HEPATOLOGY

HEPATOLOGY, VOL. 66, NO. 2, 2017

PRMT5 Restricts Hepatitis B Virus Replication Through Epigenetic Repression of Covalently Closed Circular DNA Transcription and Interference With Pregenomic RNA Encapsidation

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Chronic hepatitis B virus (HBV) infection remains a major health problem worldwide. The covalently closed circular DNA (cccDNA) minichromosome, which serves as the template for the transcription of viral RNAs, plays a key role in viral persistence. While accumulating evidence suggests that cccDNA transcription is regulated by epigenetic machinery, particularly the acetylation of cccDNA-bound histone 3 (H3) and H4, the potential contributions of histone methylation and related host factors remain obscure. Here, by screening a series of methyltransferases and demethylases, we identified protein arginine methyltransferase 5 (PRMT5) as an effective restrictor of HBV transcription and replication. In cell culture–based models for HBV infection and in liver tissues of patients with chronic HBV infection, we found that symmetric dimethylation of arginine 3 on H4 on cccDNA was a repressive marker of cccDNA transcription and was regulated by PRMT5 depending on its methyltransferase domain. Moreover, PRMT5-triggered symmetric dimethylation of arginine 3 on H4 on the cccDNA minichromosome involved an interaction with the HBV core protein and the Brg1-based human SWI/SNF chromatin remodeler, which resulted in down-regulation of the binding of RNA polymerase II to cccDNA. In addition to the inhibitory effect on cccDNA transcription, PRMT5 inhibited HBV core particle DNA production independently of its methyltransferase activity. Further study revealed that PRMT5 interfered with pregenomic RNA encapsidation by preventing its interaction with viral polymerase protein through binding to the reverse transcriptase–ribonuclease H region of polymerase, which is crucial for the polymerase–pregenomic RNA interaction. *Conclusion*: PRMT5 restricts HBV replication through a two-part mechanism including epigenetic suppression of cccDNA transcription and interference with pregenomic RNA encapsidation; these findings improve the understanding of epigenetic regulation of HBV transcription and host–HBV interaction, thus providing new insights into targeted therapeutic intervention. (HEPATOLOGY 2017;66:398-415).

Hepatitis B virus (HBV) is a hepatotropic, partially double-stranded DNA virus. HBV infection remains a serious global health problem, with more than 240 million people chronically infected. Upon infection, relaxed circular DNA in the HBV virion is delivered into the nucleus and converted into covalently closed circular DNA (cccDNA), which then serves as a template to transcribe pregenomic RNA (pgRNA) and other subgenomic viral RNAs. The 5′-ε signal within pgRNA is recognized and bound by HBV polymerase (Pol), which is required for subsequent pgRNA

Abbreviations: cccDNA, covalently closed circular DNA; ChIP, chromatin immunoprecipitation; CMV, cytomegalovirus; CoIP, coimmunoprecipitation; dHepaRG, differentiated HepaRG; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; H3/H4, histones 3 and 4; H3K9me3, H3K9 trimethylation; H4R3me2a, asymmetric dimethylation on histone 4 arginine 3; H4R3me2s, symmetric dimethylation on histone 4 arginine 3; HBV, hepatitis B virus; HBc, HBV core protein; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; HBx, hepatitis B x protein; pgRNA, pregenomic RNA; PHH, primary human hepatocyte; Pol, polymerase; PRMT5, protein arginine methyltransferase 5; RIP, RNA immunoprecipitation; RT-RH, reverse transcriptase–ribonuclease H; sh, short hairpin; WT, wild type.

Received September 19, 2016; accepted February 20, 2017.
Additional Supporting Information may be found at onlinelibrary.wiley.com/doi/10.1002/hep.29133/suppinfo.

encapsidation into a newly forming capsid with HBV core protein (HBc) in the cytoplasm. Mature nucleocapsids with HBV relaxed circular DNA which is reverse-transcribed from pgRNA are either released from the cell after being enveloped with HBV surface proteins or recycled to the cccDNA reservoir in the nucleus. The cccDNA is crucial for persistent HBV infection and recurrence upon the discontinuation of treatment. Current therapies successfully suppress viral replication, but their inhibitory effects on stably existing cccDNA are limited. Identification of the mechanisms and factors involved in silencing or elimination of cccDNA could allow the development of more precisely targeted therapeutic strategies.[1]

The cccDNA organizes into a minichromosome with histone 3 (H3) and H4 proteins and nonhistone proteins, such as HBc, hepatitis B x protein (HBx), and host transcription factors.[2-4] Accumulating evidence suggests that the host epigenetic mechanisms, including DNA methylation and DNA-bound histone modifications, play vital roles in the regulation of cccDNA transcriptional activity.[5,6] It has been suggested that the acetylation status of H3 and H4 on the HBV minichromosome is associated with cccDNA transcriptional activity and that the histone acetyltransferase CBP/p300 and histone deacetylase 1 are involved in the regulation of HBV transcription.[4,7] Moreover, it has been shown that interferon-α can result in hypoacetylation of cccDNA-bound histones and recruitment of transcriptional corepressors to cccDNA.[7-9] In addition to histone acetylation, histone methylation can regulate DNA transcription and has been shown to be related to the replication of viruses such as herpes simplex virus-1, Epstein-Barr virus, and human cytomegalovirus (CMV), whose nucleic acids also organize into nucleosomal structures.[10-12] However, the effect of histone methylation on regulation of HBV cccDNA function and the host factor(s) involved remain obscure.

Here, by screening a series of methyltransferases and demethylases, we identified protein arginine methyltransferase 5 (PRMT5) as a restrictor of HBV replication that can preferentially catalyze the symmetric dimethylation of arginine 3 on H4 (H4R3me2s) on cccDNA through its methyltransferase activity to epigenetically silence cccDNA transcription and, in a methyltransferase-independent manner, interferes with HBV pgRNA encapsidation and thus inhibits HBV DNA production.

Materials and Methods

CELL CULTURE, VIRUSES, AND INFECTION

The HBV-producing stable cell line HepG2.117 and the HepG2-NTCP cell line were kindly provided

*These authors contributed equally to this work.

Supported by the "Twelfth Five-Year" National Key Technology Research and Development Programs of China (2012ZX10002007), the National Natural Science Foundation of China (81461130019), and the German Research Foundation (SFB/Transregio TRR60).

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DOI 10.1002/hep.29133

Potential conflict of interest: Dr. Levrero consults for Roche and Assembly. He advises Gilead, Sanofi, and Galapagos. He received grants from Bristol-Myers Squibb.

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by Prof. Mengji Lu (University Hospital Essen) and Prof. Stephan Urban (University Hospital Heidelberg), respectively. HepaRG cells and primary human hepatocytes (PHHs) were purchased from Biopredic International (France) and RIDL (China), respectively. Huh7 and HEK293 cells were obtained from the Cell Bank of the Chinese Academy of Sciences. Cell cultures are described in the Supporting Information. The HBc protein–deficient virus (HBV-ΔHBc), adeno-PRMT5, adeno-PRMT5Δ, adeno-PRMT5 short hairpin RNA (shPRMT5), adeno-NES-PRMT5, and adeno-shBrg1 were produced as described in the Supporting Information. HBV infection of differentiated HepaRG (dHepaRG) cells mainly followed the published protocols.[13] dHepaRG cells were transduced by incubation with the indicated adenoviral vectors for 16 hours.

PLASMIDS AND REAGENTS

Plasmids used for transfection are listed in Supporting Table S3. The constructions of the plasmids are described in the Supporting Information. The antibodies used in this study are listed in Supporting Table S4. The small interfering RNAs targeting the indicated genes (Supporting Table S5) and the control small interfering RNA were purchased from Ribobio.

PATIENTS

The clinical and virological characteristics of the patients enrolled in this study are summarized in Supporting Table S2. Liver tissue samples were obtained by percutaneous needle biopsy, immediately frozen in liquid nitrogen, and stored at −80°C. Written informed consent was obtained from all subjects prior to participation, and the study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved and supervised by the ethics committee of the Shanghai Public Health Clinical Center of Fudan University.

HBV cccDNA AND HBV CORE PARTICLE DNA EXTRACTION AND QUANTITATION BY QUANTITATIVE PCR

HBV cccDNA was extracted according to the method described, with minor modifications.[8] An aliquot of the extracted nuclear DNA was treated with Plasmid-safe deoxyribonuclease (Epicentre) for 45 minutes at 37°C, then 30 minutes at 70°C, and then analyzed for cccDNA by TaqMan PCR amplification (Toyobo, Japan). The cytoplasmic cell lysate was digested by deoxyribonuclease I and then precipitated with PEG8000 overnight; the core particles were incubated in proteinase K buffer at 56°C for 2 hours. The precipitated core particle DNA was analyzed by quantitative PCR or Southern blot. Levels of HBV cccDNA and core particle DNA were normalized to genomic glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

HBV CORE PARTICLE RNA EXTRACTION

The HBV Core particle was purified as described in the Supporting Information, and the core particle pgRNA was extracted using TRIzol reagent and detected by northern blot.

