Hepatitis B virus (HBV) is an enveloped virus belonging to the Hepadnaviridae family (1) and possessing a partially double-stranded circular DNA genome. HBV is transmitted by blood via perinatal and sexual routes and infects more than 300 million people worldwide. HBV infection leads to chronic infection in 90% of perinatal individuals, 20 to 30% of children, and less than 1% of adults (2). Chronic infection often results in development of cirrhosis and hepatocellular carcinoma (HCC). Although HBx has been shown to bind to a number of host proteins, the molecular mechanisms by which HBx regulates HBV replication are largely unknown. In this study, we identified jumonji C-domain-containing 5 (JMJD5) as a novel binding partner of HBx interacting in the cytoplasm. DNA microarray analysis revealed that JMJD5-knockout (JMJD5KO) Huh7 cells exhibited a significant reduction in the expression of transcriptional factors involved in hepatocyte differentiation, such as HNF4A, CEBPA, and FOXA3. We found that hydroxylase activity of JMJD5 participates in the regulation of these transcriptional factors. Moreover, JMJD5KO Huh7 cells exhibited a severe reduction in HBV replication, and complementation of HBx expression failed to rescue replication of a mutant HBV deficient in HBx, suggesting that JMJD5 participates in HBV replication through an interaction with HBx. We also found that replacing Gly135 with Glu in JMJD5 abrogates binding with HBx and replication of HBV. Moreover, the hydroxylase activity of JMJD5 was crucial for HBV replication. Collectively, these results suggest that direct interaction of JMJD5 with HBx facilitates HBV replication through the hydroxylase activity of JMJD5.

**IMPORTANCE**

HBx protein encoded by hepatitis B virus (HBV) plays important roles in pathogenesis and replication of HBV. We identified jumonji C-domain-containing 5 (JMJD5) as a novel binding partner to HBx. JMJD5 was shown to regulate several transcriptional factors to maintain hepatocyte function. Although HBx had been shown to support HBV replication, deficiency of JMJD5 abolished contribution of HBx in HBV replication, suggesting that HBx-mediated HBV replication is largely dependent on JMJD5. We showed that hydroxylase activity of JMJD5 in the C terminus region is crucial for expression of HNF4A and replication of HBV. Furthermore, a mutant JMJD5 with Gly135 replaced by Glu failed to interact with HBx and to rescue the replication of HBV in JMJD5-knockout cells. Taken together, our data suggest that direct interaction of JMJD5 with HBx facilitates HBV replication through the hydroxylase activity of JMJD5.

Hepatocyte Factor JMJD5 Regulates Hepatitis B Virus Replication through Interaction with HBx

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**ABSTRACT**

Hepatitis B virus (HBV) is a causative agent for chronic liver diseases such as hepatitis, cirrhosis, and hepatocellular carcinoma (HCC). HBx protein encoded by the HBV genome plays crucial roles not only in pathogenesis but also in replication of HBV. Although HBx has been shown to bind to a number of host proteins, the molecular mechanisms by which HBx regulates HBV replication are largely unknown. In this study, we identified jumonji C-domain-containing 5 (JMJD5) as a novel binding partner of HBx interacting in the cytoplasm. DNA microarray analysis revealed that JMJD5-knockout (JMJD5KO) Huh7 cells exhibited a significant reduction in the expression of transcriptional factors involved in hepatocyte differentiation, such as HNF4A, CEBPA, and FOXA3. We found that hydroxylase activity of JMJD5 participates in the regulation of these transcriptional factors. Moreover, JMJD5KO Huh7 cells exhibited a severe reduction in HBV replication, and complementation of HBx expression failed to rescue replication of a mutant HBV deficient in HBx, suggesting that JMJD5 participates in HBV replication through an interaction with HBx. We also found that replacing Gly135 with Glu in JMJD5 abrogates binding with HBx and replication of HBV. Moreover, the hydroxylase activity of JMJD5 was crucial for HBV replication. Collectively, these results suggest that direct interaction of JMJD5 with HBx facilitates HBV replication through the hydroxylase activity of JMJD5.
degraded in a ubiquitin-independent proteasome (16), suggesting that some HBx functions may be regulated by posttranslational modifications.

Recent investigations on HCC in HBx transgenic mice generated in several laboratories have suggested that HBx participates in the pathogenesis of HBV (17–20). In addition, HBx has been shown to be involved in HBV replication in vitro and in vivo by using a recombinant HBV plasmid, pHBVΔX, possessing a stop codon in the coding region of HBx (21–24). However, the molecular mechanisms of HBx in HBV replication remain unclear.

