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ARTICLE

IFN-α inhibits HBV transcription and replication by promoting HDAC3-mediated de-2-hydroxyisobutyrylation of histone H4K8 on HBV cccDNA minichromosome in liver

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The epigenetic modification of hepatitis B virus (HBV) covalently closed circular DNA (cccDNA) plays a crucial role in cccDNA transcription and viral persistence. Interferon-α (IFN-α) is a pivotal agent against HBV cccDNA. However, the mechanism by which IFN-α modulates the epigenetic regulation of cccDNA remains poorly understood. In this study, we report that IFN-α2b enhances the histone deacetylase 3 (HDAC3)-mediated de-2-hydroxyisobutyrylation of histone H4 lysine 8 (H4K8) on HBV cccDNA minichromosome to restrict the cccDNA transcription in liver. By screening acetyltransferases and deacetylases, we identified that HDAC3 was an effective restrictor of HBV transcription and replication. Moreover, we found that HDAC3 was able to mediate the de-2-hydroxyisobutyrylation of H4K8 in HBV-expressing hepatoma cells. Then, the 2-hydroxyisobutyrylation of histone H4K8 (H4K8hib) was identified on the HBV cccDNA minichromosome, promoting the HBV transcription and replication. The H4K8hib was regulated by HDAC3 depending on its deacetylase domain in the system. The low level of HDAC3 and high level of H4K8hib were observed in the liver tissues from HBV-infected human liver-chimeric mice. The levels of H4K8hib on HBV cccDNA minichromosome were significantly elevated in the liver biopsy specimens from clinical hepatitis B patients, which was consistent with the high transcriptional activity of cccDNA. Strikingly, IFN-α2b effectively facilitated the histone H4K8 de-2-hydroxyisobutyrylation mediated by HDAC3 on the HBV cccDNA minichromosome in primary human hepatocytes and hepatoma cells, leading to the inhibition of HBV transcription and replication. Our finding provides new insights into the mechanism by which IFN-α modulates the epigenetic regulation of HBV cccDNA minichromosome.

Keywords: HBV; cccDNA minichromosome; IFN-α; epigenetic regulation; HDAC3; 2-hydroxyisobutyrylation

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INTRODUCTION

Chronic infection of hepatitis-B virus (HBV) is a global public health issue, with more than 240 million individuals infected [1]. HBV is a hepatotropic, partially double-stranded DNA virus [2]. HBV covalently closed circular DNA (cccDNA) minichromosome, serving as the template for the transcription of pregenomic RNA (pgRNA) and other subgenomic viral RNAs, is a key intermedium in HBV-replication cycle [3]. HBV cccDNA is an obstacle in clinic, leading to the persistent infection of HBV and recurrence upon the discontinuation of treatment [4]. HBV cccDNA is accumulated in the nucleus as a chromatin-like cccDNA minichromosome assembled by histones and non-histones [5–8]. Thereby, the host factors that regulate the status of cccDNA minichromosome become the potential targets of anti-HBV therapy. Interferon-α (IFN-α) displays potent antiviral activities by inducing IFN-stimulated genes (ISGs), and the critical role of IFN-α-induced signaling pathways has been demonstrated in the host antiviral responses [9–11]. IFN-α inhibits HBV transcription and replication by epigenetic regulation of the nuclear cccDNA minichromosome [12, 13]. Recently, our group has reported that IFN-α eliminates HBV cccDNA by regulating GCN5-mediated histone H3K79 succinylation in the cells, in which the human liver-chimeric mouse model was used to evaluate the cccDNA [14]. However, the roles of IFN-α in the epigenetic regulation of cccDNA minichromosome remain poorly understood.

The post-translational modifications (PTMs) of histones and nonhistone proteins that bind to cccDNA minichromosome have become a hotspot [15]. Accumulating evidence suggests that the host epigenetic modulations, including DNA methylation and DNA-bound histone modifications, play vital roles in the cccDNA transcriptional activity [12, 16, 17]. The histone modifications on cccDNA contribute to the HBV transcription. The H3 and H4 histones, HBc, HBx, the host transcription factors, and the epigenetic modification enzymes such as P300 and HDAC1 have been associated with cccDNA transcription [12, 16–18]. However, the roles of Tip60, HDAC2, HDAC3, and Sirt5 in the epigenetic regulation of cccDNA minichromosome remain unclear. The histone deacetylases (HDACs) are a superfamily of chromatin-

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modifying enzymes that silence transcription through the modification of histones, in which the histone deacetylase 3 (HDAC3) is the predominant HDAC [19]. However, whether HDAC3 is involved in the HBV transcription in liver is elusive. It has been reported that the acetylation, methylation, and succinylation status of H3 and H4 on the cccDNA minichromosome is associated with cccDNA transcriptional activity [17, 20, 21]. As a novel type of post-translational modification, lysine 2-hydroxyisobutyrylation (Khib) plays an important role in gene transcription and signal transduction [22]. However, the effect of histone 2-hydroxyisobutyrylation on regulation of cccDNA minichromosome in the liver has not been reported yet.

In this study, we are interested in the significance of IFN-α and 2-hydroxyisobutyrylation in the modulation of cccDNA minichromosome in the liver. Strikingly, we identified that HDAC3 as a restrictor of HBV replication could catalyze the H4 lysine 8 (H4K8) de-2-hydroxyisobutyrylation on cccDNA minichromosome, leading to the epigenetic silence of cccDNA transcription. Moreover, IFN-α2b effectively facilitated the histone H4K8 de-2-hydroxyisobutyrylation. Our finding provides new insights into the mechanism by which IFN-α modulates the epigenetic regulation of HBV cccDNA minichromosome.

MATERIALS AND METHODS

Cell culture, HBV production, infection, and transfection

The cell lines such as HEK 293 T, HepG2-NTCP, and HepG2.2.15 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) [23]. HepG2-NTCP and HepG2.2.15 cells were cultured in 400 μg/mL G418. HepG2 cells were transfected with hNTCP expression plasmid using TransIT-LT1 (Mirus, USA) and HepG2-NTCP cells were established according to the manufacturer's instructions [24, 25]. HepAD38 (tet-off) cell was cultured in DMEM/F12 medium supplemented with 10% FBS and 400 μg/mL G418 (for induction of HBV replication). Primary human hepatocytes (PHHs) were purchased from Shanghai RILD Inc. (Shanghai, China). The cells were cultured similarly using the same plating and incubation medium as described [26, 27]. All cell lines were treated with 100 U/mL penicillin, and 100 mg/mL streptomycin in a humidified incubator equilibrated with 5% CO_2 at 37 °C. The cells were cultured in a 6-well plate or 100 mm dishes for 12 h and then were transfected with plasmid or siRNAs. The transfections were performed using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol.

HBV particles used in this study were mainly derived from HepAD38 cells, and the preparation method was as described [28, 29]. HBV particles were concentrated from the clarified supernatant by overnight precipitation with 10% PEG8000 and 2.5% NaCl. The precipitates were washed and resuspended with a medium at 200-fold concentration. The HBV DNA was quantified by real-time PCR. HBV infections were also performed as described [21, 28, 30]. Briefly, HepG2-NTCP and PHH cells were infected with HBV virus derived from HepAD38 cells at 1000–2000 genome equivalents (GEq)/cell in the presence of 4% PEG8000 and 1.5% DMSO at 37 °C for 16 h as previously described. Under these conditions, efficient infection of HepG2-NTCP and PHH cells requires an inoculum of $>10^4$ HBV GEq/cell.

Plasmids and reagents

The plasmids of HDAC3, Tip60, HDAC2, H4, and their mutants were amplified by PCR from the cDNA of HepG2.2.15 cells using specific primers and were cloned into the pCMV-3Tag-1a vector. The primers for constructing these vectors were listed in Supplementary Table S1. Plasmids used for transfection were listed in Supplementary Table S2. The small-interfering RNAs targeting the indicated genes (Supplementary Table S3) and the control small-interfering RNA were purchased from Sangon Biotech (Shanghai, China). Recombinant human IFN-α2b (Kawin, Beijing, China) was used at 1000 IU/mL.