ANALYSIS OF CORE CAPSIDS

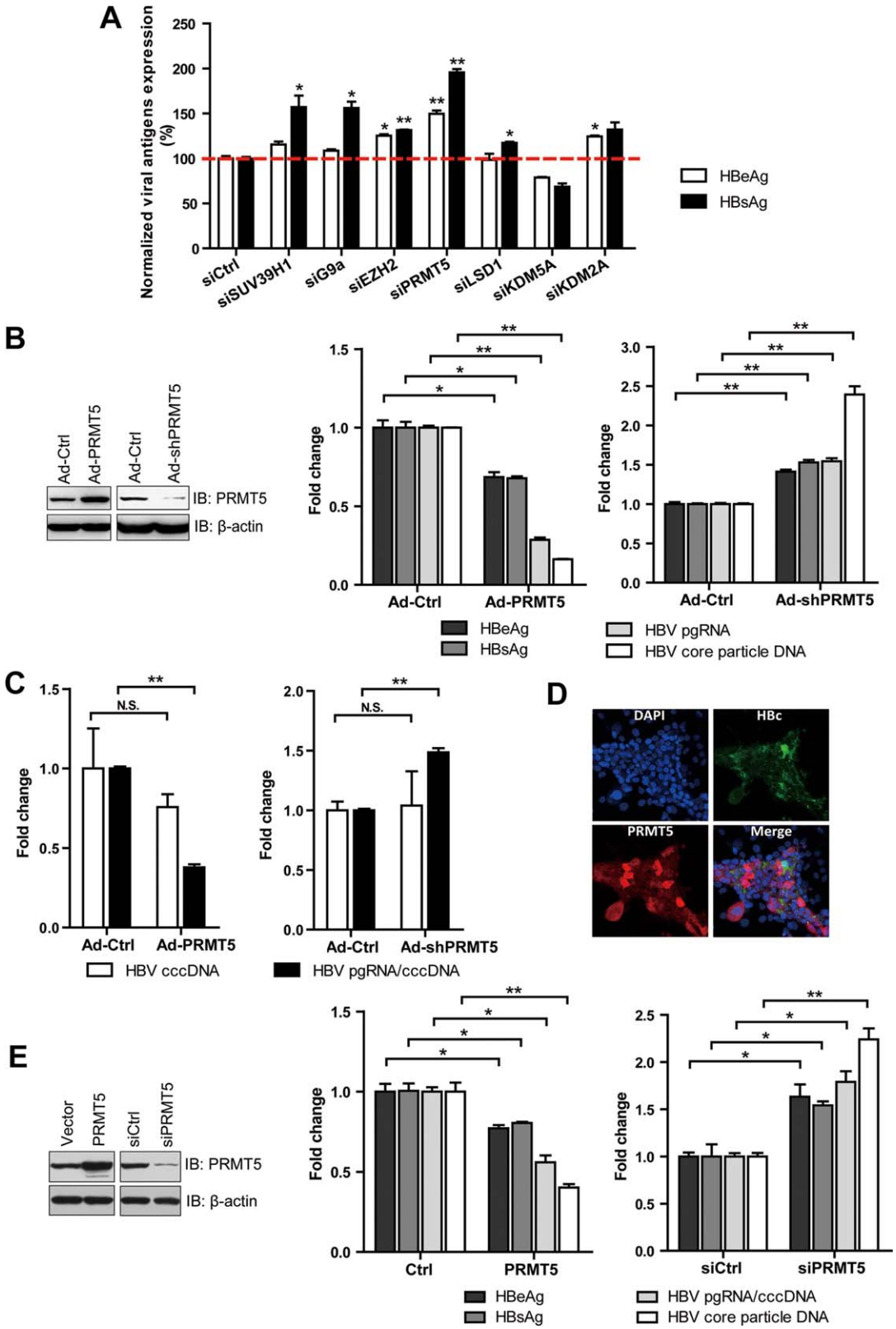
Cytoplasmic cell lysates containing HBV capsids were directly resolved on a 1% agarose gel in 0.5 × trishydroxymethylaminomethane–borate–ethylene diamine tetraacetic acid buffer and transferred to nitrocellulose membranes. Proteins were then detected by western blot using anti-HBc antigen antibody (Dako).[13]

SOUTHERN BLOT/NORTHERN BLOT

Extracted DNA and extracted RNA were loaded on a 1.2% agarose gel in 1 × trishydroxymethylaminomethane–acetic acid–ethylene diamine tetraacetic acid buffer and on formaldehyde denaturing agarose gel in 1× MOPS buffer, respectively, and then transferred onto a positively charged nylon membrane (Roche, Switzerland) using a vacuum blotter (Bio-Rad). HBV DNA and RNA were hybridized and detected using a DIG Northern Starter Kit (Roche) according to the manufacturer's instructions.[14]

CHROMATIN IMMUNOPRECIPITATION ASSAY

Chromatin immunoprecipitation (ChIP) assays were performed using the method described, with minor modifications.[8] Protein–DNA complexes were immunoprecipitated with the indicated antibodies, and normal immunoglobulin G was conducted in each experiment to exclude nonspecific binding. Precipitated DNA was analyzed by quantitative PCR, and the


A
Normalized viral antigens expression (%)
250
200
150
100
50
0
siCtrl
siSUV39H1
siG9a
siEZH2
siPRMT5
siLSD1
siKDM5A
siKDM2A
HBeAg
HBsAg
B
Ad-Ctrl
Ad-PRMT5
Ad-Ctrl
Ad-shPRMT5
IB: PRMT5
IB: β-actin
Fold change
Ad-Ctrl
Ad-PRMT5
Ad-shPRMT5
HBeAg
HBsAg
HBV pgRNA
HBV core particle DNA
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HBV cccDNA
HBV pgRNA/cccDNA
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IB: PRMT5
IB: β-actin
Ctrl
PRMT5
siCtrl
siPRMT5
HBeAg
HBsAg
HBV pgRNA/cccDNA
HBV core particle DNA

FIG. 1

results are presented as percentages of input DNA. Strategies for quantification and calculation are described in Supporting Fig. S4.

REAL-TIME REVERSE-TRANSCRIPTION PCR

Total RNA was extracted using TRIzol reagent and reverse-transcribed using ReverTra Ace qPCR RT Master Mix with the gDNA Remover kit, followed by quantitative PCR using the Thunderbird SYBR qPCR Mix (both from Toyobo, Japan). The primers used are listed in Supporting Table S6. The pgRNA primers can also amplify HBV preC RNA. Quantification of HBV pgRNA was normalized to 28S and 18S ribosomal RNA.

COIMMUNOPRECIPITATION AND WESTERN BLOT

Cells were lysed on ice in coimmunoprecipitation (CoIP) lysis buffer. After centrifugation, the supernatant was precleared with protein A/G-agarose beads. Protein complexes were then immunoprecipitated with the indicated antibodies and analyzed by western blot using the specific antibodies (Supporting Table S4).

IMMUNOFLUORESCENCE ASSAY

The immunofluorescence assay was performed as described.[15] Fluorescence was observed under a confocal fluorescence microscope (Leica; TCS SP2).

RNA IMMUNOPRECIPITATION ASSAY

The RNA immunoprecipitation (RIP) assay was performed using the method described, with minor modifications.[16] Immunoprecipitated pgRNA was quantified by quantitative PCR and represented as percentages of input pgRNA.

STATISTICS

The data are presented as the mean ± standard error of the mean. Statistical analyses were performed using the Student *t* test or Mann-Whitney U test. $P < 0.05$ was considered statistically significant.

Results

IDENTIFICATION OF PRMT5 AS AN ANTI-HBV HOST FACTOR THAT RESTRICTS cccDNA TRANSCRIPTION AND CORE PARTICLE DNA PRODUCTION

To examine whether HBV transcription and replication are associated with histone methylation and to identify the host factors involved, we applied the RNA interference–based approach to screen a panel of the known histone methyltransferases or demethylases, including SUV39H, G9a, EZH2, PRMT5, LSD1, KDM5A, and KDM2A (Supporting Fig. S1A and Table S1), which have been reported to catalyze histone methylation or demethylation to result in transcriptional repression.[17-19] The monomeric linear HBV DNA-transfected model was used,[4] in which the HBV minichromosome is proved to be formed and the expression levels of hepatitis B e antigen (HBeAg) and hepatitis B surface antigen (HBsAg) could serve as surrogate markers for the transcription of cccDNA. Because the result suggested that PRMT5 knockdown led to significant up-regulation of both HBeAg and HBsAg (Fig. 1A) and PRMT5 inhibits the production of pgRNA and core particle DNA in a dose-dependent manner (Supporting Fig. S1B-D), we further focused on the PRMT5-mediated suppression of HBV. By using the HepaRG model for HBV infection, we showed that overexpression or knockdown of PRMT5 significantly decreased or increased the level of HBeAg and HBsAg in the supernatant, intracellular pgRNA and HBV core particle DNA in the cytoplasm

FIG. 1. PRMT5 inhibits HBV transcription and core particle DNA production. (A) The indicated small interfering RNAs were cotransfected with monomeric linear HBV DNA into Huh7 cells. Culture supernatants were collected to detect HBeAg and HBsAg by enzyme-linked immunosorbent assay. (B-D) PRMT5 was transduced into HBV-infected dHepaRG cells for 6 days, and shPRMT5 was transduced for 5 days. Expression of PRMT5 was detected by western blot. Levels of HBV antigens in the supernatant were detected by enzyme-linked immunosorbent assay. HBV pgRNA, core particle DNA, and cccDNA were analyzed by quantitative PCR. Levels of pgRNA and cccDNA were used to calculate the ratio of pgRNA/cccDNA (B,C). HBV core protein and endogenous PRMT5 were observed by immunofluorescence assay using the specific antibodies (D). (E) PRMT5 expression plasmids or 100 nM of siPRMT5 were transfected into HBV-infected HepG2-NTCP cells. Cells were collected to examine the level of HBV antigens, core particle DNA, and pgRNA/cccDNA at day 4 posttransfection. $^{*}P < 0.05$, $^{**}P < 0.01$. Abbreviations: Ad, adenovirus; DAPI, 4′,6-diamidino-2-phenylindole; IB, immunoblot; N.S., not significant; si, small interfering.

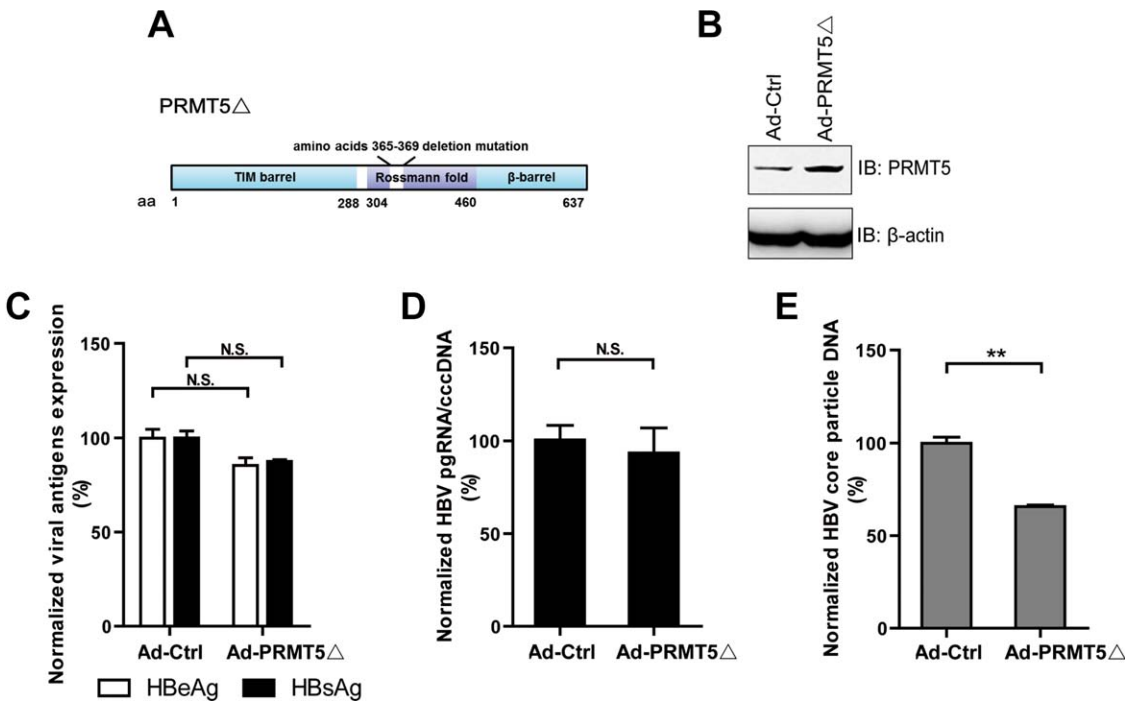


FIG. 2. Effects of PRMT5Δ on HBV transcription and core particle DNA production. (A) A schematic representation of the catalytically inactive form of human PRMT5 (PRMT5Δ) and the deleted amino acids in PRMT5Δ are shown. (B-E) PRMT5Δ was transduced into HBV-infected dHepaRG cells for 6 days. (B) Expression of PRMT5Δ was detected by western blot. (C) Culture supernatants were collected to detect HBeAg and HBsAg by enzyme-linked immunosorbent assay. (D) HBV pgRNA and cccDNA were extracted and quantified by quantitative PCR for calculating the ratio of pgRNA/cccDNA. (E) The level of core particle DNA was analyzed by quantitative PCR. $^{**}P < 0.01$. Abbreviations: Ad, adenovirus; IB, immunoblot; N.S., not significant.