A number of host proteins have been identified as binding partners for HBx, including HBx-interacting protein (22), p53 (25), COP9 signalosome (4), apolipoprotein A1 (26), Bc-2/Bcl-x (11), nuclear receptor coactivator 3 (27), protein arginine methyl-transferase 1 (28), peptidylprolyl cis/trans isomerase, NIMA-interacting 1 (29), IPS-1 (30), and S-phase kinase-associated protein 2 (31). However, the biological significance of the interaction of HBx with these host factors in the life cycle of HBV is still unclear.

In addition, HBx has been shown to interact with DDB1 (32), leading to enhancement of the stability of HBx (33) and competing with the interaction between DDB1 and CUL4-associated factor-1 (DCCAF1). Intervention in the interaction between DDB1 and DCAF1 by HBx results in hijacking of the cellular E3 ubiquitin ligase complex of DDB1/CUL4 (34) and participates in HBV replication and HBx-induced cell death (35). These findings suggest that interaction of HBx and DDB1 is required for HBV replication; however, precise mechanisms underlying the interaction of the proteins on HBV replication remain unknown.

In this study, we identified jumonji C-domain (JmjC)-containing 5 (JMJD5) as a novel binding partner for HBx and revealed that JMJD5 participates in HBV replication. Structural analyses have shown that JMJD5 exhibits hydroxylation activity rather than demethylase activity (36, 37). The structure of the jumonji C domain of JMJD5 was conserved with the C-terminal transactivation domains of the factor inhibiting hypoxia-inducible 1 alpha (FIH-1) and JMJD6 (36, 38). The C-terminal transactivation domain of FIH-1 exhibits an asparaginyl hydroxylase activity to catalyze hydroxylation of Asp803 of hypoxia-inducible factor (39). The hydroxylation activity of FIH-1 is dependent on Fe(II), and mutation of His399 to Ala abolished both the interaction with Fe(II) and the hydroxylation activity (39). Although a mutation of His321 to Ala in JMJD5, which corresponds to a mutation of His399 to Ala in FIH (37), abrogates the enzymatic activity of JMJD5 in cultured cells (40), the biological functions and substrates of JMJD5 in hepatocytes remain unknown.

By using the CRISPR/Cas9 system, we established a JMJD5-knockout (JMJD5KO) human hepatoma (High) cell line and revealed that JMJD5 is essential for HBx-mediated HBV replication. Moreover, the deficiency of JMJD5 suppressed the expression of several transcriptional factors involved in hepatocyte differentiation, such as HNF4A, CEBPA, and FOXA3. Expression of a mutant JMJD5 in which His321 was replaced with Ala failed to rescue the expression of HNF4A in JMJD5-knockout Huh7 cells, suggesting that JMJD5 is involved in the maintenance of hepatocyte homeostasis. Further, a random mutagenesis experiment identified Gly135 in JMJD5 as a critical amino acid residue for the interaction with HBx. Replacing Gly135 with Glu in JMJD5 abrogated the interaction with HBx and failed to rescue the replication of HBV in JMJD5-knockout Huh7 cells. We also demonstrated that mutation of His321 to Ala in JMJD5 failed to rescue the replication of HBV. Collectively, these data suggest that direct interaction of JMJD5 with HBx through its Gly135 and hydroxylase activity of JMJD5 facilitates HBV replication.