Western blot analysis

Total protein lysates were extracted from hepatoma cells or tissue with RIPA buffer. Protein concentrations were measured using the Bradford Assay, and 20–50 μg of protein extracts were subjected to SDS-PAGE. Then proteins were transferred to a nitrocellulose membrane, blocked with 5% non-fat milk, and incubated with primary antibodies overnight at 4 °C. After incubation with secondary antibody against mouse (1:10,000) or rabbit (1:10,000) for 1 h at 37 °C, the membrane was visualized by Super ECL Detection Reagent (Yeasen Biotech, Shanghai, China). The primary antibodies used for Western blot analysis were listed in Supplementary Table S4.

RNA extraction and quantitative real-time PCR (RT-qPCR)

Total RNA was extracted from cells (or liver tissues from human liver-chimeric mice) using TRIzol reagent (Solarbio, Beijing, China). First-strand cDNA was synthesized using the Hifair III 1st strand cDNA synthesis supermix (Yeasen Biotech, Shanghai, China). Quantitative real-time PCR was performed on StepOnePlus real-time PCR machine (Bio-Rad), using qPCR SYBR Green Master Mix (Yeasen Biotech, Shanghai, China). Relative transcriptional folds were calculated as $2^{-\Delta\Delta Ct}$. GAPDH was used as an internal control for normalization. The primers used were listed in Supplementary Table S1.

HBV antigens and serum HBV DNA quantification

Hepatitis-B virus surface antigen (HBsAg) and hepatitis-B virus e antigen (HBeAg) in culture supernatants were assayed by commercial enzyme-linked immunosorbent assay (ELISA) (Kehua Bio-engineering, Shanghai, China). The HBV DNA in the supernatants of HepAD38 cells, HepG2.2.15, and HBV-infected HepG2-NTCP cells was quantified by using a diagnostic kit with a lower limit of detection of 400 copies/mL (Sansure Biotech, Changsha China).

Isolation of HBV cccDNA from cells and liver tissues and quantification of cccDNA

HBV cccDNA can be selectively extracted from HBV-infected HepG2-NTCP and HBV-replicating HepG2.2.15 cells by the improved method described previously [17, 21, 30]. For nuclei isolation, cells were lysed in 500 μL of homogenization buffer (50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.2% NP-40, and 150 mM NaCl), and centrifuged for 10 min at 10,000 r/min at 4 °C. Nuclei were then treated with 500 μL of lysis buffer (6% SDS, 100 mM NaOH), and the reaction was mixed thoroughly and incubated for 30 min at 37 °C. After neutralization with 3 M of sodium acetate (pH 5.2), lysates were cleared for 20 min at 10,000 r/min at 4 °C. HBV DNA was extracted from the supernatant with phenol/chloroform, precipitated with ethanol, and dissolved in 50 μL of 10 mM Tris-HCl (pH 7.5) and 1 mM EDTA (pH 8.0), and digested with DNase-free RNase and treated with Plasmid-safe deoxyribonuclease (Epicentre) for 45 min at 37 °C, then 30 min at 70 °C before being analyzed. Quantitative PCR was performed using HBV cccDNA-specific primers (Supplementary Table S1). The viral genome equivalent copies were calculated based on a standard curve generated with known copy numbers of a plasmid containing a HBV genome of greater than unit length.

Immunofluorescence assays

Cells in 6-well plates were washed three times with precooled PBS and fixed by 4% paraformaldehyde for 10 min, followed by permeabilization for 10 min at room temperature with 0.5% Triton X-100. After incubation for 1 h with 5% BSA for blockade of nonspecific binding, primary antibodies were added for incubation for overnight at 4 °C. The bound antibodies were visualized by

incubation with secondary antibodies. Images were acquired using a fluorescence Microscopy. The antibodies used for immunofluorescence analysis were listed in Supplementary Table S4.

Generation of human liver-chimeric mice

Human liver-chimeric mice were generated by VITALSTAR Inc. (Beijing, China). As described, primary human hepatocytes (PHHs) were transplanted into 3-week-old mouse model (male and female) of Tet-uPA Rag2-$^{-/-}$ IL2RG-$^{-/-}$ (URG) that had been doxycycline (Dox)-induced liver injury [28, 31–33], resulting in a human liver-chimeric mouse model (Huhep-URG). Human serum albumin was measured by enzyme-linked immunosorbent assays (Human Albumin ELISA kit, Immunology Consultants Lab, Portland, USA) for assessing the engraftment and viability of PHHs. Then, the Huhep-URG mice were infected with 5.0×10^8 IU/mL (0.2 mL/mouse) HBV particles from the supernatant of HepAD38 cells (tet-off) and sacrificed 8 weeks after virus inoculation. Serum HBV DNA load in the mice was determined by quantitative PCR (Da An Gene, Guangzhou, China) before sacrifice. The information of human liver-chimeric mice was shown in Supplementary Table S5.

All animal experiments were in accordance with the Guide for the Care and Use of Laboratory Animals (NIH publications 86-23 revised 1985) and were performed according to the institutional ethical guidelines. The Institute Research Ethics Committee at the Nankai University approved the study protocol.

Chromatin immunoprecipitation (ChIP) and Re-ChIPs

ChIP assays were performed by using simple CHIP(R) plus sonication CHIP kit 4 C and RT Reagents according to the manufacturer's instructions (Cell Signaling Technology, MA, USA) [21, 34]. After the reverse cross-linking, DNA was extracted and further treated with Plasmid-Safe ATP-Dependent DNase (Epicentre Biotechnologies, Madison, WI, USA) to degrade contaminating RC and single-stranded forms of HBV DNA. Re-ChIP assays were performed by using Re-ChIP-IP kit (Active Motif, Shanghai, China). Briefly, the immunoprecipitated chromatin was removed from the magnetic beads. The chromatin was then desalted and a second ChIP was performed using a different antibody from the first. The cross-links of these sequentially immunoprecipitated protein−DNA complexes were then reversed and the DNA was analyzed by quantitative PCR. Precipitated DNA was analyzed by quantitative PCR, and the primers used were listed in Supplementary Table S1.

Patient samples

The clinical and virological characteristics of the patients enrolled in this study are summarized in Supplementary Table S6. Liver-tissue samples were obtained by percutaneous needle biopsy, immediately frozen in liquid nitrogen, and stored at −80 °C. Written informed consents approving the use of their tissue for research purposes after operation were obtained from patients, and the Institute Research Ethics Committee at the Nankai University approved the study protocol.

Statistical analysis

The data are presented as the mean ± standard deviation (SD) of at least three replicate experiments. Statistical analyses were performed by the Student *t* test or one-way analysis of variance. Statistical significance was assumed for $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

RESULTS

HDAC3 restricts the cccDNA transcription and HBV replication

The epigenetic modulations play vital roles in the cccDNA transcriptional activity, in which the epigenetic modification enzymes such as P300 and HDAC1 are involved in the events [12, 16, 17]. To identify novel host factors involved in the regulation of cccDNA transcription, we applied the RNA interference-based approach to screen a group of known histone acetyltransferases and deacetylases, including P300, Tip60, HDAC1, HDAC2, HDAC3, and Sirt5 (Fig. 1a and Supplementary Table S3) [35–37]. The expression levels of HBsAg and HBeAg were used as surrogate markers for the transcription of cccDNA [17]. Surprisingly, we found that HDAC3 significantly affected the expression of HBsAg and HBeAg among the above factors (Fig. 1a). The interference efficiencies for all siRNAs were displayed in Supplementary Fig. S1a. Next, we generated human liver-chimeric mice by using the protocol shown in Fig. 1b. Interestingly, the HDAC3 mRNA levels were significantly lower in the HBV-infected human liver-chimeric mice than those in the control human liver chimeric mice (Fig. 1c), and the mRNA levels of HDAC3 were significantly lower in human liver-biopsy specimens with high hepatitis-B viral load than those in human liver-biopsy specimens with low viral-load specimens (Fig. 1d), suggesting that HDAC3 is negatively associated with HBV in the liver. Next, we found that the overexpression (or knockdown) of HDAC3 could downregulate (or upregulate) the levels of HBsAg, HBeAg, and HBV DNA in the supernatant and intracellular pregenomic RNA (pgRNA) in the HBV-infected HepG2-NTCP and HBV-expressing HepG2.2.15 cells (Fig. 1e, f, Supplementary Fig. S1b, c), but the mutant of HDAC3Y298F without enzymatic activity [38] failed to work. Immunofluorescence staining showed that the overexpression (or knockdown) of HDAC3 could downregulate (or upregulate) HBc in the cells (Fig. 1g, Supplementary Fig. S1d), suggesting that HDAC3 inhibits the HBV transcription. The overexpression efficiency of HDAC3 and HDAC3Y298F was validated by Western blot analysis in the cells (Supplementary Fig. S1e). The interference efficiencies of two siHDAC3 fragments were confirmed by Western blot analysis in HEK293T cells (Supplementary Fig. S1f, g). We further found that HDAC3 inhibited the transcriptional activity of HBV cccDNA in the cells (defined as pgRNA/cccDNA ratio), but not the level of HBV cccDNA (Fig. 1h, i, Supplementary Fig. S1h, i), suggesting that HDAC3 is able to suppress the transcriptional activity of HBV cccDNA. Thus, we conclude that HDAC3 restricts the cccDNA transcription and HBV replication.