(Fig. 1B), respectively, and regulated the cccDNA transcriptional activity (Fig. 1C), which was calculated as the ratio of pgRNA to cccDNA. Considering that the efficacy of HBV infection in dHepaRG cells was relatively low, which might lead to an underestimated anti-HBV effect of PRMT5 when we analyzed at the whole-cell lysate level, we further evaluated the effect at the single-cell level by immunofluorescence assay (Fig. 1D; Supporting Fig. S1F) and in HBV-infected HepG2-NTCP cells (Fig. 1E) and PHHs (Supporting Fig. S1E). The results confirmed the role of PRMT5 in the suppression of cccDNA transcription and core particle DNA production and suggested that the efficacy of PRMT5 for HBV suppression is >80% at the single-cell level.

Considering that PRMT5, a member of the type II protein arginine methyltransferases, epigenetically regulates gene expression mainly through methylating histone and nonhistone targets, we next examined whether the enzymatic activity of PRMT5 is essential for its inhibitory effect on cccDNA transcription and HBV DNA production. The mutation of the methyltransferase domain of PRMT5 (PRMT5Δ; Fig. 2A; Supporting Fig. S2A) markedly diminished the inhibitory effect of PRMT5 on viral antigen expression and cccDNA transcription (Fig. 2B-D; Supporting Fig. S1G). Surprisingly, PRMT5Δ retained the majority of its capability to decrease the level of cytoplasmic core particle DNA as the wild-type (WT) PRMT5 (Fig. 2E; Supporting Fig. S2B), suggesting that PRMT5 inhibits cccDNA transcription, dependent on its methyltransferase activity, while inhibiting HBV core particle DNA production in a methyltransferase-independent manner.

PRMT5 INCREASES THE SYMMETRIC DIMETHYLATION OF cccDNA-BOUND H4R3 TO INHIBIT cccDNA TRANSCRIPTION IN A METHYLTRANSFERASE ACTIVITY–DEPENDENT MANNER

Because H3R8me2s and H4R3me2s are reported to be catalyzed by PRMT5,[17,20] we speculated whether

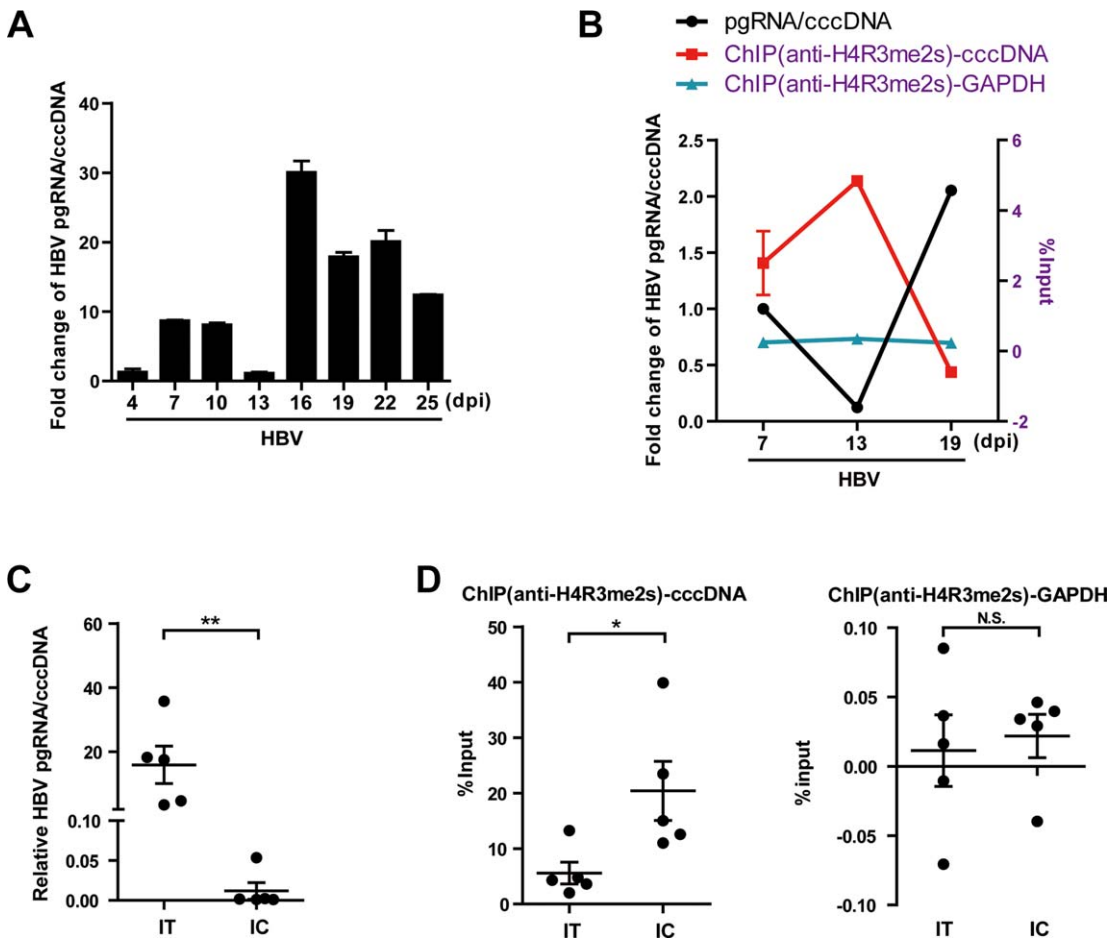


FIG. 3. cccDNA transcriptional activity was negatively correlated with cccDNA-bound H4R3me2s. (A,B) HBV-infected dHepaRG cells in 100-mm dishes were harvested by trypsinization on the indicated days. One eighth of the cells were used to extract HBV cccDNA, and one sixteenth of the cells were used to extract HBV pgRNA, followed by quantitative PCR analysis. (A) The ratio of pgRNA to cccDNA at 4 dpi was set as 1. (B) Three quarters of HBV-infected dHepaRG cells harvested at 7, 13, and 19 dpi were used for ChIP assays with the indicated antibody. Levels of the specific modified histones on HBV cccDNA or the host gene GAPDH were analyzed by quantitative PCR. The ratio of pgRNA to cccDNA at 7 dpi was set as 1. (C,D) Each liver biopsy was used to conduct RNA extraction and ChIP assay with the indicated antibody. Relative HBV pgRNA/cccDNA was calculated as the ratio of normalized HBV pgRNA to cccDNA. Levels of H4R3me2s on HBV cccDNA or the host GAPDH DNA were examined by quantitative PCR. $^{*}P < 0.05$, $^{**}P < 0.01$. Abbreviations: dpi, days postinfection; IC, inactive carrier; IT, immune-tolerant; N.S., not significant.

the status of H3R8me2s and H4R3me2s on cccDNA correlates with the cccDNA transcriptional activity. In the HBV-infected dHepaRG model, the cccDNA transcriptional activity (pgRNA/cccDNA) was shown to fluctuate during natural HBV infection (Fig. 3A; Supporting Fig. S3A,B). By ChIP analysis, we found that the status of H4R3me2s on the HBV minichromosome was negatively correlated with cccDNA transcriptional activity during HBV replication (Fig. 3B, red line and black line), whereas H3R8me2s was almost undetectable on the HBV minichromosome throughout the period of viral infection (Supporting Fig. S3C). As the control, the status of H4R3me2s at the transcription start site of host genes such as GAPDH was relatively stable (Fig. 3B, blue line). We repeated this experiment three times with sera from different chronic HBV patients. Although the kinetics of cccDNA transcriptional activity varied a bit in the three experiments, they were always negatively associated with the status of H4R3me2s on the HBV minichromosome.

To further validate the effect of cccDNA-bound H4R3me2s on HBV transcription *in vivo*, we collected liver biopsies from 10 chronic HBV patients

