between RBM25 and core protein maintains HBV replication.

Our results showed that depletion of RBM25 inhibited HBV replication, while its overexpression enhanced viral replication. These findings indicated that RBM25 was a key host factor involved in HBV replication. RBM25 has primarily been associated with mRNA stability and

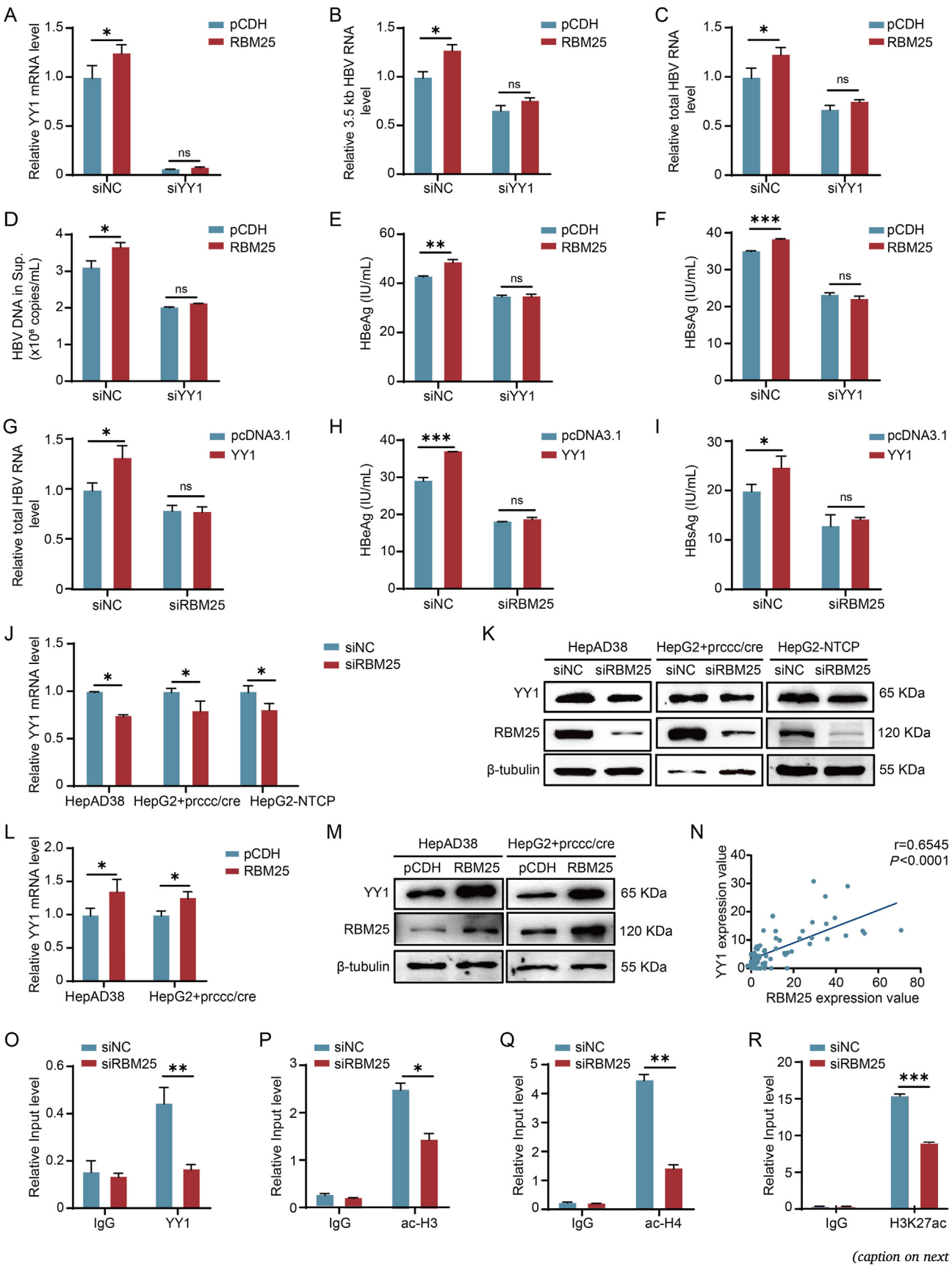

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Fig. 5. RBM25 promotes YY1 expression and facilitates HBV transcription through YY1-mediated acetylation of cccDNA-associated histones. **A–F** HepG2 cells with YY1 knockdown were transfected with pCDH-RBM25-Flag plasmids and prcccDNA/pCMV-Cre plasmids. **A–C** YY1 mRNA, 3.5 kb HBV RNA and total HBV RNA levels were quantified by RT-qPCR. **D–F** HBV DNA, HBeAg and HBsAg levels in the supernatants were measured by qPCR and CLIA. **G–I** HepG2 cells with RBM25 knockdown were co-transfected with pcDNA3.1-YY1 and prcccDNA/pCMV-Cre plasmids. **G** Total HBV RNA levels were quantified by RT-qPCR. **H, I** HBeAg and HBsAg levels in the supernatants were measured by CLIA. **J, K** RBM25 was knocked down in HepAD38, HepG2 and HepG2-NTCP cells, followed by transfection of prcccDNA/pCMV-Cre plasmids into HepG2 cells. **L, M** RBM25 was stably overexpressed in HepAD38 and HepG2 cells, followed by transfection of prcccDNA/pCMV-Cre plasmids into HepG2 cells. YY1 expression levels were measured by RT-qPCR and Western blot. **N** Pearson's correlation analysis was performed on a published dataset of 69 CHB patients (GEO: GSE230397) to assess the relationship between RBM25 and YY1 mRNA levels. **O–R** HepG2 cells with RBM25 knockdown were transfected with prcccDNA/pCMV-Cre plasmids, followed by cccDNA ChIP assays using anti-YY1, anti-ac-H3, anti-ac-H4, and anti-H3K27ac antibodies. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, ns, not significant.

alternative splicing regulation (Kanno et al., 2017; Zhou et al., 2008). It has been reported to modulate the splicing of key genes related to apoptosis, ion channel function, and cytokine activity (Sénéchal et al., 2023). In human prostate cancer, RBM25 binding directly to an Amotl1-derived circRNA (circAMOTL1L) has been observed to impact the stability of mRNAs associated with apoptosis (Yang et al., 2019,b). We performed CCK-8 assays, which showed that neither RBM25 knockdown nor overexpression affected cell proliferation in HepG2 cells transiently transfected with prcccDNA/pCMV-Cre plasmids. Taken together, these data suggested that RBM25 did not exert its effects on HBV replication through apoptosis and/or proliferation. In our study, although RBM25 bound to HBV RNA, we found that the interaction between RBM25 