HDAC3-mediated 2-hydroxyisobutyrylation of histone H4K8 is anchored on the HBV cccDNA minichromosome

Lysine 2-hydroxyisobutyrylation (Khib) is a newly identified histone modification conserved from yeast to humans [39]. However, the site of 2-hydroxyisobutyrylation of histone H4 removed by HDAC3 remains unclear. Considering that the 2-hydroxyisobutyrylation can be removed by HDAC3 and HDAC2 [39] and that Tip60 as a writer catalyzed the H4K8hib [40], we were interested in whether Tip60 and HDAC2 affected the levels of Pan-Khib on the cccDNA minichromosome. Strikingly, our data showed that the overexpression (or knockdown) of Tip60 increased (or reduced) the binding of Tip60 to cccDNA, leading to the increase (or decrease) of 2-hydroxyisobutyrylation in HBV-infected HepG2-NTCP and HBV-expressing HepG2.2.15 cells (Supplementary Fig. S2-1a–d), suggesting that Tip60 is able to induce the 2-hydroxyisobutyrylation on the HBV cccDNA minichromosome. However, HDAC2 displayed the opposite roles in the system (Supplementary Fig. S2-1e–h). Moreover, we asked whether HDAC3 had an effect on H4K8hib. Our data showed that the overexpression of HDAC3 resulted in the H4K8hib reduction in HBV-infected HepG2-NTCP and HBV-expressing HepG2.2.15 cells, but the mutant of HDAC3Y298F failed to work (Fig. 2a). Further, Western blot analysis validated that the levels of histone H4K8 2-hydroxyisobutyrylation were observably enhanced in the HBV-infected human liver-chimeric mice relative to those in the control group (Fig. 2b). Next, we supposed that 2-hydroxyisobutyrylation of histone H4K8 mediated by HDAC3 might influence the epigenetic regulation of cccDNA minichromosome. Surprisingly, ChIP assays and Re-ChIP assays revealed that the 2-hydroxyisobutyrylation of proteins, the 2-hydroxyisobutyrylation

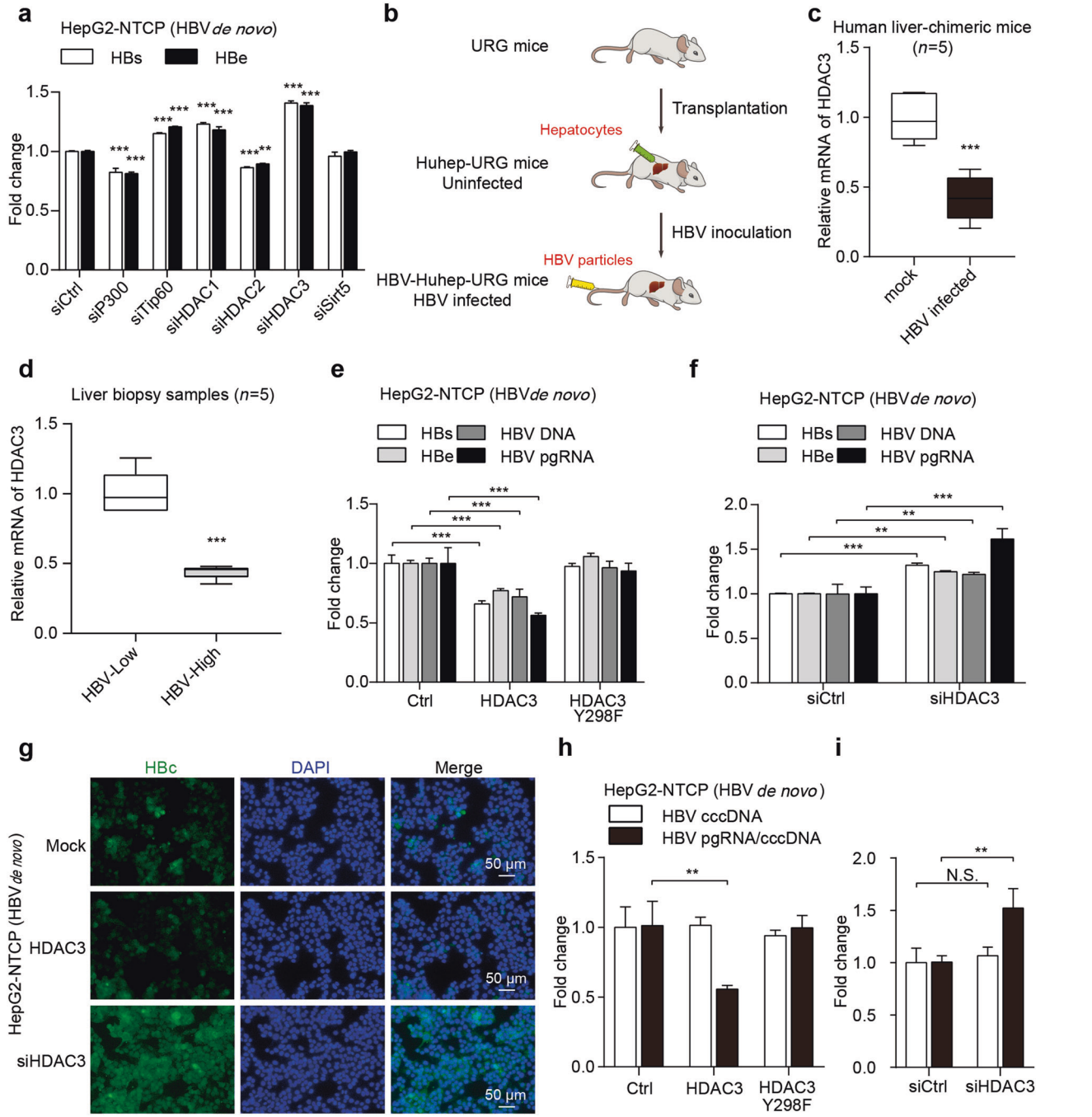

Fig. 1 HDAC3 restricts the cccDNA transcription and HBV replication. a HepG2-NTCP cells were infected with HBV *de novo* and transfected with indicated short interfering RNA (siRNA) at -2, 1, and 4 dpi (days post infection). Levels of HBsAg and HBeAg in the supernatant were detected by ELISA 7 days post-inection. **b** Schematic diagram of human liver-chimeric mouse model construction. **c** The mRNA levels of HDAC3 were analyzed by RT-qPCR in the livers of HBV uninfected ($n = 5$) and infected ($n = 5$) human liver-chimeric mice. **d** The mRNA levels of HDAC3 were analyzed by RT-qPCR in the liver-biopsy specimens from clinical hepatitis-B patients with low ($n = 5$) and high ($n = 5$) HBV viral load. **e–i** HBV–infected HepG2-NTCP cells were transfected with HDAC3/HDAC3Y298F or siHDAC3. The levels of HBsAg, HBeAg, and HBV DNA were measured by ELISA and qPCR in the supernatant, respectively. HBV pgRNA and cccDNA were extracted and quantified by qPCR. HBV cccDNA transcription activity was assessed by calculating the ratio of pgRNA/cccDNA in the cells. HBV core proteins were observed by immunofluorescence staining using the specific antibodies in the cells. The mean ± SD of at least three experiments is shown. Statistically, significant differences are indicated as follows: $^{**}P < 0.01$, $^{***}P < 0.001$. N.S. not significant.