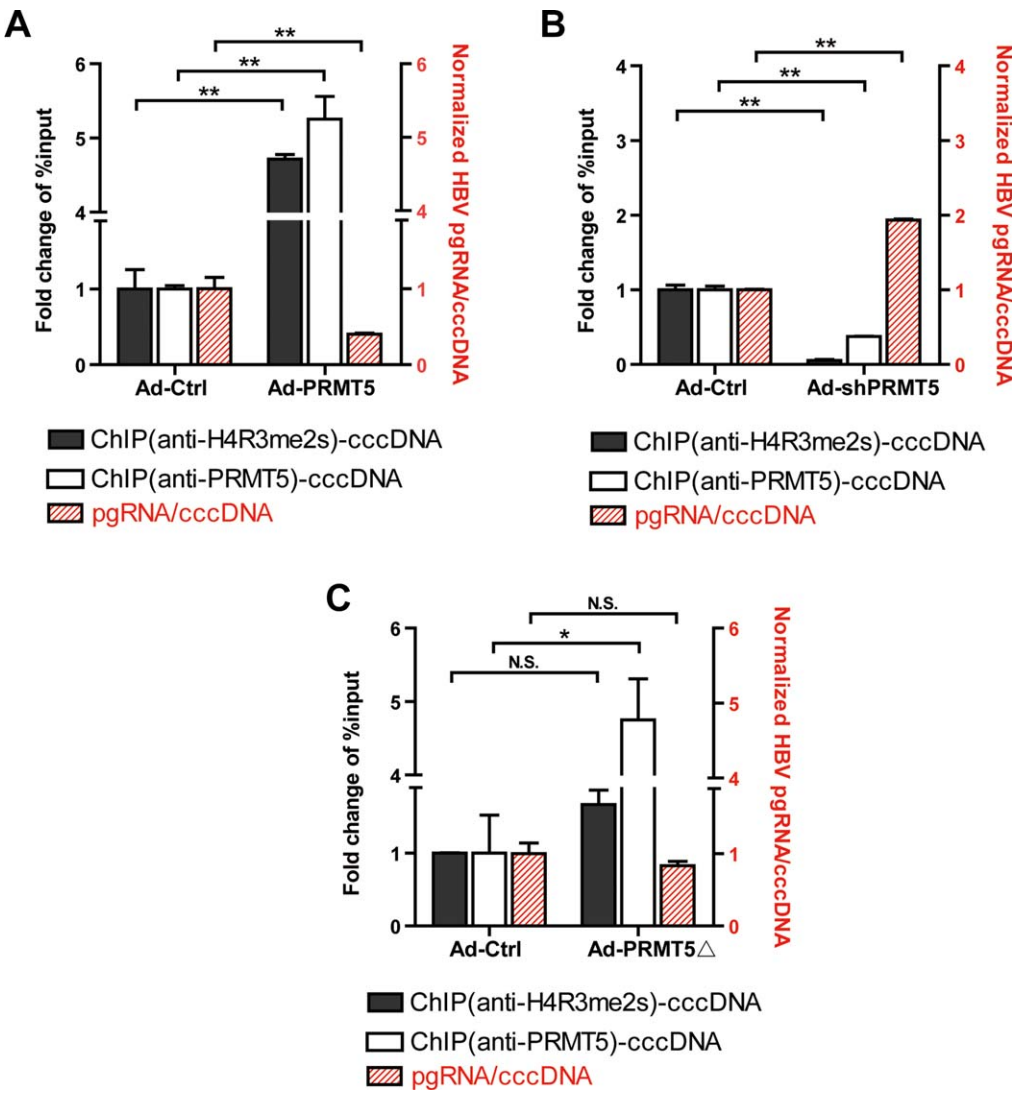


FIG. 4. PRMT5 increases the symmetric dimethylation of cccDNA-bound H4R3 to inhibit cccDNA transcription. (A,C) PRMT5 or PRMT5Δ was transduced into HBV-infected dHepaRG cells in 100-mm dishes for 6 days. (B) shPRMT5 was transduced into HBV-infected dHepaRG cells in 100-mm dishes for 5 days. One eighth of the cells were used to extract HBV cccDNA, and one sixteenth of the cells were used to extract total RNA, followed by quantitative PCR analysis. Three quarters of the cells were harvested and used for ChIP assays with the indicated antibodies. Levels of the specific proteins on HBV cccDNA were analyzed by quantitative PCR. $^{*}P < 0.05$, $^{**}P < 0.01$. Abbreviation: N.S., not significant.

including 5 HBeAg-positive patients in immune-tolerant phase and 5 HBeAg-negative inactive carriers to analyze the association of the status of cccDNA-bound H4R3me2s with the levels of cccDNA transcriptional activity. The level of pgRNA/cccDNA in inactive carrier patients was, as expected, significantly lower than that in immune-tolerant patients (Fig. 3C), whereas the average level of cccDNA-bound H4R3me2s was about 3-fold higher in inactive carriers than in immune-tolerant patients. Moreover, the level of H4R3me2s on cccDNA varied more than that on the host GAPDH DNA (Fig. 3D). The level of H3 acetylation on cccDNA, which was reported to be involved in the regulation of HBV replication in the liver tissue,[7] showed no significant correlation with cccDNA transcriptional activity in the 10 patients (Supporting Fig. S5A), which might be attributed to different inclusion criteria for HBeAg status and the alanine aminotransferase level of the patients.

We further examined whether alteration of PRMT5 expression affects the status of cccDNA-bound H4R3me2s. Overexpression or knockdown of PRMT5 led to increased or reduced binding of PRMT5 to cccDNA and accordingly increased or reduced the level of H4R3me2s on the minichromosome and cccDNA transcription activity, respectively (Fig. 4A,B). In addition, although the level of cccDNA-bound PRMT5Δ was comparable with that of cccDNA-bound WT PRMT5, neither the status of H4R3me2s nor the cccDNA transcriptional activity was affected by the increased expression of PRMT5Δ (Fig. 4C). Together, these results indicate that cccDNA-bound H4R3me2s is a repressive marker of cccDNA transcription and that PRMT5 can be recruited to cccDNA and, in a methyltransferase-dependent manner, catalyze H4R3me2s modification on cccDNA to repress cccDNA transcription.

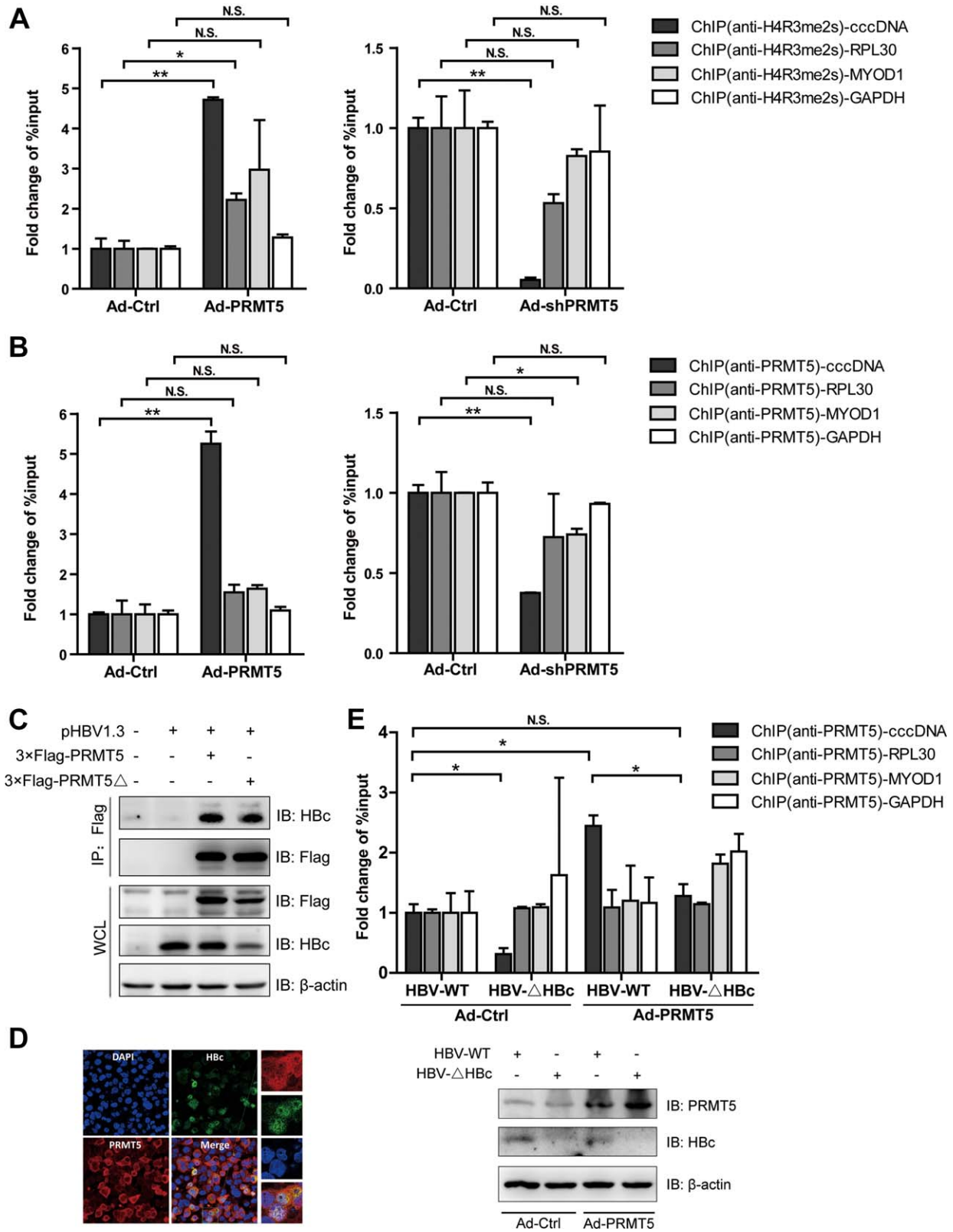
PRMT5 PREFERENTIALLY REGULATES H4R3me2s ON cccDNA AND INTERACTS WITH HBc

Because H4R3me2s was present not only on the cccDNA minichromosome but also on the host genome, we next compared the effect of PRMT5 on cccDNA-bound or host gene–bound H4R3me2s. Histone–DNA complexes were immunoprecipitated using antibodies specifically against H4R3me2s, followed by quantitative PCR analysis with primers designed to detect cccDNA and three host genes—RPL30, MYOD1, and GAPDH. The results showed that overexpression or knockdown of PRMT5 significantly elevated or reduced the level of H4R3me2s as well as PRMT5 on HBV cccDNA minichromosome, respectively, but had much less impact on the host genes studied here (Fig. 5A,B), implying that PRMT5 can, to some extent, preferentially bind to cccDNA and catalyze cccDNA-bound H4R3me2s.

The HBc and HBx proteins have been associated with the HBV minichromosome[3,4,21] (Supporting Fig. S8A) and thus were candidates to mediate the preferential binding of PRMT5 to cccDNA. Therefore, we further clarified whether there is any interaction between PRMT5 and the HBc or HBx protein. While no interaction between PRMT5 and HBx (Supporting Fig. S8B) was observed, the CoIP results suggested that the ectopically expressed PRMT5 and PRMT5Δ and the endogenous PRMT5 interacted with HBc (Fig. 5C; Supporting Fig. S8C-F). In addition, ectopically expressed HBc was mainly distributed in the nucleus and partially colocalized with the nucleus-distributed PRMT5 (Fig. 5D). To further examine the role of HBc in the binding of PRMT5 to cccDNA, we constructed an HBc-deficient virus (HBV-ΔHBc) and found that less PRMT5 was bound to the cccDNA of HBV-ΔHBc compared to that of HBV-WT. Moreover, overexpression of PRMT5 resulted in significant up-regulation of the level of cccDNA-bound PRMT5 in the HBV-WT group but not in the HBV-ΔHBc group (Fig. 5E). These results provide a possibility that PRMT5 is targeted to HBV cccDNA through its interaction with the viral core protein.