**MATERIALS AND METHODS**

**Cell lines and viruses.** HEK293T, Huh7, HepG2, HepG2.2.15 (41), and Vero cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 μM penicillin, and 100 μg/ml streptomycin. Hepatitis C virus (HCV) derived from the genotype 2a JFH-1 strain mutated in E2, p7, and NS5, as shown previously (42), was prepared by serial passages in Huh7.5.1 cells. Japanese encephalitis virus (JEV) (Ae_US strain) (43) was prepared in C6/36 cells. Herpes simplex virus 1 (HSV-1) (KOS strain) was prepared in Vero cells. Infectious titers of HCV and JEV were determined by focus-forming assay. Mouse monoclonal antibodies against HCV NS5A (5A27) (44) and JEV NS3 (1791) (45) were used to visualize their foci. The titers of HSV-1 were determined by plaque assay. HBV was prepared from the culture supernatant of HepAD38.7 cells (41). HepAD38.7 cells were cultured in DMEM/F-12 medium supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 18 μg/ml hydrocortisone (Sigma), 5 μg/ml insulin (Sigma), 400 μg/ml G418 (Nacalai Tesque), and 400 ng/ml tetracycline (Nacalai Tesque). To obtain the HBV-containing culture supernatants, HepAD38.7 cells (1 × 10⁶ cells) were seeded on a 10-cm dish (Greiner) coated with collagen type 1 (Nitta Gelatin, Inc.) and cultured with tetracycline-free medium. Culture supernatants were replaced with fresh medium every 3 days postpassage and collected. HBV was concentrated by 15% polyethylene glycol (PEG) solution (molecular weight [MW], 8,000; 0.75 M NaCl and 60 mM EDTA) at 3,000 rpm for 20 min. After PEG precipitation, the pellets were dissolved in primary maintenance medium consisting of Williams E medium supplemented with 5 μg/ml transferrin, 10 ng/ml epidermal growth factor (EGF), 3 μg/ml insulin, 2 mM l-glutamine, 18 μg/ml hydrocortisone, 40 ng/ml dexamethasone, 5 ng/ml sodium selenite, 2% dimethyl sulfoxide (DMSO), 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% FBS (46). For HBV infection, HepG2-lntNPC-C4 cells (2 × 10⁶) (47) were seeded on 24-well plates (Greiner) coated with collagen type 1 and incubated overnight. For HBV infection, 1,000 genome equivalents (GEq)/ml of HBV in the primary maintenance medium containing 4% PEG 8000 (Nacalai Tesque) was inoculated, and the culture medium was replaced every 2 days.

**Plasmids.** HBV expression plasmids, pUC19 HBV Ae_US, pUC19 HBV Bj_JPN35, and pUC19 HBV IPNAT (48), were used for amplification of the regions of HBs. PCR products were cloned into pEFL FLAG pgk puro (49), pGEX6P-1 (GE Healthcare), and pGBK7 (Clontech) vectors. cDNA of JMJD5 was cloned into pEFL HA pgk hygro (where HA is hemagglutinin and hygro is hygromycin) and pAGADT7 (Clontech) vectors. pEFL OSF-HBx was generated by insertion of a One-Step-FLAG (OSF) tag and HBx DNA into pEFL puro vector (where puro is puromycin) vector. cDNAs of DDB1, STUB1, SMC4, PSME3, and TIF1B were amplified by using cDNA derived from Huh7 cells and cloned into pAGADT7 vector. A DNA of HNF4A was amplified by using cDNA derived from Huh7 cells and cloned into pEFL FLAG pgk puro vector. To express HBV pregenomic RNA (pgRNA), full-length HBV DNA (C_IPNAT strain) was amplified by PCR and cloned into pcDNA3.1 (+), with the resulting construct named pcMV pgRNA (where CMV is cytomegalovirus). A lentiviral vector that expresses a short hairpin RNA (shRNA) was generated by using pFTRE3g_RK2_gF (where RK2 is geen fluorescent protein (50). Briefly, pFTRE3g_RK2_gF was cut by PacI/Ascl and cloned into the U6 promoter and pGK_puro cassette, with the resulting product designated Fu6_pGKpuro. The shRNA target sequence of JMJD5 was 5'G CCTGACATGCAGACAGATT-3' and was located in the 5' noncoding region. The shRNA sequence of LacZ was 5'-CTACACAAATTCGGCAT TIC-3'. The reporter plasmids of the nucleoside promoter of HBV were generated by insertion of a DNA fragment of the HBV nucleoside promoter (Ac_U5 strain). DNA fragments of the core upstream regulatory sequence (CURS) and basic core promoter (BCP) were cloned into
Immunoprecipitation and immunoblotting. Cells were lysed with lysis buffer consisting of 20 mM Tris-HCl (pH 7.4), 135 mM NaCl, 1% Triton X-100, 1% glycerol, and protease inhibitor cocktail tablets (Roche), incubated for 30 min at 4°C, and subjected to centrifugation at 14,000 × g for 15 min at 4°C. The supernatants were boiled at 95°C for 5 min and then incubated with anti-FLAG antibody at 4°C for 90 min. After incubation with protein G-Sepharose 4B (GE Healthcare) at 4°C for 90 min, the beads were washed three times by lysis buffer and boiled at 95°C for 5 min. The proteins were resolved by SDS-PAGE (Novex gels; Invitrogen), transferred onto nitrocellulose membranes (iBlot; Life Technologies), blocked with phosphate-buffered saline (PBS) containing 0.05% Tween 20 and 5% skim milk, incubated with primary antibody at room temperature for 12 h, and then incubated with horseradish peroxidase (HRP)-conjugated secondary antibody at room temperature for 1 h. The immune complexes were visualized with Super Signal West Femto substrate (Pierce) and detected by an LAS-3000 image analyzer system (Fuji-film).