of histone H3, and the 2-hydroxyisobutyrylation of histone H4K8 were observed on the cccDNA minichromosome in HBV-infected human liver-chimeric mice, HBV-expressing HepG2.2.15, and HBV-infected HepG2-NTCP cells using the Pan-Khib antibodies, anti-H3 antibodies, and anti-H4K8hib antibodies, respectively. The pan-acetylation was used as positive control in the system (Fig. 2c–h, Supplementary Fig. S2-2a–c), suggesting that the histone H4K8 2-hydroxyisobutyrylation is associated with the epigenetic regulation of cccDNA minichromosome in a model (Supplementary Fig. S2-2d). Thus, we conclude that HDAC3-mediated 2-hydroxyisobutyrylation of histone H4K8 is anchored on the HBV cccDNA minichromosome.

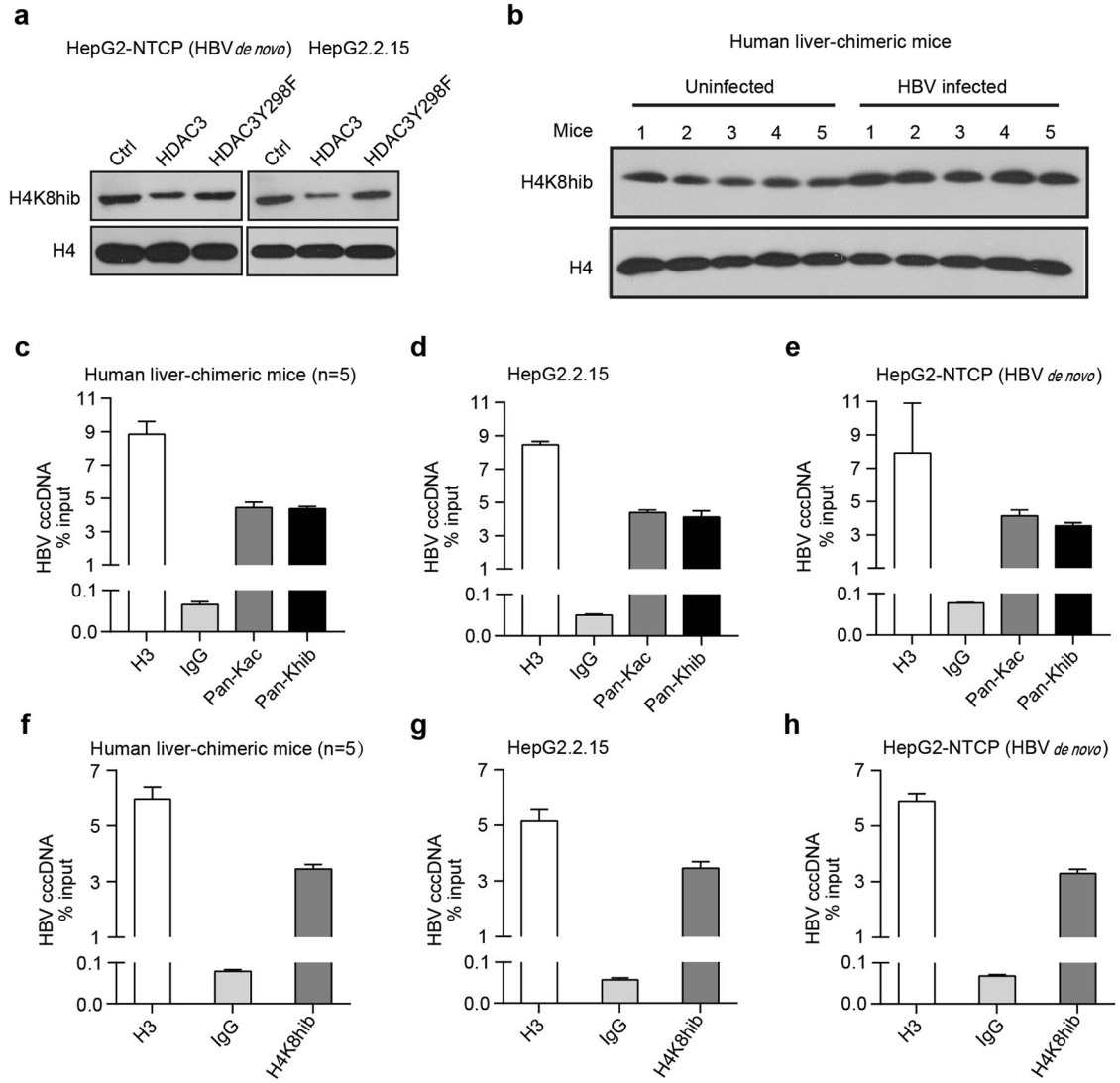

Fig. 2 HDAC3-mediated 2-hydroxyisobutyrylation of histone H4K8 is anchored on the HBV cccDNA minichromosome. a HBV-infected HepG2-NTCP and HepG2.2.15 cells were transfected with HDAC3/HDAC3Y298F plasmid. The levels of H4K8hib were examined by Western blot analysis in the cells. **b** The levels of H4K8 2-hydroxyisobutyrylation were determined by Western blot analysis in the liver from HBV uninfected and infected human liver chimeric mice (each, $n = 5$). **c** The levels of acetylation and 2-hydroxyisobutyrylation on the cccDNA minichromosome were determined by ChIP-qPCR in the liver from HBV-infected human liver-chimeric mice ($n = 5$). **d**, **e** The levels of acetylation and 2-hydroxyisobutyrylation on the cccDNA minichromosome were examined by ChIP-qPCR in HepG2.2.15 and HBV-infected HepG2-NTCP cells. **f** The level of H4K8 2-hydroxyisobutyrylation on cccDNA minichromosome was analyzed by ChIP-qPCR in liver from HBV-infected human liver-chimeric mice ($n = 5$). **g**, **h** The levels of H4K8 2-hydroxyisobutyrylation on the cccDNA minichromosome were analyzed by ChIP-qPCR in HepG2.2.15 and HBV-infected HepG2-NTCP cells. The mean ± SD of at least three experiments is shown.

H4K8 2-hydroxyisobutyrylation contributes to the cccDNA transcription and HBV replication

Given that the H4K8hib was associated with the regions of active gene transcription by ChIP-seq, gene expression analysis, and immunodetection [40], we speculated that the 2-hydroxyisobutyrylation of histone H4K8 might be necessary for the epigenetic regulation of cccDNA minichromosome. Interestingly, we observed that the overexpression of histone H4 and the 2-hydroxyisobutyrylation-mimic H4K8T mutant (K−T) increased the levels of HBV DNA, HBsAg, and HBeAg in the supernatant and pgRNA/cccDNA from HBV-expressing HepG2.2.15 and HBV-infected HepG2-NTCP cells, but not HBV cccDNA, whereas the mutant of histone H4K8R (K−R) failed to work in the system (Fig. 3a–d, Supplementary Fig. S3a), suggesting that H4K8hib can promote cccDNA transcription and HBV replication, rather than the level of HBV cccDNA. The efficiency of overexpression of histone H4, H4K8R, and H4K8T was verified in the system (Supplementary Fig. S3b). Moreover, we observed that the cccDNA transcriptional activity (pgRNA/cccDNA) was fluctuated during the HBV infection in the HBV-infected HepG2-NTCP models (Fig. 3e). The level of H4K8hib on cccDNA minichromosome was consistent with the cccDNA transcriptional activity (pgRNA/cccDNA) in the cells. However, the status of H4K8hib was relatively stable at the transcription-start site of host gene GAPDH (Fig. 3f), suggesting that H4K8hib is responsible for the cccDNA transcription and HBV replication. Then, we verified that the levels of H4K8hib on HBV cccDNA minichromosome were significantly elevated in the liver-biopsy specimens from clinical hepatitis-B patients ($n = 5$), which was consistent with the high transcriptional activity of cccDNA, but not the levels of H4 on HBV cccDNA minichromosome and the levels of H4K8hib on GAPDH (Fig. 3g–i, Supplementary Fig. S3c). Thus, we conclude that H4K8 2-hydroxyisobutyrylation contributes to the cccDNA transcription and HBV replication.