PRMT5-MEDIATED SUPPRESSION OF cccDNA TRANSCRIPTION INVOLVES THE Brg1-BASED HUMAN SWI/SNF CHROMATIN REMODELER AND RNA POL II

It has been reported that PRMT5 acts as a part of a variety of complexes to regulate gene transcription and cell signal transduction.[22] Among these complexes, the human SWI/SNF chromatin remodeler is mainly involved in PRMT5-catalyzed histone methylation, which results in epigenetic gene silencing.[23] The human SWI/SNF chromatin remodeler is a multisubunit complex that can be divided into two distinct complexes, called PBAF and BAF. The central core of the PBAF complex is the Brg1 adenosine triphosphatase subunit, and the BAF complex contains either hBrm or Brg1 adenosine triphosphatase subunits.[24] Because HBV cccDNA transcriptional activity is suppressed by PRMT5-catalyzed H4R3me2s, we speculated whether the human SWI/SNF chromatin remodeler is involved in the inhibition of cccDNA transcription by PRMT5. We thus evaluated the changes in the association of Brg1 and hBrm with cccDNA when we altered the expression levels of PRMT5 in HBV-infected dHepaRG cells. The results showed that the binding of Brg1 to cccDNA significantly increased or decreased when PRMT5 was overexpressed or knocked down, respectively (Fig. 6A). Unlike Brg1, the binding of hBrm to cccDNA did not significantly change with the alteration of PRMT5 expression (Supporting Fig. S9). Furthermore, knockdown of Brg1 significantly restored the PRMT5-mediated suppression of cccDNA transcription (Fig. 6B), which confirms the role of Brg1 in PRMT5-mediated repression of HBV cccDNA transcription.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Fold change of %input
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Ad-PRMT5
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ChIP(anti-H4R3me2s)-cccDNA
ChIP(anti-H4R3me2s)-RPL30
ChIP(anti-H4R3me2s)-MYOD1
ChIP(anti-H4R3me2s)-GAPDH
B
ChIP(anti-PRMT5)-cccDNA
ChIP(anti-PRMT5)-RPL30
ChIP(anti-PRMT5)-MYOD1
ChIP(anti-PRMT5)-GAPDH
C
pHBV1.3
3×Flag-PRMT5
3×Flag-PRMT5△
IP: Flag
WCL
IB: HBc
IB: Flag
IB: β-actin
D
DAPI
HBc
PRMT5
Merge
E
HBV-WT
HBV-△HBc
IB: PRMT5

FIG. 5

Because cccDNA is transcribed by the host RNA Pol II,[25] whose enrichment on cccDNA is reported to be associated with the active posttranscriptional modifications on cccDNA-bound histones,[26] we further examined whether PRMT5 could regulate its binding to cccDNA. As expected, the binding of RNA Pol II to cccDNA was negatively regulated by PRMT5 in HBV-infected dHepaRG cells, while the enrichment of RNA Pol II on the three host genes was not much affected (Fig. 6C). Together, the above data suggest that PRMT5 regulates the binding of the host Brg1-based human SWI/SNF chromatin remodeler and RNA Pol II to cccDNA, which may contribute to the PRMT5-mediated epigenetic suppression of cccDNA transcription.

PRMT5 INHIBITS HBV CORE PARTICLE DNA PRODUCTION IN A METHYLTRANSFERASE ACTIVITY–INDEPENDENT MANNER

As indicated above, PRMT5Δ maintained the activity of inhibiting the production of core particle DNA but did not affect cccDNA transcription (Fig. 2). To confirm this, we further tested the levels of HBV core capsids, RNAs, and core particle DNA in an HBV-replicating cell model, in which the viral pgRNA is transcribed under the CMV promoter. Southern blot results showed that PRMT5Δ expression or knockdown of endogenous PRMT5 decreased or increased the level of core particle DNA in a dose-dependent manner, respectively, while it did not markedly affect the levels of HBV total RNA and core capsids (Fig. 7A; Supporting Fig. S10A). Similar results were obtained in the stable HBV-replicating cell line HepG2.117 (Fig. 7B). To further clarify the step at which PRMT5 inhibits HBV core particle DNA production, we constructed cytoplasm-localized or nucleus-localized PRMT5Δ. Expression of both NES-PRMT5Δ and NLS-PRMT5Δ in CMV-HBV-replicating Huh7 cells did not reduce the levels of HBV RNAs and core capsids; however, the cytoplasm-localized, but not the nucleus-localized, PRMT5Δ significantly decreased the core particle DNA levels (Fig. 7C). In HBV-infected dHepaRG cells, the cytoplasm-localized PRMT5 decreased the level of viral core particle DNA but did not affect the production of viral antigens and pgRNA and the level of cccDNA-bound H4R3me2s and PRMT5 proteins (Fig. 7D, E). These results suggest that PRMT5 does not affect the HBV RNA transcription or the formation of the core capsid itself but inhibits HBV core particle DNA production at the cytoplasmic level in a methyltransferase activity–independent manner.

PRMT5 INTERFERES WITH pgRNA ENCAPSIDATION BY COUNTERACTING THE INTERACTION BETWEEN pgRNA AND HBV POL THROUGH BINDING TO THE REVERSE TRANSCRIPTASE–RIBONUCLEASE H REGION OF POL

Because pgRNA encapsidation is an essential step before core particle DNA synthesis, we further evaluated whether PRMT5 prevents pgRNA encapsidation to block viral DNA synthesis. To address this, we transfected Huh7 cells with the pCMV-HBV YMHD vector, which lacks the Pol catalytic active site and thus leads to the accumulation of RNA-containing capsids in the cells. Northern blot results showed that while the levels of HBV total RNA and capsids were not affected by PRMT5Δ, it dramatically reduced the level of encapsidated RNA in a dose-dependent manner (Fig. 8A), indicating that PRMT5Δ inhibits pgRNA encapsidation rather than directly targeting the cytoplasmic viral pgRNA for degradation.

FIG. 5. PRMT5 preferentially regulates H4R3me2s on cccDNA and interacts with HBc. (A,B) PRMT5 was transduced into HBV-infected dHepaRG cells in 100-mm dishes for 6 days. shPRMT5 was transduced into HBV-infected dHepaRG cells in 100-mm dishes for 5 days. Three quarters of the cells were used for ChIP assays with the indicated antibodies. Levels of H4R3me2s or PRMT5 on HBV cccDNA or the host RPL30, MYOD1, and GAPDH genes were analyzed by quantitative PCR. (C) Huh7 cells were cotransfected as indicated, and the cells were subjected to CoIP assay with the indicated antibody. Expression of the indicated proteins was analyzed by western blot (β-actin as a loading control). (D) Huh7 cells were cotransfected with plasmids encoding PRMT5 and 1.3-fold HBV genome. Cells were harvested for the immunofluorescence assay. PRMT5 and HBc were detected by the specific antibodies. (E) dHepaRG cells in 100-mm dishes were infected by HBV WT and HBc protein–deficient virus (HBV-ΔHBc). Three quarters of the cells were used for ChIP assays with the anti-PRMT5antibody. The rest of the cells were subjected to analysis of the expression levels of HBc and PRMT5 by western blot. $^{*}P < 0.05$, $^{**}P < 0.01$. Abbreviations: Ad, adenovirus; DAPI, 4′,6-diamidino-2-phenylindole; IB, immunoblot; IP, immunoprecipitation; N.S., not significant; WCL, whole-cell lysate.

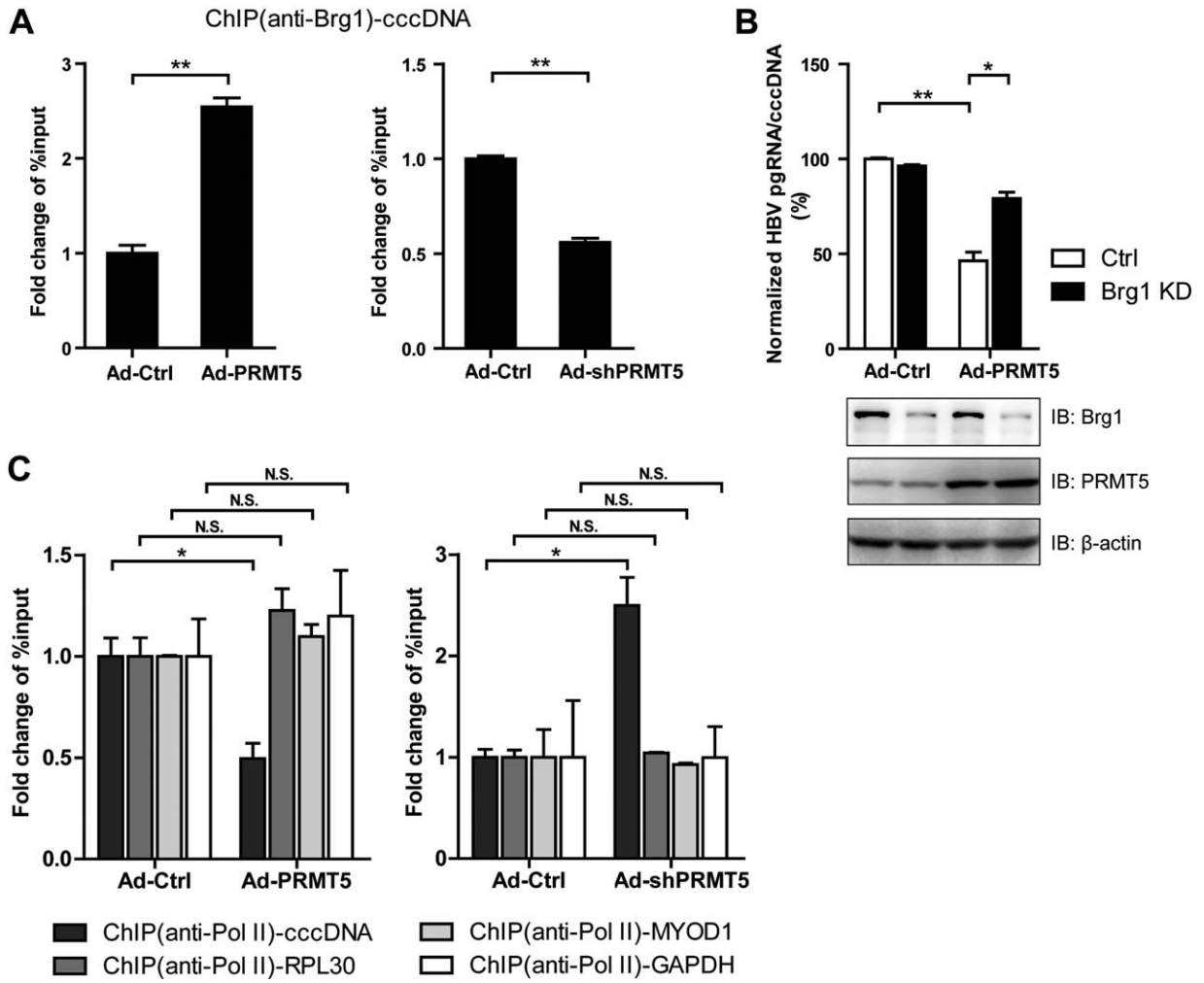


FIG. 6. Effects of PRMT5 on the binding of Brg1 and RNA Pol II to cccDNA. (A,C) PRMT5 was transduced into HBV-infected dHepaRG cells in 100-mm dishes for 6 days. shPRMT5 was transduced into HBV-infected dHepaRG cells in 100-mm dishes for 5 days. Three quarters of the cells were used for ChIP assay with the indicated antibodies. Levels of Brg1 or Pol II on the indicated genes were analyzed by quantitative PCR. (B) PRMT5 was transduced into HBV-infected dHepaRG cells at day 7 post–HBV infection. Adeno-shBrg1 was added 1 day later, and the cells were cultured for another 5 days before being subjected to quantitative PCR analysis. Expression of PRMT5 and Brg1 was detected by western blot. HBV pgRNA and cccDNA were quantified by quantitative PCR for calculating the ratio of pgRNA/cccDNA. $^{*}P < 0.05$, $^{**}P < 0.01$. Abbreviations: Ad, adenovirus; IB, immunoblot; KD, knockdown.