and HBV RNA did not lead to any detectable changes in RNA stability. Furthermore, RNA-Seq analysis revealed that RBM25 knockdown significantly reduced the ratio of the HBV splice variant Sp1 relative to total 3.5 kb HBV RNA (data not shown), suggesting that RBM25 promoted HBV RNA splicing. However, the spliced HBV RNA transcript Sp1 is known to inhibit HBV replication (Sozzi et al., 2022), which contrasts with our observations. This implies that the role of RBM25 in regulating HBV replication may operate independently of its effects on HBV RNA splicing.

The transcription of cccDNA is regulated by various transcription factors, histone-modifying enzymes, non-coding RNAs, RNA-binding proteins, and other regulatory molecules (Ren et al., 2020; Teng et al., 2021; Yang et al., 2019,b). RBM25 has recently been reported to predominantly localize in active chromatin regions, where it directly binds to DNA to regulate gene transcription. Our findings demonstrated that RBM25 enhanced HBV promoter activity, leading to an increase in cccDNA transcription. Additionally, through DNA pull-down and ChIP assays, we further confirmed that RBM25 bound to cccDNA in an HBV DNA sequence-independent manner through its RE/RD and PWI domains, enhancing the activity of multiple promoters. We also ruled out the global transcriptional enhancement effect of the transcriptional co-regulatory factor HBx. YY1 is a widely expressed transcription regulator that modulates promoter activity through histone modifications (Dandri, 2020; Wang et al., 2023). RBM25 is known to mediate YY1 function in chromatin binding, DNA looping, and transcription. In the liver tissues of CHB patients, RBM25 mRNA exhibited a significant positive correlation with YY1 mRNA. Further investigations revealed that RBM25 enhanced YY1 expression, whereas YY1 did not significantly affect RBM25 expression levels. We observed that YY1 depletion attenuated the regulatory effect of RBM25 on cccDNA transcription, indicating that RBM25 regulated HBV transcription in a YY1-dependent manner. A recent study by Xiao et al. demonstrated that RBM25 could directly interact with YY1 through co-immunoprecipitation experiments (Xiao et al., 2019). According to previous literature on the functional domains of RBM25, the RE/RD domain is primarily involved in protein-protein interactions (Fortes et al., 2007), while the PWI domain is mainly responsible for interactions with RNA and DNA (Gong et al., 2013; Szymczyna et al., 2003). Based on these findings, we hypothesized that RBM25 may bind to cccDNA via its PWI domain, while simultaneously interacting with YY1 through its RE/RD domain, thereby mediating YY1's regulation of cccDNA transcription.