Mass spectrometry analysis. 293T cells transfected with pEF OSF-HBs or empty vector were incubated for 48 h and lysed by lysis buffer. The cell lysates were incubated with Strep-Tactin Sepharose (IBM GmbH) for 90 min and washed with lysis buffer three times, and then the precipitants were boiled in sample buffer at 95°C for 5 min. Copurified proteins were subjected to SDS-PAGE, stained with Coomassie brilliant blue, and digested by trypsin. The extracted peptides from the SDS-PAGE gel were subjected to LC-MS/MS using an LTQ-Orbitrap Velos mass spectrometer (Thermo Scientific). Tandem mass spectra were acquired automatically and then searched against a human database from Swiss-Prot with the Mascot software (Matrix Science).

Antibodies. pGEX6p-1 JMJD5 was transfomed into Escherichia coli [E. coli BL21(DE3)/pLySs; Novagen] and glutathione S-transferase (GST) fused with JMJD5 was purified by glutathione-Sepharose 4B (GE Healthcare). Recombinant JMJD5 proteins were immobilized into BALB/c mice (4-week-old females; SLC Japan), and hybridomas were generated by fusion with the myeloma cell line Pau. We successfully obtained a clone, named A6, as an anti-JMJD5 monoclonal antibody. Antibodies to the following proteins were purchased from the indicated manufacturers: anti-FLAG mouse monoclonal antibody (clone M2; Sigma), anti-HA rat monoclonal antibody (clone 3F10; Roche), horseradish peroxidase (HRP)-conjugated anti-FLAG mouse monoclonal antibody (clone M2; Sigma), and anti-β-actin mouse monoclonal antibody (A2282; Sigma).

Yeast two-hybrid assays. The interaction of HBx and host proteins was analyzed by using a Matchmaker 3 two-hybrid system (Clontech) according to the manufacturer's protocol. Saccharomyces cerevisiae (AH109) cells were transformed with pGBK7 HBx and pGADT7 host proteins and plated onto yeast medium (synthetic dextrose [SD]) depleted of Leu and Trp (SD/LW) or depleted of Leu, Trp, Ade, His (SD/LWAH).

Production of lentiviruses. HEK293T cells (2 × 10^6) were seeded on a 10-cm dish and incubated at 37°C for 1 day. A lentiviral transfer vector (Fu6lingtonpuro; 1.5 µg), 2.5 µg of pCMV-dR8.2vdp, and 1 µg of pCMV-VSV-G were mixed with 500 µl of Opti-MEM and 40 µl of polyethylenimine (PEI), 1 mg/ml MW, 25,000 (Polysciences, Inc.) and incubated for 15 min. DNA complex was inoculated into HEK293T cells, and the culture medium was changed at 4 h posttransfection. Culture supernatants at 3 days posttransfection were passed through a 0.45-µm pore-size filter. For the infection of lentivirus, 2 × 10^6 HepG2.2.15-7 cells seeded on a six-well plate were incubated for 1 day, and the virus-containing culture supernatants (2 ml) and hexadimethrine bromide (polybrene; 4 mg/ml; Sigma) were inoculated onto cells and centrifuged at 2,500 rpm for 45 min at 32°C. Puromycin was added at 2 days postinfection to select stable cell lines.