Because HBc, Pol and pgRNA are the three key factors involved in the process of pgRNA encapsidation and we found that PRMT5 interacts with the HBc, we speculated whether PRMT5 affects the associations of HBc with Pol and with pgRNA. The CoIP assay suggested that the interaction between HBc and Pol was not affected by PRMT5Δ (Supporting Fig. S10B). In addition, the RIP assay showed that in the absence of Pol, the interaction between HBc and pgRNA was weak, even without PRMT5Δ expression (Supporting Fig. S10C), which is consistent with previous studies showing that the binding between HBV Pol and the 5′-ε signal on pgRNA is a prerequisite for pgRNA encapsidation and subsequent viral DNA production.[27,28] These results suggest that the interaction between PRMT5 and HBc has little contribution to PRMT5-mediated inhibition of pgRNA encapsidation. We then investigated the impact of PRMT5 on the binding of Pol to pgRNA. The RIP assay clearly suggested that ectopically-expressed PRMT5 and PRMT5Δ disrupted the interaction between Pol and pgRNA, whereas knockdown of

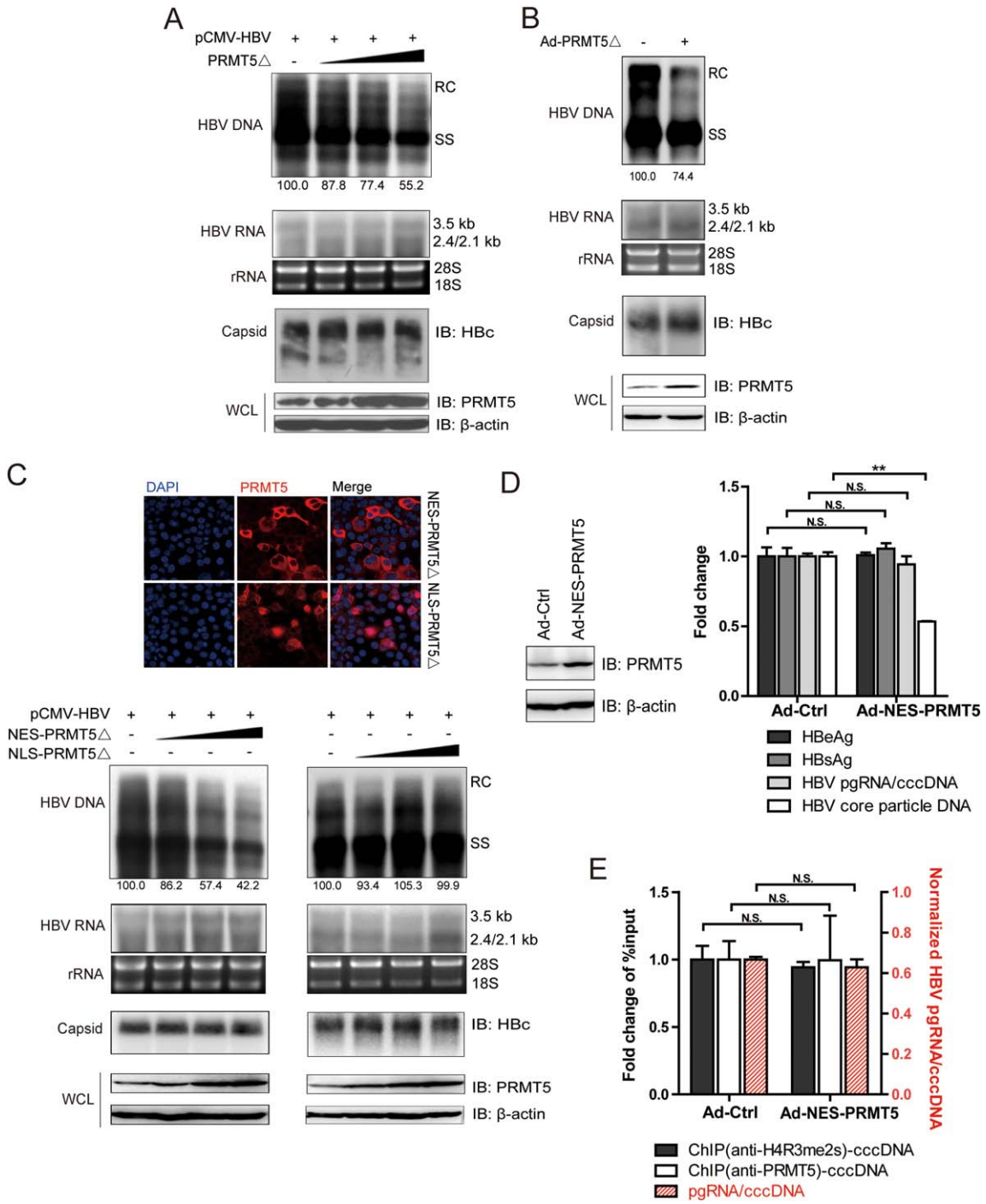
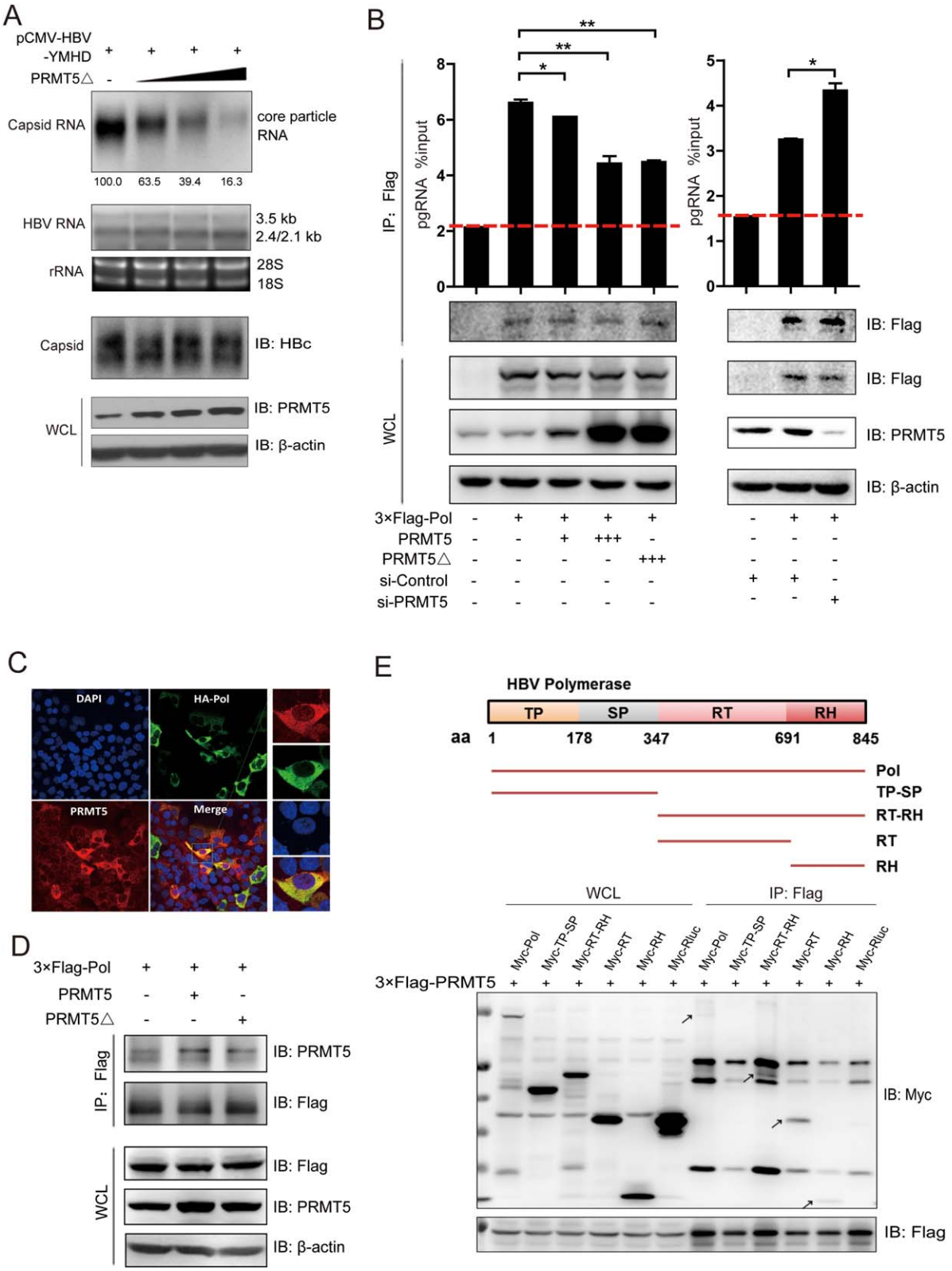


FIG. 7. Cytoplasmic PRMT5Δ reduces HBV core particle DNA production. (A) PRMT5Δ expression plasmids were cotransfected with pCMV-HBV into Huh7 cells. The pcDNA3.1a vector was used as a negative control. Cells were harvested at 72 hours post-transfection. (B) PRMT5Δ was transduced into HepG2.117 cells, and the cells were harvested at 5 days after transduction. Core particle DNAs were analyzed by Southern blot. The positions of HBV relaxed circular DNA and single-stranded DNA are indicated. Numbers below the lanes indicate the intensity of viral DNA bands relative to the vector group. HBV RNA was analyzed by northern blot. The 28S and 18S ribosomal RNAs served as loading controls. The positions of the 3.5-kb and 2.4/2.1-kb HBV RNAs are indicated. An aliquot of cell lysates was loaded onto an agarose gel to detect cytoplasmic core capsids. Overexpression of PRMT5Δ, NES-PRMT5Δ, and NLS-PRMT5Δ was detected by western blot; and *β*-actin was used as a loading control. (C) Huh7 cells transfected with NES-PRMT5Δ and NLS-PRMT5Δ, followed by an immunofluorescence assay with anti-PRMT5 antibody. (D,E) NES-PRMT5 was transduced into HBV-infected dHepaRG cells in 100-mm dishes for 6 days. Expression of NES-PRMT5 was detected by western blot. Levels of HBV antigens in the supernatant were detected by enzyme-linked immunosorbent assay. HBV pgRNA, core particle DNA, and cccDNA were analyzed by quantitative PCR. Three quarters of the cells were harvested and used for ChIP assays with the indicated antibodies. Levels of the specific proteins on HBV cccDNA were analyzed by quantitative PCR. $^{**}P < 0.01$. Abbreviations: Ad, adenovirus; DAPI, 4′,6-diamidino-2-phenylindole; IB, immunoblot; N.S., not significant; RC, relaxed circular DNA; rRNA, ribosomal RNA; SS, single-stranded DNA; WCL, whole-cell lysate.