Histone modifications shape cccDNA structure and function, influencing HBV expression at various stages of the viral life cycle (Hensel et al., 2017; Tropberger et al., 2015). Among these modifications, H3K4me3, ac-H3, ac-H4, and H3K27ac are known to relax and open the minichromosomal structure, promoting transcriptional activity (Gao et al., 2020; Pollicino et al., 2006). Our findings also showed that RBM25 facilitated the recruitment of ac-H3, ac-H4, and H3K27ac binding to cccDNA by YY1, thereby activating cccDNA transcription.

We identified an unexpected finding that core protein levels did not correlate with trends observed in other virological markers. Core protein is essential for nearly every stage of the HBV life cycle, including nucleocapsid formation and pgRNA packaging (Zlotnick et al., 2015). However, recent studies have shown that the core protein does not affect histone modifications, transcription factors, or transcriptional activity. It is considered to be present in excess (Zhong et al., 2022). This may explain why decreased core protein levels following RBM25 overexpression did not impact cccDNA transcription or HBV replication. Mechanistic analysis revealed that RBM25 promoted core protein degradation. The degradation pathways of the HBV core protein include the ubiquitin-proteasome pathway, the autophagy-lysosome pathway, and endoplasmic reticulum-associated degradation (Miyakawa et al., 2022; Qian et al., 2012; Wang et al., 2019). Among these, the ubiquitin-proteasome pathway is the primary pathway for HBV core protein degradation. RBM25 is known to regulate transcription, pre-mRNA splicing, mRNA stability, and translation, thereby influencing the expression of target genes (Zhou et al., 2008). We speculated that, although RBM25 did not directly interact with the core protein (Supplementary Fig. S9A), it may indirectly regulate core protein degradation by regulating the expression of an intermediate host factor. Additionally, we found that the core protein, but not other proteins, transcriptionally promoted RBM25 expression, which may contribute to the RBM25 recognition by HBV. During chronic infection, the viral core protein or other factors, such as inflammatory factors, may induce RBM25 upregulation, thereby facilitating the establishment of chronic HBV infection and contributing to persistent HBV infection. In summary, our study highlights the interaction between core protein and RBM25 in maintaining HBV replication.

This study highlights the complex and dynamic interactions between HBV replication and host factor regulation. Upon infection, RBM25 expression was upregulated and translocated to the nucleus following core protein expression and accumulation. In the nucleus, RBM25 upregulated YY1 expression and facilitated YY1 recruitment to cccDNA. Subsequently, YY1 recruited ac-H3, ac-H4 and H3K27ac to cccDNA, thereby enhancing HBV transcription. However, the continuous accumulation of RBM25 initiated a negative feedback mechanism through accelerating core protein degradation. Furthermore, RBM25 knockdown significantly reduced YY1 expression and its binding to cccDNA. This, in turn, diminished YY1-mediated recruitment of ac-H3, ac-H4, and H3K27ac to cccDNA, ultimately decreasing HBV transcription, while simultaneously inhibiting the degradation of the core protein (Fig. 8).