Purification of intracellular and supernatant HBV relaxed circular DNA (rcDNA) and cellular RNA. Extracellular and intracellular HBV DNA was extracted as reported previously (48). Briefly, the cell pellets were lysed by using lysis buffer (50 mM Tris-HCl [pH 7.4], 1 mM EDTA, 1% NP-40) at 4°C for 15 min. After centrifugation at 15,000 rpm for 5 min, supernatants were incubated with 7 mM magnesium acetate (MgOAc), 0.2 mg/ml of DNase I (Roche), and 0.1 mg/ml of RNase A (Sigma) at 37°C for 3 h. After the addition of 10 mM EDTA, the lysates were digested by protease K (0.3 mg/ml; Life Technologies) and 2% SDS at 37°C for 12 h. Extracted HBV DNA was purified by phenol-chloroform-isooamyl alcohol, precipitated with ethanol, and resolved in pure water. Total RNA was extracted by using Isogen I (Nippon Gene) according to the manufacturer’s protocol.

qPCR. Quantitative PCR (qPCR) for HBV DNA was performed by using Fast SYBR green master mix (Applied Biosystems). The following primers were used for detection of HBV DNA: 5'–GGAGGGATACATAGAGGTCTCTTGA-3' and 5'–GGTGGCCTTTGTCTCTAATTG-3' (H11002). Total RNA was used for quantification of mRNA expression by using a Power SYBR green RNA-to-Ct 1-Step kit (Applied Biosystems). The PCR was performed by using a ViaA7 real-time PCR system (Life Technologies). The following primers were used for: HNF4A, 5'–GAGTGCTGTTGTGAGCAAAAC-3' and 5'–CTTCTGAGTCTGATCCCCTG-3' (H11002); for FoxA3, 5'–GTGAAGATGGAGGCCCATGAC-3' and 5'–GAGGATCTCAGCTGATGAGATT-3' (Cebpa, 5'–GAGGATCTCAGCTGATGAGATT-3' and 5'–GTCATTGTTCACGG-3' (CACTG-3'); for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5'–TGTTGTTGAAGCTGTCAGGG-3' and 5'–ACATGCGCATGACGATGACG-3'. The expression level of each gene was determined by the ΔΔCt (where Ct is threshold cycle) method using GAPDH as an internal control.

HbAg measurement. The supernatants from HBV-infected HepG2-hNtcp/C4 cells were collected at 10 days postinfection, and the amounts of HBV core antigen (HbcAg) were determined by Lumipulse assay (Fuirebio, Inc.) according to the manufacturer’s protocol.

Immunofluorescence microscopy. HepG2 cells cultured on glass slides for 1 day were transfected with plasmids for the expression of HBx and JMJD5 and fixed with 4% paraformaldehyde in PBS for 2 h at 1 day posttransfection; they were then washed with PBS three times and permeabilized by incubation with 0.2% Triton X-100 in PBS for 15 min. After cells were washed with PBS three times, they were incubated with anti-HA and anti-FLAG antibodies (1/1,000) diluted by 2% FBS in PBS at room temperature for 1 h. The cells were again washed with PBS three times and then incubated with Alexa Fluor 488 (AF488)-conjugated anti-mouse antibody (1/2,000) and AF488-conjugated anti-rat antibody (1/2,000) diluted by 2% FBS in PBS at room temperature for 1 h. The stained cells were covered with Prolong Gold AntiFade reagent with 4',6-diamidino-2-phenylindole (DAPI; Life Technologies) and observed under a Fluoview FV1000 confocal microscope (Olympus).

Generation of JMJD5-knockout Huh7 cells. The gene knockout Huh7 cell line was constructed by using CRISPR/Cas9 as described previously (51, 52). Huh7 cells were transfected with pX330 and pCAG EgsxFP and incubated for 1 week. GFP-positive Huh7 cells were sorted by...
JMJD5 Interacts with HBx

fluorescence-activated cell sorting (FACS) and formed single colonies. Gene deficiency was confirmed by sequencing and Western blotting.

**Reporter assay.** Huh7 cells were transfected with either CURS BCP-Luc or BCP-Luc together with pRL-SV40 (where SV40 is simian virus 40), and luciferase activity in the cell lysate was assayed by using a Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer’s protocol at 24 h posttransfection.

**Southern blotting.** HBV core-incorporated DNA was electrophoresed in 1% agarose with Tris-acetate EDTA (TAE) buffer and transferred onto a nylon membrane (Hybond-N; GE Healthcare). The pUC19 HBV (C_JPNAT) was digested by AatII and AclI, and the DNA fragment was extracted and purified by using a QiAquick gel extraction kit (Qiagen). Labeling probes, hybridization, and detection were performed by using a Gene Images AlkPhos Direct Labeling and Detection system (Amersham), according to the manufacturer’s protocol.