A
pCMV-HBV -YMHD
PRMT5△
Capsid RNA
core particle RNA
100.0 63.5 39.4 16.3
HBV RNA
3.5 kb 2.4/2.1 kb
rRNA
28S 18S
Capsid
IB: HBc
WCL
IB: PRMT5
IB: β-actin
B
IP: Flag
pgRNA %input
WCL
IB: Flag
IB: PRMT5
IB: β-actin
3×Flag-Pol
PRMT5
PRMT5△
si-Control
si-PRMT5
C
DAPI
HA-Pol
PRMT5
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D
3×Flag-Pol
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IP: Flag
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IB: Flag
WCL
IB: Flag
IB: PRMT5
IB: β-actin
E
HBV Polymerase
TP SP RT RH
aa 1 178 347 691 845
Pol
TP-SP
RT-RH
RT
RH
WCL
IP: Flag
Myc-Pol
Myc-TP-SP
Myc-RT-RH
Myc-RT
Myc-RH
Myc-Rluc
3×Flag-PRMT5
IB: Myc
IB: Flag

FIG. 8

endogenous PRMT5 significantly enhanced the Pol–pgRNA interaction (Fig. 8B). Thus, we further investigated how PRMT5 counteracted the Pol–pgRNA interaction. While there was no direct interaction between PRMT5 and pgRNA (Supporting Fig. S10D), a colocalization of PRMT5 with HBV Pol in the cytoplasm (Fig. 8C) and a physical interaction between HBV Pol and endogenous or ectopic PRMT5 or PRMT5Δ (Fig. 8D) were observed. Further studies indicated that PRMT5 interacted with the reverse transcriptase–ribonuclease H (RT-RH) region of Pol (Fig. 8E), which has been recognized as the domain that is responsible for the Pol–pgRNA interaction.[28] Together, these results suggest that PRMT5 interferes with pgRNA encapsidation through disrupting the Pol–pgRNA interaction, possibly by competitively binding to the RT-RH region of Pol, which provides a mechanism of how PRMT5 inhibits core particle DNA production.

Discussion

Here, we identified PRMT5 as a potent host restrictor of HBV replication. We demonstrate that PRMT5, in a methyltransferase-dependent manner, epigenetically silences cccDNA transcription by triggering H4R3me2s on the cccDNA minichromosome, which involved the regulation of the binding of the Brg1-based human SWI/SNF chromatin remodeler and RNA Pol II to cccDNA. Importantly, the negative correlation between the cccDNA-bound H4R3me2s and cccDNA transcription was validated *in vitro* and *in vivo*. In addition, PRMT5, in a methyltransferase-independent manner, disrupted pgRNA encapsidation by competitively binding to the RT-RH region of HBV Pol, which further inhibited HBV DNA production and may affect cccDNA replenishment (Supporting Fig. S11).

In recent years, a number of epigenetic markers on the HBV cccDNA minichromosome associated with viral transcription have been identified. While high levels of posttranscriptional modifications of the cccDNA-bound histones associated with active transcription are observed across the HBV genome and the role of acetylation of cccDNA-bound histones in the regulation of HBV transcription has been recognized,[7,9,26] the posttranscriptional modifications associated with transcriptional repression, including trimethylation of H3K9 (H3K9me3) and H3K27me3, were hardly detected on HBV DNA[26]; thus, the role of histone methylation remains elusive and controversial. Here, we report that, both *in vitro* and *in vivo*, cccDNA-bound H4R3me2s could be detected, was significantly associated with repressed cccDNA transcription, and could be manipulated by the alteration of PRMT5 expression. Because the previous study with a ChIP-sequencing approach did not include H4R3me2s, it will be interesting to map its distribution on cccDNA in the future. In addition to the present study, the role of methyltransferases including PRMT1 and SETDB1 in regulating HBV transcription has been reported.[29,30] PRMT1, a member of the type I protein arginine methyltransferase family, was found to be a binding partner of HBx and could inhibit HBV transcription. Considering that the asymmetric dimethylation on H4R3 (H4R3me2a), known to be catalyzed by PRMT1, has been recognized as a gene activation marker,[19] and that our results from an HBV infection system revealed that there was no positive correlation between the status of H4R3me2a and cccDNA transcriptional activity (Supporting Fig. S3C), the inhibitory effect of PRMT1 on HBV transcription may act not through its catalysis of cccDNA-

FIG. 8. PRMT5 interferes with pgRNA encapsidation by counteracting the interaction between pgRNA and HBV Pol through binding to the RT-RH region of Pol. (A) PRMT5Δ was cotransfected with pCMV-HBV YMHD into Huh7 cells. Cells were harvested at 72 hours posttransfection. Core particle RNAs were analyzed by northern blot. Numbers below the lanes indicate the intensity of the core particle RNA bands relative to the vector group. Total HBV RNA was analyzed by northern blot. An aliquot of cell lysates was loaded onto an agarose gel to detect the cytoplasmic core capsids. Overexpression of PRMT5Δ was detected by western blot. (B) Huh7 cells were cotransfected as indicated, and pCMV-HBV-ΔPol was transfected in all groups to transcribe pgRNA. At 36 hours posttransfection, cells were treated with 10 μM of MG132 for 9 hours and harvested for RIP assay. (C) PRMT5 was cotransfected with hemagglutinin Pol into Huh7 cells. After 48 hours, cells were harvested for an immunofluorescence assay. Anti-PRMT5 and anti-HA antibodies were used. (D) Huh7 cells were cotransfected as indicated. At 36 hours posttransfection, cells were treated with 10 μM of MG132 for 9 hours and then harvested for CoIP assay with anti-Flag antibody. (E) Schematic diagram of the full-length and truncated constructs of HBV Pol (upper panel). Huh7 cells were cotransfected with Flag-PRMT5 and the indicated Myc-tagged HBV Pol constructs or the negative control, Myc-Rluc. At 36 hours posttransfection, cells were treated with 10 μM of MG132 for 9 hours and then harvested for CoIP assay with anti-Flag antibody (lower panel). $^{*}P < 0.05$, $^{**}P < 0.01$. Abbreviations: aa, amino acid; DAPI, 4′,6-diamidino-2-phenylindole; HA, hemagglutinin; IB, immunoblot; IP, immunoprecipitation; si, small interfering; SP, spacer; TP, terminal protein; WCL, whole-cell lysate.

bound H4R3me2a but through other target proteins. Riviere et al.[30] suggested that histone methyltransferase SETDB1-mediated H3K9me3 was involved in the transcriptional repression of HBV cccDNA. Notably, this repressive effect could only be observed in the absence of HBx (HBVX$^-$),[26,30] which might explain why we did not observe H3K9me3-associated transcriptional repression of cccDNA in dHepaRG cells infected with WT HBV (Supporting Fig. S3C), thus arguing for a role of H3K9me3 in regulating cccDNA transcription in the presence of the HBx protein during natural HBV infection. Together, these data suggest that, compared with H4R3me2a and H3K9me3, PRMT5-catalyzed H4R3me2s may play a vital role in the restriction of HBV transcription during HBV infection. However, whether other factor(s) that can regulate the status H4R3me2s and factor(s) that modulate the activity of PRMT5 remains to be investigated. Besides, from the perspective of the virus, such an epigenetic mechanism with a repressive effect might be a strategy for maintaining chronic infection.

Previous studies have suggested that PRMT5 methylates histones H4R3 and H3R8 as a part of the Brg1-based or hBrm-based SWI/SNF chromatin remodeling complex[23] and that this, in turn, facilitates the binding of Brg1 to the target gene for subsequent chromatin remodeling. Here, we showed that altering PRMT5 expression accordingly regulated the binding of Brg1, but not hBrm, to cccDNA. We thus speculated that the Brg1-based human SWI/SNF chromatin remodeler complex may be associated with PRMT5 to promote H4R3me2s on cccDNA as well as to remodel the nucleosome structure of the HBV minichromosome to coordinately impede the binding of RNA Pol II to cccDNA.[31] Because PRMT5-mediated methylation of H4R3 can recruit the DNA methyltransferase DNMT3A[20] and HBV DNA methylation has been shown to suppress cccDNA transcription, it will be interesting to further determine whether the methylation of cccDNA is increased along with the PRMT5-mediated up-regulation of cccDNA-bound H4R3me2s and coordinately contributes to the repression of HBV transcription by PRMT5.

Although both the host chromosomes and the HBV cccDNA minichromosome are organized with the host histone proteins, we showed that the status of H4R3me2s varied more on cccDNA than on the host GAPDH gene during HBV infection and that the alteration of the expression of PRMT5 significantly changed the level of cccDNA-bound, but not host gene–bound, H4R3me2s and PRMT5, implying that PRMT5 can, to some extent, specifically bind to cccDNA and catalyze cccDNA-bound H4R3me2s to repress cccDNA transcription. Based on the evidence that PRMT5 interacted and colocalized with HBc in the nucleus and knockdown of HBc decreased the binding of PRMT5 to cccDNA, we proposed that HBc plays a role in targeting PRMT5 to cccDNA. The factors that have been reported to mediate the binding of PRMT5 to host chromosomes, such as the histone-binding protein COPR5, may also be involved in the binding of PRMT5 to cccDNA, which remains to be further studied. The preference of PRMT5 could also be attributed to the differences in chromatin protein composition and the chromosome structure between cccDNA and host genes,[3,32] which may lead to different sensitivities to the alteration of PRMT5 expression levels.

Until now, a number of host restrictors that target different steps of HBV replication have been identified, including the APOBEC cytidine deaminases,[33] the interferon-induced MyD88 and zinc finger protein,[14,34] the tumor necrosis factor-α-induced cIAP2,[35] and RIG-I.[16] Here, we report that PRMT5, in addition to suppressing HBV transcription, has a novel inhibitory effect on HBV pgRNA encapsidation through binding to the RT-RH region of Pol, which is crucial for the pgRNA–Pol interaction as well as for the subsequent RNA reverse transcription.[28,36] Because the pgRNA is reverse-transcribed within the capsids, which are subsequently used for the formation of mature virions or recycling to the nucleus for cccDNA maintenance, there is a possibility that by binding to the viral Pol, PRMT5 can also be incorporated into the viral nucleocapsids to interfere with HBV reverse transcription and prevent the replenishment of cccDNA, which requires further investigation. Another possibility we cannot exclude is that the binding of PRMT5 to core protein affects the packaging of the Pol–pgRNA ribonucleoprotein complex into capsid.