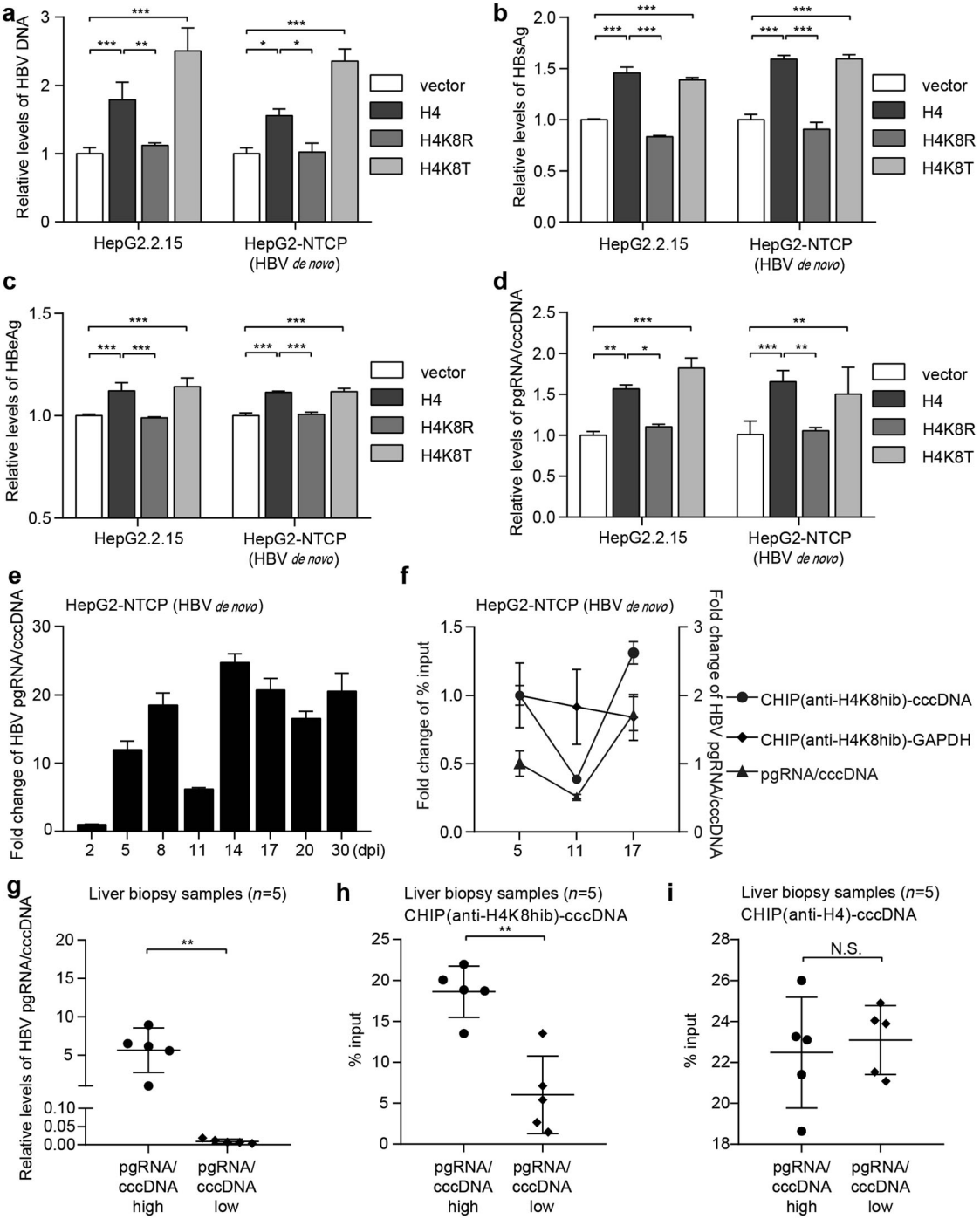

Fig. 3 H4K8 2-hydroxyisobutyrylation contributes to the cccDNA transcription and HBV replication. a–d HBV-infected HepG2-NTCP and HepG2.2.15 cells were transfected with H4/H4K8R/H4K8T. The levels of HBsAg and HBeAg (or HBV DNA) in the supernatant were measured by ELISA (or qPCR), respectively. HBV pgRNA and cccDNA were extracted and quantified by qPCR. HBV cccDNA transcription activity was assessed by calculating the ratio of pgRNA/cccDNA in the cells. **e**, **f** HepG2-NTCP cells were infected with HBV *de novo*. HBV pgRNA and cccDNA were extracted and quantified by qPCR. HBV cccDNA transcription activity was assessed by calculating the ratio of pgRNA/cccDNA in the cells. The levels of H4K8 2-hydroxyisobutyrylation on the cccDNA or host gene GAPDH DNA were examined by ChIP-qPCR in the cells at indicated days. **g–i** The liver biopsy specimen was used for RNA extraction and ChIP assay using H4 or H4K8hib antibody. HBV pgRNA and cccDNA were extracted and quantified by qPCR. HBV cccDNA transcription activity was assessed by calculating the ratio of pgRNA/cccDNA. Levels of H4K8hib and H4 on cccDNA were examined by qPCR. The mean ± SD of at least three experiments is shown. Statistically significant differences are indicated as follows: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. N.S. not significant.

HDAC3 is responsible for the de-2-hydroxyisobutyrylation of H4K8 on cccDNA minichromosome

Based on that HDAC3 and H4K8 2-hydroxyisobutyrylation contributed to the cccDNA transcription and HBV replication, we supposed that HDAC3 might be involved in the deacetylation of histones H3 and H4, de-2-hydroxyisobutyrylation of histone H3, and de-2-hydroxyisobutyrylation of H4K8 on cccDNA minichromosome to modulate the cccDNA transcription and HBV replication.