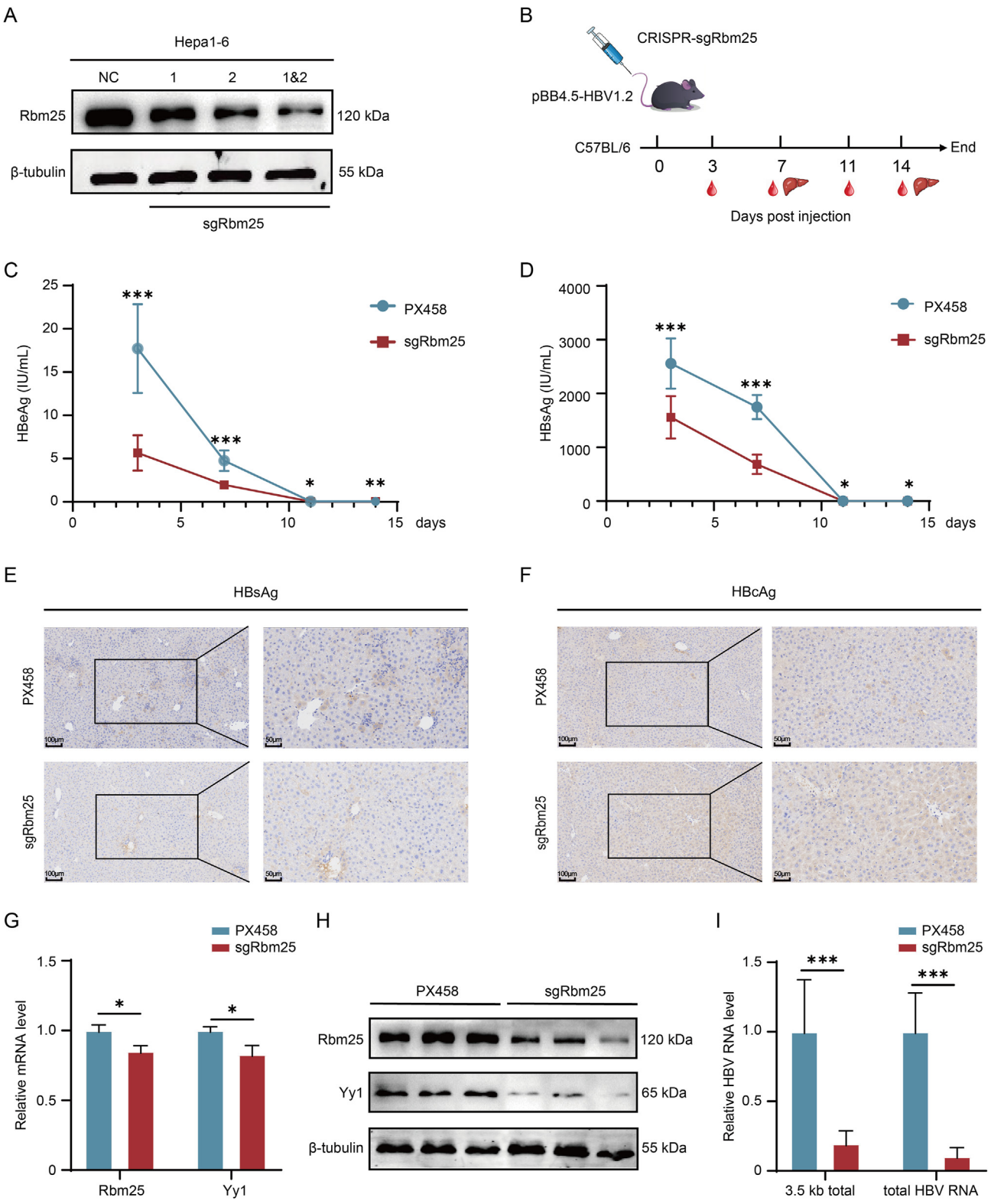

Fig. 6. Silencing of RBM25 inhibits HBV replication in HBV-replicating mouse model. **A** Hepa1-6 cells were transfected with CRISPR-sgRbm25 plasmids, and RBM25 protein levels were analyzed by Western blot. **B** Schematic representation of the experimental procedure. C57BL/6 mice were hydrodynamically injected with pBB4.5-HBV1.2 plasmid and CRISPR-sgrbm25-1/2 plasmids, or the PX458 vector (n = 10 per group). Blood and liver samples were collected at the indicated time points for analysis. **C, D** Serum levels of HBeAg and HBsAg were measured by CLIA. **E, F** Hepatic HBsAg and HBcAg expression levels were analyzed by immunohistochemical staining. Scale bar: 50 μm or 100 μm. **G, I** Hepatic Rbm25 mRNA, Yy1 mRNA, 3.5 kb HBV RNA, and total HBV RNA levels were quantified by RT-qPCR. **H** Intracellular protein levels were analyzed by Western blot. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