Besides histones, PRMT5 can target a series of other host proteins and has been implicated in various cellular processes, including pluripotency, tumorigenesis,[37] and regulation of viral replication and pathogenesis by targeting a variety of cellular substrates and viral proteins.[38] We showed that the anti-HBV effect of PRMT5 is not dependent on nuclear factor-κB activation (Supporting Fig. S7)[39] or alteration of the expression levels of HBV transcription-related host transcription factors like hepatocyte nuclear factor 4 and CCAAT/enhancer binding protein α (Supporting Figs. S5B and S6), but other possibilities remain to be

further studied, such as whether PRMT5 could directly methylate HBV Pol or HBc to influence their function or whether PRMT5 inhibits HBV replication through an indirect mechanism(s). Moreover, although the level of PRMT5 mRNA is not affected by HBV infection (Supporting Fig. S5C,D), it is much higher in hepatoma HepG2-NTCP cells than PHHs (data not shown), which is consistent with reports that PRMT5 expression is up-regulated in tumor tissues. Considering that HBV replication is often robust in PHHs but low in tumor cells, we determined whether there is any association between the anti-HBV effect of PRMT5 and the low HBV replication efficacy in tumor-derived cells. Our recent data showed that the HBV transcription activity was lower in liver tumor cells than in paired paratumor tissues, while, interestingly, the levels of PRMT5 mRNA and the cccDNA-bound PRMT5 and H4R3me2s were higher in tumor cells (unpublished). Although more validation is required, these data have provided more insight into the physiological significance of PRMT5-mediated restriction of HBV replication *in vitro* and *in vivo*.

In conclusion, the present study revealed a two-part mechanism of the regulation of HBV gene transcription and viral replication by PRMT5, which promotes the understanding of the epigenetic modulation of cccDNA transcription and the control of HBV replication and may provide new insight into noncytolytic mechanisms of cccDNA elimination.

Acknowledgment: We thank Prof. Ningshao Xia (Xiamen University) for providing mouse antibodies against HBc; Prof. Jiming Zhang (Huashan Hospital affiliated to Fudan University), Dr. Zhanqing Zhang (Shanghai Public Health Clinical Center affiliated to Fudan University), and Prof. Jian Zhou (Zhongshan Hospital affiliated to Fudan University) for patient recruitment and providing clinical samples; Dr. Yinghui Liu for preparing several of the truncations used; Jin Li, Cong Wang, and Ke Qiao for technical assistance; and Dr. Yuchen Xia for critical reading of the manuscript.

REFERENCES

1) Ganem D, Prince AM. Hepatitis B virus infection—natural history and clinical consequences. N Engl J Med 2004;350:1118-1129.
2) Bock CT, Schranz P, Schroder CH, Zentgraf H. Hepatitis B virus genome is organized into nucleosomes in the nucleus of the infected cell. Virus Genes 1994;8:215-229.
3) Bock CT, Schwinn S, Locarnini S, Fyfe J, Manns MP, Trautwein C, et al. Structural organization of the hepatitis B virus minichromosome. J Mol Biol 2001;307:183-196.
4) Belloni L, Pollicino T, De Nicola F, Guerrieri F, Raffa G, Fanciulli M, et al. Nuclear HBx binds the HBV minichromosome and modifies the epigenetic regulation of cccDNA function. Proc Natl Acad Sci USA 2009;106:19975-19979.
5) **Guo YH, Li YN**, Zhao JR, Zhang J, Yan Z. HBc binds to the CpG islands of HBV cccDNA and promotes an epigenetic permissive state. Epigenetics 2011;6:720-726.
6) Zhang Y, Mao R, Yan R, Cai D, Zhu H, Kang Y, et al. Transcription of hepatitis B virus covalently closed circular DNA is regulated by CpG methylation during chronic infection. PLoS One 2014;9:e110442.
7) Pollicino T, Belloni L, Raffa G, Pediconi N, Squadrito G, Raimondo G, et al. Hepatitis B virus replication is regulated by the acetylation status of hepatitis B virus cccDNA-bound H3 and H4 histones. Gastroenterology 2006;130:823-837.
8) Belloni L, Allweiss L, Guerrieri F, Pediconi N, Volz T, Pollicino T, et al. IFN-alpha inhibits HBV transcription and replication in cell culture and in humanized mice by targeting the epigenetic regulation of the nuclear cccDNA minichromosome. J Clin Invest 2012;122:529-537.
9) Liu F, Campagna M, Qi Y, Zhao X, Guo F, Xu C, et al. Alpha-interferon suppresses hepadnavirus transcription by altering epigenetic modification of cccDNA minichromosomes. PLoS Pathog 2013;9:e1003613.
10) **Huang J, Kent JR**, Placek B, Whelan KA, Hollow CM, Zeng PY, et al. Trimethylation of histone H3 lysine 4 by Set1 in the lytic infection of human herpes simplex virus 1. J Virol 2006;80:5740-5746.
11) Murata T, Kondo Y, Sugimoto A, Kawashima D, Saito S, Isomura H, et al. Epigenetic histone modification of Epstein-Barr virus BZLF1 promoter during latency and reactivation in Raji cells. J Virol 2012;86:4752-4761.
12) Nitzsche A, Steinhausser C, Mucke K, Paulus C, Nevels M. Histone H3 lysine 4 methylation marks postreplicative human cytomegalovirus chromatin. J Virol 2012;86:9817-9827.
13) Liu Y, Li J, Chen J, Li Y, Wang W, Du X, et al. Hepatitis B virus polymerase disrupts K63-linked ubiquitination of STING to block innate cytosolic DNA-sensing pathways. J Virol 2015;89:2287-2300.
14) Li JH, Lin SS, Chen QY, Peng L, Zhai JW, Liu YH, et al. Inhibition of hepatitis B virus replication by MyD88 involves accelerated degradation of pregenomic RNA and nuclear retention of pre-S/S RNAs. J Virol 2010;84:6387-6399.
15) **Yu S, Chen J**, Wu M, Chen H, Kato N, Yuan Z. Hepatitis B virus polymerase inhibits RIG-I- and Toll-like receptor 3–mediated beta interferon induction in human hepatocytes through interference with interferon regulatory factor 3 activation and dampening of the interaction between TBK1/IKKepsilon and DDX3. J Gen Virol 2010;91:2080-2090.
16) Sato S, Li K, Kameyama T, Hayashi T, Ishida Y, Murakami S, et al. The RNA sensor RIG-I dually functions as an innate sensor and direct antiviral factor for hepatitis B virus. Immunity 2015;42:123-132.
17) Greer EL, Shi Y. Histone methylation: a dynamic mark in health, disease and inheritance. Nat Rev Genet 2012;13:343-357.
18) Klose RJ, Zhang Y. Regulation of histone methylation by demethylimination and demethylation. Nat Rev Mol Cell Biol 2007;8:307-318.

19) Peterson CL, Laniel MA. Histones and histone modifications. Curr Biol 2004;14:R546-R551.
20) Zhao Q, Rank G, Tan YT, Li H, Moritz RL, Simpson RJ, et al. PRMT5-mediated methylation of histone H4R3 recruits DNMT3A, coupling histone and DNA methylation in gene silencing. Nat Struct Mol Biol 2009;16:304-311.
21) Nassal M. HBV cccDNA: viral persistence reservoir and key obstacle for a cure of chronic hepatitis B. Gut 2015;64:1972-1984.
22) Karkhanis V, Hu YJ, Baiocchi RA, Imbalzano AN, Sif S. Versatility of PRMT5-induced methylation in growth control and development. Trends Biochem Sci 2011;36:633-641.
23) Pal S, Vishwanath SN, Erdjument-Bromage H, Tempst P, Sif S. Human SWI/SNF-associated PRMT5 methylates histone H3 arginine 8 and negatively regulates expression of ST7 and NM23 tumor suppressor genes. Mol Cell Biol 2004;24:9630-9645.
24) Euskirchen G, Auerbach RK, Snyder M. SWI/SNF chromatin-remodeling factors: multiscale analyses and diverse functions. J Biol Chem 2012;287:30897-30905.
25) Rall LB, Standring DN, Laub O, Rutter WJ. Transcription of hepatitis B virus by RNA polymerase II. Mol Cell Biol 1983;3:1766-1773.
26) Tropberger P, Mercier A, Robinson M, Zhong W, Ganem DE, Holdorf M. Mapping of histone modifications in episomal HBV cccDNA uncovers an unusual chromatin organization amenable to epigenetic manipulation. Proc Natl Acad Sci USA 2015;112:E5715-E5724.
27) Bartenschlager R, Schaller H. Hepadnaviral assembly is initiated by polymerase binding to the encapsidation signal in the viral RNA genome. EMBO J 1992;11:3413-3420.
28) Jones SA, Hu J. Hepatitis B virus reverse transcriptase: diverse functions as classical and emerging targets for antiviral intervention. Emerg Microbes Infect 2013;2:e56.
29) Benhenda S, Ducroux A, Riviere L, Sobhian B, Ward MD, Dion S, et al. Methyltransferase PRMT1 is a binding partner of HBx and a negative regulator of hepatitis B virus transcription. J Virol 2013;87:4360-4371.
30) Riviere L, Gerossier L, Ducroux A, Dion S, Deng Q, Michel ML, et al. HBx relieves chromatin-mediated transcriptional repression of hepatitis B viral cccDNA involving SETDB1 histone methyltransferase. J Hepatol 2015;63:1093-1102.
31) Wilson BG, Roberts CW. SWI/SNF nucleosome remodellers and cancer. Nat Rev Cancer 2011;11:481-492.
32) Shi L, Li S, Shen F, Li H, Qian S, Lee DH, et al. Characterization of the nucleosome positioning in hepadnaviral cccDNA minichromosome. J Virol 2012;86:10059-10069.
33) **Lucifora J, Xia Y**, Reisinger F, Zhang K, Stadler D, Cheng X, et al. Specific and nonhepatotoxic degradation of nuclear hepatitis B virus cccDNA. Science 2014;343:1221-1228.
34) **Mao R, Nie H**, Cai D, Zhang J, Liu H, Yan R, et al. Inhibition of hepatitis B virus replication by the host zinc finger antiviral protein. PLoS Pathog 2013;9:e1003494.
35) Wang Z, Ni J, Li J, Shi B, Xu Y, Yuan Z. Inhibition of hepatitis B virus replication by cIAP2 involves accelerating the ubiquitin-proteasome-mediated destruction of polymerase. J Virol 2011;85:11457-11467.
36) Hu J, Boyer M. Hepatitis B virus reverse transcriptase and epsilon RNA sequences required for specific interaction *in vitro*. J Virol 2006;80:2141-2150.
37) Stopa N, Krebs JE, Shechter D. The PRMT5 arginine methyltransferase: many roles in development, cancer and beyond. Cell Mol Life Sci 2015;72:2041-2059.
38) Shire K, Kapoor P, Jiang K, Hing MN, Sivachandran N, Nguyen T, et al. Regulation of the EBNA1 Epstein-Barr virus protein by serine phosphorylation and arginine methylation. J Virol 2006;80:5261-5272.
39) Wei H, Wang B, Miyagi M, She Y, Gopalan B, Huang DB, et al. PRMT5 dimethylates R30 of the p65 subunit to activate NF-kappaB. Proc Natl Acad Sci USA 2013;110:13516-13521.

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Supporting Information

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