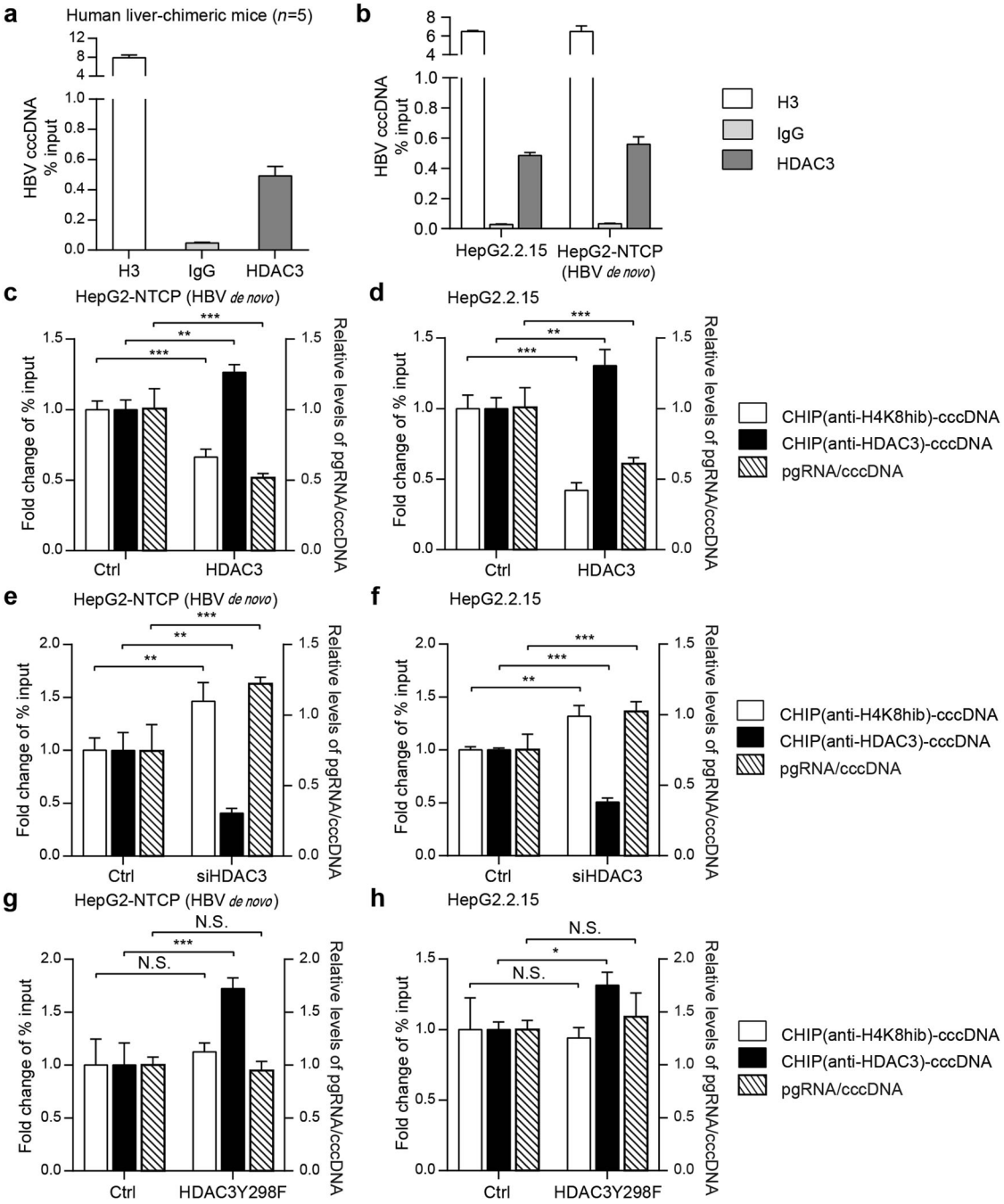

Fig. 4 HDAC3 is responsible for the de-2-hydroxyisobutyrylation of H4K8 on cccDNA minichromosome. a The level of HDAC3 on cccDNA minichromosome was analyzed by ChIP-qPCR in the liver from HBV-infected human liver-chimeric mice ($n = 5$). **b** The levels of HDAC3 on the cccDNA minichromosome were analyzed by ChIP-qPCR in HepG2.2.15 and HBV-infected HepG2-NTCP cells. **c**–**h** HBV-infected HepG2-NTCP and HepG2.2.15 cells were transfected with HDAC3/HDAC3Y298F or siHDAC3. The levels of H4K8 2-hydroxyisobutyrylation and HDAC3 on the cccDNA were examined by ChIP-qPCR. HBV pgRNA and cccDNA were extracted and quantified by qPCR. HBV cccDNA transcription activity was assessed by calculating the ratio of pgRNA/cccDNA in the cells. The mean ± SD of at least three experiments is shown. Statistically, significant differences are indicated as follows: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. N.S. not significant.

Interestingly, ChIP assays revealed that the HDAC3 bound to the HBV cccDNA in the liver from HBV-infected human liver-chimeric mice, HBV-expressing HepG2.2.15, and HBV-infected HepG2-NTCP cells (Fig. 4a, b), suggesting that HDAC3 is anchored on the HBV cccDNA minichromosome. We further examined whether the expression of HDAC3 affected the status of cccDNA-bound AcH3, AcH4, H3Khib, and H4K8hib. Strikingly, our data showed that the overexpression (or knockdown) of HDAC3 increased (or reduced) the binding of HDAC3 to cccDNA, leading to the decrease (or increase) of acetylation of histone H3 and H4, 2-hydroxyisobutyrylation of histone H3 and H4K8 2-hydroxyisobutyrylation and cccDNA transcription activity in HBV-infected HepG2-NTCP and HBV-expressing HepG2.2.15 cells (Fig. 4c–f, Supplementary Fig. S4a, b), suggesting that HDAC3 is able to induce the de-2-hydroxyisobutyrylation of H4K8 on the HBV cccDNA minichromosome, which inhibits the cccDNA transcription. The mutant of HDAC3 (Y298F) increased the binding of HDAC3 to cccDNA, but failed to work in the system (Fig. 4g, h, Supplementary Fig. S4c),

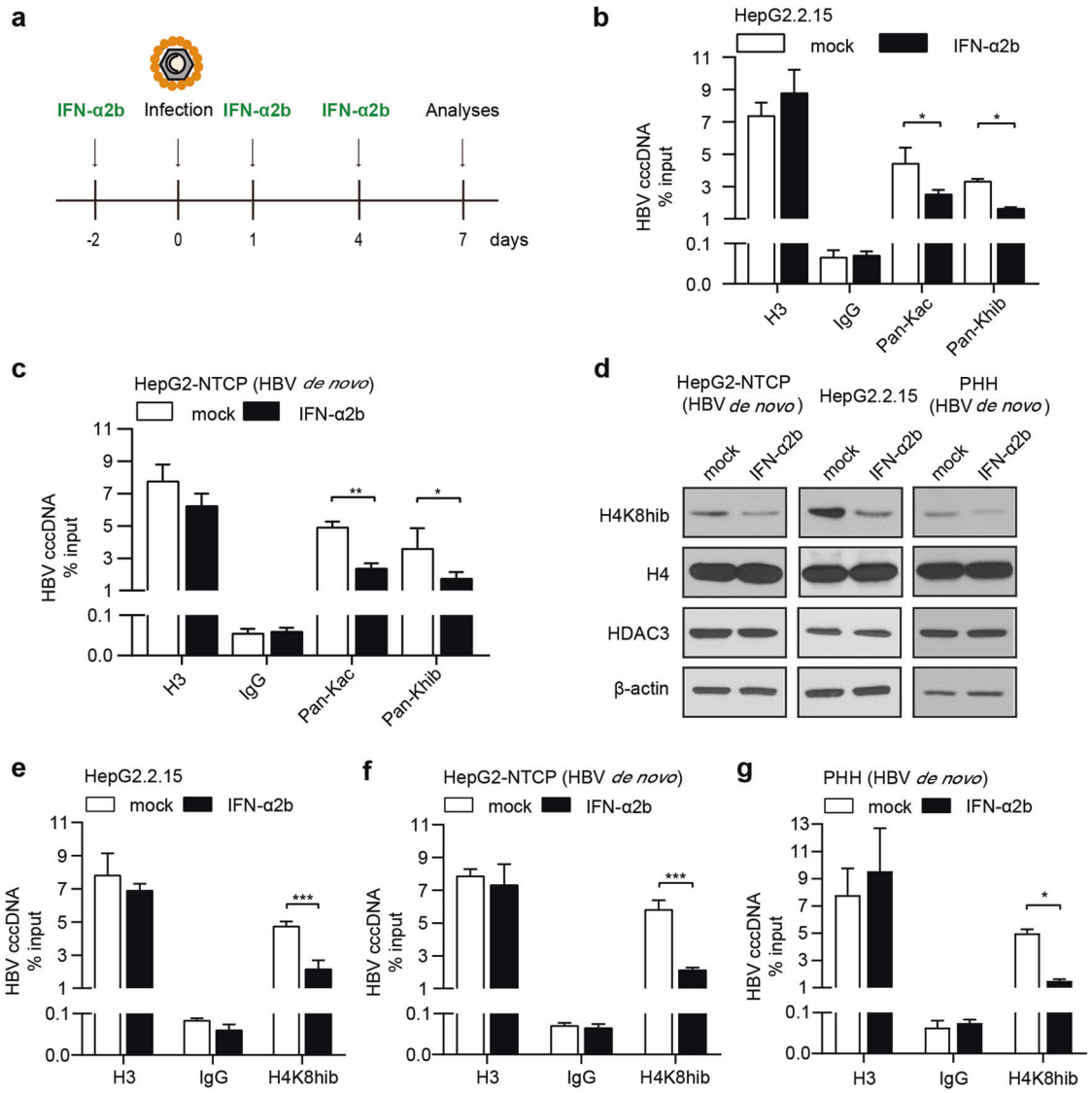

Fig. 5 IFN-α2b confers the 2-hydroxyisobutyrylation of H4K8 on the HBV cccDNA minichromosome. a A process schematic diagram of the following experiment. **b**–**f** HepG2.2.15 and HBV-infected HepG2-NTCP cells were treated without or with IFN-α for 7 days at 1000 IU/mL. **b**, **c** The levels of acetylation and 2-hydroxyisobutyrylation on the cccDNA minichromosome were examined by ChIP-qPCR in the cells. **d** The levels of H4K8 2-hydroxyisobutyrylation and HDAC3 were examined by Western blot analysis in the HepG2.2.15, HBV-infected HepG2-NTCP, and HBV-infected PHH cells. **e**–**g** The level of H4K8 2-hydroxyisobutyrylation on cccDNA minichromosome was analyzed by ChIP-qPCR in the cells. The mean ± SD of at least three experiments is shown. Statistically, significant differences are indicated as follows: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