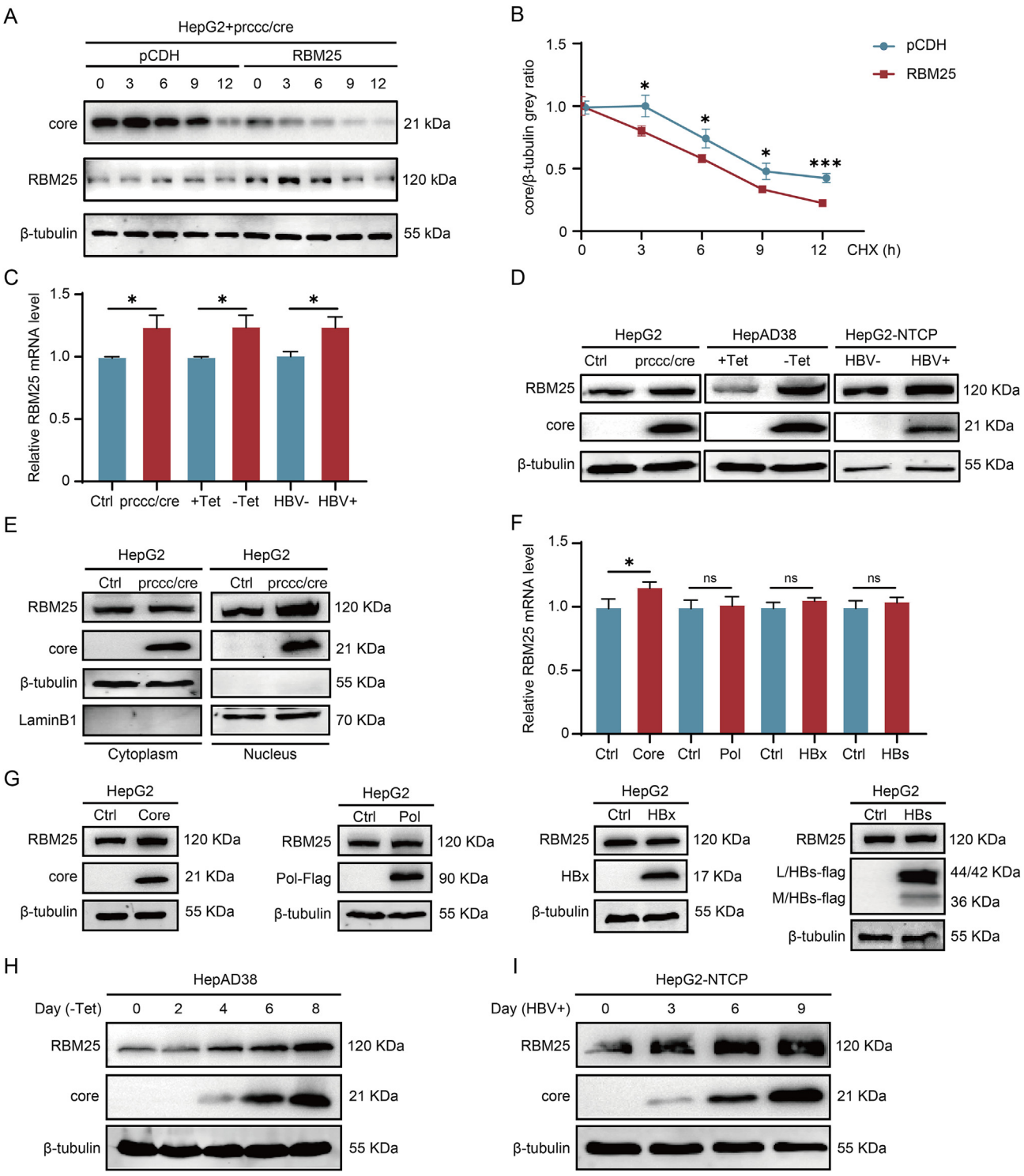

Fig. 7. HBV core protein upregulates RBM25 expression. **A, B** HepG2 cells overexpressing RBM25 were transfected with prcccDNA/pCMV-Cre plasmids for 48 h, followed by treatment with cycloheximide (CHX) for 0, 3, 6, 9, and 12 h. The dynamic changes in core protein levels were analyzed by Western blot, and the corresponding gray value curves were quantified. Core protein levels in both the RBM25 overexpression and control group were normalized to their respective baseline (0 h) levels for comparison. **C, D** HepG2 cells were transfected with prcccDNA/pCMV-Cre plasmids, HepAD38 cells were treated with Tet removal to induce HBV replication, and HepG2-NTCP cells were infected with HBV virions. RBM25 expression was quantified by RT-qPCR and Western blot. **E** HepG2 cells transfected with prcccDNA/pCMV-Cre plasmids were analyzed for RBM25 protein levels in the cytoplasm and nucleus by Western blot. **F, G** HepG2 cells were transfected with plasmids expressing different viral proteins or a control vector. RBM25 mRNA and protein levels were determined by RT-qPCR and Western blot. **H** HepAD38 cells were harvested at 0, 2, 4, 6, and 8 days following Tet removal to induce HBV replication. The expression levels of RBM25 and HBV core protein were analyzed by Western blot. **I** HepG2-NTCP cells were infected with concentrated HBV virions. Cells were collected at 0, 3, 6, and 9 days post-infection for analyzing the expression levels of RBM25 and core protein by Western blot. $^{*}P < 0.05$, $^{***}P < 0.001$, ns, not significant.

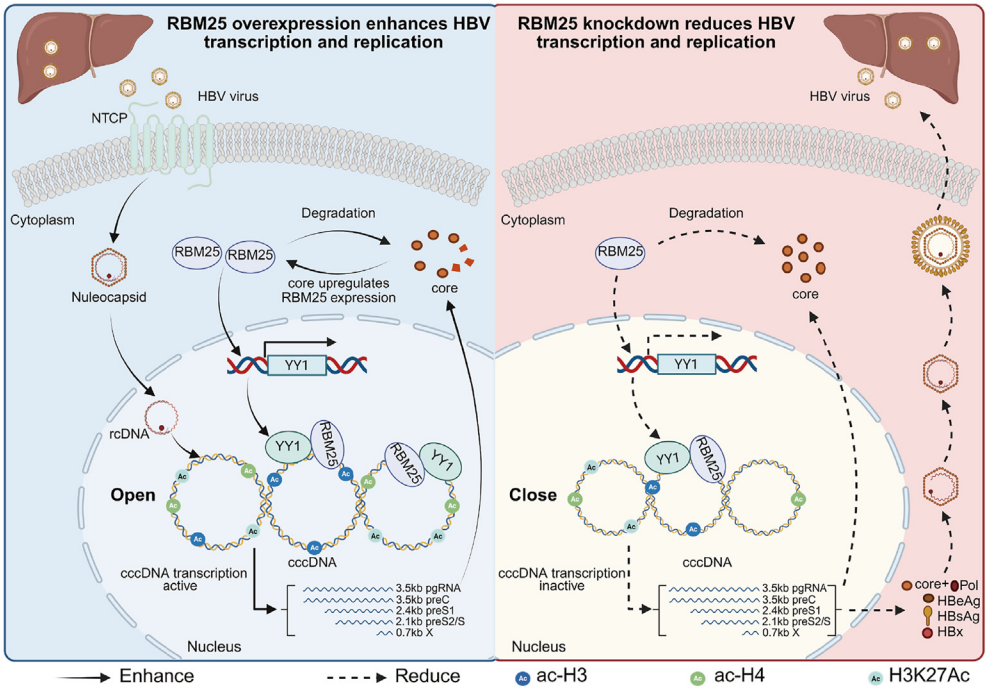

Fig. 8. Schematic diagram of the proposed mechanism. RBM25 promotes HBV cccDNA transcription and replication by regulating YY1.