suggesting that HDAC3 works in a HDAC3 (Y298)-dependent manner. Together, we conclude that HDAC3 is responsible for the de-2-hydroxyisobutyrylation of H4K8 on cccDNA minichromosome in a model (Supplementary Fig. S4d).

IFN-α2b confers the 2-hydroxyisobutyrylation of H4K8 on the HBV cccDNA minichromosome

Considering that IFN-α2b could achieve the effect on HBV by targeting epigenetic regulation of nuclear cccDNA minichromosome [12, 14], we concerned that IFN-α2b might affect the 2-hydroxyisobutyrylation of histone H4K8 on the cccDNA minichromosome. HepG2.2.15 and HepG2-NTCP cells were treated with IFN-α2b and HepG2-NTCP cells were infected with HBV, as depicted in the schematic illustration in a model (Fig. 5a). At the outset, ChIP assays demonstrated that IFN-α2b reduced the levels of 2-hydroxyisobutyrylation on cccDNA minichromosome in the HBV-expressing HepG2.2.15 and HBV-infected HepG2-NTCP cells using pan-antibody. The pan-acetylation was used as positive control [12] in the system (Fig. 5b, c). Notably, Western blot analysis revealed that the treatment with IFN-α2b inhibited the levels of intracellular 2-hydroxyisobutyrylation on histone H4K8 in the HBV-expressing HepG2.2.15, HBV-infected HepG2-NTCP, and HBV-infected PHH cells, but not the levels of HDAC3, respectively (Fig. 5d). Moreover, ChIP assays validated that IFN-α2b attenuated the levels of H4K8 2-hydroxyisobutyrylation on the cccDNA minichromosome in the cells (Fig. 5e–g). Thus, we conclude that IFN-α2b decreases the levels of H4K8 2-hydroxyisobutyrylation on the HBV cccDNA minichromosome.

IFN-α2b restricts cccDNA transcription and HBV replication by inducing de-2-hydroxyisobutyrylation of H4K8 on the HBV cccDNA minichromosome

Given that IFN-α2b could inhibit the HBV transcription and replication in the cells and humanized mice [12, 41, 42], we

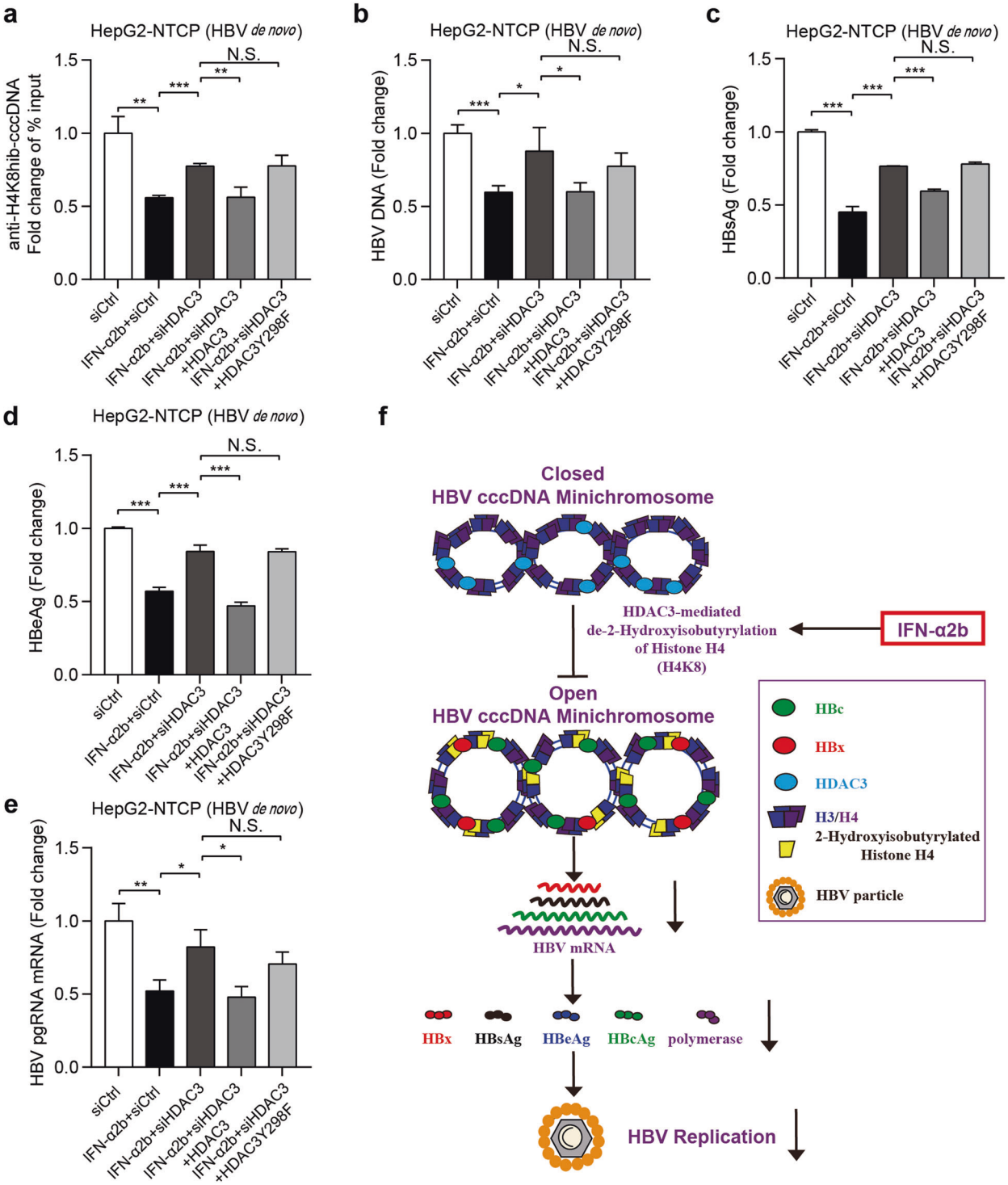

Fig. 6 IFN-α2b restricts cccDNA transcription and HBV replication by inducing de-2-hydroxyisobutyrylation of H4K8 on the HBV cccDNA minichromosome. a The level of H4K8 2-hydroxyisobutyrylation on cccDNA minichromosome was analyzed by ChIP-qPCR in HBV-infected HepG2-NTCP cells. **b**–**d** The levels of HBV DNA, HBsAg, and HBeAg in the supernatant were measured by ELISA and qPCR in the cells, respectively. **e** HBV pgRNA and cccDNA were extracted and quantified by qPCR in the cells. HBV cccDNA transcription activity was assessed by calculating the ratio of pgRNA/cccDNA in the cells. **f** Schematic representation of IFN-α modulating epigenetic regulation of cccDNA minichromosome by promoting HDAC3-mediated de-2-hydroxyisobutyrylation. In this model, histone H4K8 2-hydroxyisobutyrylation modulates epigenetic regulation of cccDNA minichromosome, promoting cccDNA transcription and HBV replication. HDAC3 is responsible for the histone H4K8 de-2-hydroxyisobutyrylation on cccDNA minichromosome. IFN-α inhibits the histone H4K8 2-hydroxyisobutyrylation on cccDNA minichromosome. The mean ± SD of at least three experiments is shown. Statistically significant differences are indicated as follows: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. N.S. not significant.