CONCLUSIONS

In conclusion, these findings identified RBM25 as a novel host factor that supported HBV replication and revealed a previously uncharacterized role of RBM25 in regulating HBV cccDNA transcription. These results provide new insights into the interaction between host factors and HBV.

MATERIALS AND METHODS

Cell culture

HEK293T cells (ATCC), HepG2 cells (ATCC), HepG2-NTCP cells (Michailidis et al., 2017) (gift from professor Kuanhui Xiang), and HepAD38 cells (Ladner et al., 1997) (gift from professor Ningshao Xia) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Gibco) and 1% penicillin/streptomycin (Gibco), and cultured at 37 °C with 5% CO_2. DNA and siRNA transfections were performed using Lipofectamine 2000 and Lipofectamine RNAiMAX (Invitrogen), respectively. The sequences of siRNAs were listed in Supplementary Table S1.

Plasmids

Plasmids pBB4.5–1.2 × HBV (genotype C, p-HBV1.2), pGL3-Basic, pGL3-BCP-Luc, pGL3-CP-Luc, pGL3-XP-Luc, pGL3-SpII-Luc were previously maintained and constructed in our laboratory. The prcccDNA and pCMV-Cre plasmids were gifts from Professor Qiang Deng (Qi et al., 2014). The sgRNA/Cas9 dual expression vector pSpCas9(BB)-2A-GFP (PX458) was obtained from Addgene (Cambridge, MA). The sequence for sgRNA targeting mouse Rbm25 was inserted into PX458 to generate CRISPR-Cas9-sgRbm25. The RBM25 expression plasmid (pCDH-RBM25-Flag) and YY1 expression plasmid (pcDNA3.1-YY1-Flag) were constructed by cloning their coding sequence into pCDH-Flag or pcDNA3.1-Flag vector. RBM25 coding sequence with deletions in the RRM, RE/RD, and PWI domains was individually cloned into the pCDH-Flag vector to generate RBM25 truncation plasmids (pCDH-ΔRRM-Flag, pCDH-ΔRE/RD-Flag, pCDH-ΔPWI-Flag).

Lentiviral production and establishment of stable cell lines

To produce lentivirus, HEK293T cells were co-transfected with packaging plasmids and either the pCDH-RBM25-Flag or pCDH-Flag vector plasmid. The supernatants containing lentivirus were collected 48 h post-transfection. The cells were then incubated with lentivirus in the presence of 2 μg/mL polybrene (Sigma) for 24 h, followed by the addition of fresh medium containing puromycin (Sigma). The stably transduced cell lines were designated as HepAD38-RBM25 and HepG2-RBM25, respectively.

Virus preparation and cell infection

HBV particles were collected from the supernatants of HepAD38 cells by PEG8000 (Sigma) precipitations, as previously described (Michailidis et al., 2017). HepG2-NTCP cells were pre-cultured in medium containing 2% Dimethyl Sulfoxide (DMSO) (Sigma) and 3% FBS for 24 h. HBV particles were then added to the cells and incubated for 24 h.

Western blot

Western blot was performed as previously described (Gu et al., 2024). Cells were homogenized in lysis buffer (Beyotime) to extract total protein. Proteins were separated via SDS-PAGE gels and transferred to PVDF membranes (Millipore Crop). The membranes were blocked with 5% skim milk for 2 h before incubation with primary antibodies. The antibodies used were listed in Supplementary Table S2.

Quantification of HBsAg and HBeAg

The levels of HBsAg and HBeAg in the supernatants and serum were measured by chemiluminescent immunoassay (CLIA) using the corresponding kit (Shenzhen New Industries Biomedical Engineering Co., Ltd).

Extraction and quantification of HBV DNA and RNA

Supernatant HBV DNA was extracted using the Viral DNA/RNA Kit (TransGen). Total HBV RNA was extracted using FreeZol Reagent

(Vazyme) according to the manufacturer's instructions and reverse transcribed to cDNA with HiScript III 1st Strand cDNA Synthesis Kit (Vazyme). Supernatant HBV DNA and total HBV RNA were quantified by Roche LightCycler system and SYBR green system (Genstar). The primers sequences were listed in Supplementary Table S3.

Northern and Southern blotting analysis

Northern and Southern blotting analyses were performed as previously described (Tan et al., 2024; Wang et al., 2016). Briefly, total RNA and DNA samples were separated by electrophoresis and transferred onto nylon membranes. After UV crosslinking and prehybridization, the membranes were hybridized with HBV probe. Following blocking and incubation with secondary antibody, the membranes were incubated with CDP-star (Roche) and exposed using a DIG-Northern Starter kit (Roche).

Luciferase activity assay

The pGL3-Basic plasmids containing the HBV promoter were transfected into HepG2 cells. Luciferase activity was measured using the Dual-Luciferase® Reporter Assay System Kit (Promega).

DNA pull-down experiment

The HBVcircle was biotinylated using the Label IT® Nucleic Acid Labeling Kits (Mirus) following the manufacturer's instructions. Biotin-labeled HBVcircle was incubated with nuclear extracts from HepG2 cells and subsequently recovered using streptavidin-coated magnetic beads. Proteins potentially binding to the HBVcircle were identified by Western blot.

Chromatin immunoprecipitation (ChIP)

ChIP assays were performed following manufacturer's instructions (ABclonal). Cells were crosslinked with formaldehyde, followed by MNase (Cell Signaling Technology) digestion or sonication. The nuclear extracts were incubated with the antibodies overnight, followed by incubation with Protein A/G magnetic beads (Invitrogen). The immunoprecipitated chromatin was analyzed by qPCR and electrophoresis. The primers for qPCR and PCR, as well as the antibodies, were listed in Supplementary Tables S2 and S3.

Mouse experiments

C57BL/6 mice were obtained from Peking University Health Science Center. The mice were subjected to hydrodynamic injection (HDI) via the tail vein with pBB4.5-HBV1.2 plasmid and CRISPR-sgRbm25, or the vector control PX458 (n = 10 per group). Blood and liver samples were collected at the indicated time points. All animal experiments were conducted according to the relevant regulations and guidelines, and were approved by the Animal Ethics Committee of Peking University Health Science Center, Beijing, China (Approval No. LA2022396).

Immunohistochemical staining

Immunohistochemical staining for HBsAg and HBcAg was performed on all liver tissue samples following previously described methods (Gu et al., 2024). Mouse anti-HBs and anti-HBc antibodies were obtained from Maixin Biotechnology Development Ltd (Fuzhou).

Statistical analysis

Statistical analysis was performed using GraphPad Prism 7.0 software (GraphPad Software, USA). The results were analyzed using Student's unpaired two-tailed *t*-test or one-way ANOVA. The nonparametric Spearman's rank test was employed for correlation analysis. Significant difference was defined as $P < 0.05$.

DATA AVAILABILITY

All the data generated during the current study are included in the manuscript. Original data and materials will be available upon requests.

ETHICS STATEMENT

All animal experiments in this study was approved by the Institutional Review Board of Peking University (Approval No. LA2022396).

AUTHOR CONTRIBUTIONS

Fengmin Lu, Xiangmei Chen and Yukun Li: study concept and design and interpretation of data; Fengmin Lu, Xiangmei Chen, Yukun Li, Ziyuan Ma, Guochao Wei, Lin Wang, Yongzhen Liu, Xiaojing Zhang and Shourong Liu: drafting of the manuscript; Yukun Li, Tianhao Mao, Liwei Zheng, Zhao Zhou, Qianqian Jiang, Xinyu Du, Xin Liu, Ting Zhang: experiments; Fengmin Lu, Xiangmei Chen and Guochao Wei: obtained funding. All authors have read and agreed to the published version of the manuscript.

DECLARATION OF COMPETING INTEREST

The authors declare no conflicts of interest. Prof. Fengmin Lu is an editorial board member for *Virologica Sinica* and was not involved in the editorial review or the decision to publish this article.

ACKNOWLEDGEMENTS

This study was supported by the National Key R&D Program of China (No.2022YFA1303600 and No. 2023YFC2306800), the National Natural Science Foundation of China (No. 82372235 and No. 82272315), and the Sanming Project of Medicine in Shenzhen (No. SZSM202311032). In addition, we thank Professor Qiang Deng for providing the prcccDNA and pCMV-Cre plasmids.

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data to this article can be found online at https://doi.org/10.1016/j.virs.2025.05.004.

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