supposed that IFN-α2b might attenuate the cccDNA transcription and HBV replication by inducing the de-2-hydroxyisobutyrylation of H4K8 on the HBV cccDNA minichromosome. Interestingly, our data showed that IFN-α2b reduced the levels of 2-hydroxyisobutyrylation of H4K8 on the cccDNA minichromosome, HBV DNA, HBsAg, HBeAg, and pgRNA in HBV-expressing HepG2.2.15 and HBV-infected HepG2-NTCP cells (Fig. 6a–e, Supplementary Fig. S5a–e). Subsequently, the siHDAC3 enhanced the binding of H4K8 2-hydroxyisobutyrylation to the cccDNA minichromosome, which rescued the IFN-α2b-mediated inhibition of cccDNA transcription and HBV replication (Fig. 6a–e, Supplementary Fig. S5a–e), suggesting that both IFN-α2b and HDAC3 contribute to the de-2-hydroxyisobutyrylation of H4K8, leading to the inhibition of cccDNA transcription and HBV replication. As expected, our data showed that the HDAC3Y298F failed to reverse the rescue of H4K8 2-hydroxyisobutyrylation mediated by siHDAC3 relative to HDAC3Y298 in the system (Fig. 6a–e, Supplementary Fig. S5a–e), suggesting that HDAC3Y298 is required for the HDAC3-mediated de-2-hydroxyisobutyrylation of H4K8 on the cccDNA minichromosome. Thereby, we conclude that IFN-α2b restricts cccDNA transcription and HBV replication by inducing de-2-hydroxyisobutyrylation of H4K8 on the HBV cccDNA minichromosome (Fig. 6f).

DISCUSSION

The epigenetic regulation of HBV cccDNA minichromosome plays a crucial role in the viral persistence [3, 27]. IFN-α can inhibit cccDNA transcription and HBV DNA replication [12–14, 43]. However, the effect of IFN-α on the epigenetic regulation of cccDNA minichromosome is poorly understood. In the present study, we investigated the mechanism by which IFN-α modulated the HDAC3-mediated de-2-hydroxyisobutyrylation of histone H4K8 on cccDNA minichromosome.

Similar to the host nucleosome assembly, HBV cccDNA exists in the nucleus of liver cells as a stable minichromosome composed of cellular histones and nonhistone proteins [5–8]. Recently, the research on histone PTM of cccDNA minichromosome has become a hotspot, including histone acetylation, succinylation, methylation, etc. [15, 44]. Many epigenetic markers related to viral transcription have been found to bind on the cccDNA minichromosome [14, 17, 21, 27, 45–47]. Accumulating evidence shows that the epigenetic modification of cccDNA minichromosome mediated by various post-translational modifying enzymes affects cccDNA transcription and HBV replication, such as the acetylation of H3K27 and H3K122 (H3K27ac and H3K122ac) mediated by acetylation-modifying enzymes [27, 45, 46], methylation-modifying enzyme-mediated trimethylation of H3K9 (H3K9me3) and H3K27 (H3K27me3) [21, 47], PRMT5-mediated symmetric methylation of H4R3 (H4R3me2s) [17], and GCN5-mediated succinylation of H3K79 (H3K79suc) [14]. We are interested in whether there are other host factors involved in the epigenetic modification of cccDNA. The effect of HDAC3 as an eraser to remove histone 2-hydroxyisobutyrylation on HBV has not been reported yet. In this study, we screened and identified that HDAC3 could inhibit the cccDNA transcription and HBV replication in the HBV-infected HepG2-NTCP and HBV-expressing HepG2.2.15 cells, in which the HDAC3Y298 was required for the enzyme activity of HDAC3. It suggests that HDAC3 is involved in the modulation of cccDNA transcription and HBV replication in the liver.

Histone lysine 2-hydroxyisobutyrylation is an evolutionarily conserved and dynamic marker [39]. However, unlike the various epigenetic modifications that have been well documented, new histone modifications such as histone 2-hydroxyisobutyrylation on the cccDNA minichromosome remain unclear. Considering that H4K8 2-hydroxyisobutyrylation was associated with high transcriptional activity of genes [39], we asked whether the histone 2-hydroxyisobutyrylation played a role in the epigenetic regulation of cccDNA minichromosome. Given that the 2-hydroxyisobutyrylation could be removed by HDAC3 [39, 40], in this study, we demonstrated that HDAC3 had an effect on H4K8hib reduction in HBV-infected HepG2-NTCP and HBV-expressing HepG2.2.15 cells, in which the HDAC3Y298 was required for the H4K8 de-2-hydroxyisobutyrylation. Surprisingly, in vivo and in vitro, the 2-hydroxyisobutyrylation and 2-hydroxyisobutyrylation of histone H3 were observed on the cccDNA minichromosome. We further identified that the 2-hydroxyisobutyrylation of H4K8 was involved in the event, which was validated in the model of HBV-infected human liver-chimeric mice. It suggests that the 2-hydroxyisobutyrylation of histone H4K8 plays an important role in the epigenetic regulation of cccDNA minichromosome. Functionally, our data showed that the 2-hydroxyisobutyrylation of histone H4K8 promoted the cccDNA transcription and HBV replication in HBV-expressing cells. In the liver-biopsy specimens of clinical hepatitis-B cases, the histone H4K8 2-hydroxyisobutyrylation level of samples with high transcriptional activity was significantly increased. It strongly suggests that the 2-hydroxyisobutyrylation of histone H4K8 on the cccDNA minichromosome can enhance the cccDNA transcription. Our data suggest that HDAC3 can bind to the cccDNA minichromosome to induce the deacetylation of histones H3 and H4, de-2-hydroxyisobutyrylation of histone H3, and H4K8 de-2-hydroxyisobutyrylation, leading to the inhibition of cccDNA transcription and HBV replication in the liver. We first report that the histone H4K8 2-hydroxyisobutyrylation mediated by HDAC3 is also required for the epigenetic regulation of cccDNA minichromosome beside histone acetylation, methylation and succinylation.

It has been reported that IFN-α inhibits HBV transcription and replication in cell culture and humanized mice through epigenetic regulation of targeted nuclear cccDNA minichromosome [12–14, 43]. IFN-α can inhibit cccDNA transcription by reducing the acetylated histone H3 lysine 9 (H3K9) and 27 (H3K27) on the cccDNA minichromosome [13]. Our group reported that IFN-α eliminates the HBV cccDNA by regulating GCN5-mediated histone H3K79 succinylation, thereby performing epigenetic regulation of cccDNA minichromosome [14]. In this study, we further investigated the effect of IFN-α on other epigenetic regulation of cccDNA minichromosome. Strikingly, we demonstrated that IFN-α2b was able to attenuate the levels of 2-hydroxyisobutyrylation and histone H4K8 2-hydroxyisobutyrylation of cccDNA minichromosome in the cells. Moreover, IFN-α2b was able to reduce the levels of H4K8hib on cccDNA minichromosome not only in hepatoma cells but also in PHH cells. It suggests that IFN-α also modulates the histone H4K8 2-hydroxyisobutyrylation beside histone acetylation and succinylation. We first report that IFN-α can promote HDAC3-mediated H4K8 de-2-hydroxyisobutyrylation on cccDNA minichromosome, leading to inhibiting cccDNA transcription and HBV replication. Taken together, we summarize a model that IFN-α restricts cccDNA transcription and HBV replication through HDAC3-mediated epigenetic regulation of H4K8 de-2-hydroxyisobutyrylation on cccDNA minichromosome (Fig. 6f). Clinically, our finding provides new insights into the mechanism by which IFN-α eliminates HBV.

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AUTHOR CONTRIBUTIONS

XDZ and LNZ conceived the project, designed research, analyzed the data, and wrote the paper. LNZ, HFY, YFW, HLY, WZ, YY, YG, MZ, and LYF performed the experiments. All authors have approved the final version of the paper.

ADDITIONAL INFORMATION

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