**Northern blotting.** Total RNA (5 μg) was subjected to electrophoresis in 1.2% agarose-formaldehyde gel and morpholinopropanesulfonic acid (MOPS) buffer. RNA was visualized by ethidium bromide staining, and electrophoresed RNA was transferred onto a positively charged nylon membrane (Roche). A pBluescriptII (SK) – HBV-chloromphenicol acetyltransferase (CAT) construct was linearized by HindIII, an RNA probe was synthesized by a digoxigenin (DIG) RNA labeling kit (Roche), and hybridization and detection were performed by using a DIG Northern Starter kit (Roche) according to the manufacturer’s protocols. The signals were detected by an LAS-3000 image analyzer system (Fujifilm).

**Microarray analyses of Huh7 and JMJD5KO cells.** Total RNA was extracted from Huh7 cells and from JMJD5KO Huh7 cells and purified with an miRNeasy kit (Qiagen) according to the manufacturer’s instructions. Microarray analysis was performed as described previously (54). Briefly, 200 ng of total RNA was reverse transcribed, and the resulting cDNAs were used for in vitro transcripion. Each cRNA sample was hybridized using a SurePrint G3 human gene expression (8 by 60,000) microarray kit, version 2.0 (model 039494; Agilent Technologies). Raw data were imported into a Subio platform, version 1.18 (Subio), for database management and quality control. Gene expression profiling was compared between Huh7 and JMJD5KO cells.

**Random mutagenesis assay.** Random mutagenesis against JMJD5 was introduced by using a GeneMorph II random mutagenesis kit (Agilent Technologies) according to the manufacturer’s protocol. The primers used were 5' - ATCGATACGGGATCCATATGGCTGGAGACACCCACT G-3' and 5' - TCATCTGCAGCTCGAGCTACGACCACCAGAAGCTGA TGCTGA-3'. PCR products were electrophoresed, purified by using a QiAquick gel extraction kit (Qiagen), and cloned into pGADT7 vector by using an In-Fusion HD cloning kit (TAKaRa). After transformation, the bacterial colonies were picked up, and their plasmids were extracted and used to transform yeasts into yeasts expressing HBx, which were then plated on SD/L-W or SD/-LWAH medium.

**JMJD5 structural modeling.** The JMJD5 secondary structure and the disorder regions were predicted from the amino acid sequence using the programs PSIPRED (35) and DISOPRED (56), respectively. The putative structure of the N-terminal region (residues 1 to 150) of JMJD5 was modeled using I-TASSER (57). The electrostatic potential was calculated by the APBS tool, and figures were rendered by PyMol (The PyMol Molecular Graphics System, version 1.7.6, Schrödinger, LLC).

**Microarray data accession number.** The microarray data have been deposited in the NCBI Gene Expression Omnibus (GEO) database under accession number GSE73491.

**RESULTS**

**Identification of JMJD5 as a binding partner for HBx.** To identify a novel HBx-interacting protein, we performed coimmunoprecipitation and MS analyses. 293T cells transfected with an empty vector or a One-Strep-FLAG (OSF) tag HBx expression vector and HBx and HBx-binding proteins were purified using Strep-Tactin beads and subjected to SDS-PAGE, and the resolving gel was visualized by Coomassie brilliant blue solution (Fig. 1A, left). Proteins that had entered the running gel were identified by using LC-MS/MS (Fig. 1A, right). We identified the DDB1/CUL4 complex, which was previously reported as HBx-binding proteins (33), CSN5, a component of the COP9 signalosome, was also reported to be an interacting protein with HBx by using yeast two-hybrid screening (4). In addition, all of the COP9 signalosome components, CSN1 to CSN7, were communoprecipitated with HBx in this assay. HBx has been reported to be involved in the ubiquitin proteasome system (58) and in the regulation of gene expression (39). Therefore, we selected STUB1, TIF4, and PSME3, which are known to be involved in the induction of proteasome degradation, and nuclear proteins such as SMC4 and JMJD5. We next determined whether these candidate proteins directly bind to HBx in yeasts. Yeast cells were transformed by using a GAL4-activated domain fused with DDB1, STUB1, SMC4, PSME3, TIF1B, or JMJD5 and a GAL4-binding domain fused with HBx. The transformed yeast cells were plated on yeast medium depleted of Leu and Trp (SD/–LW) or depleted of Leu, Trp, Ade, and His (SD/–LWAH). DDB1 was originally identified as an HBx-binding protein by using yeast two-hybrid screening (60), and in this study we used DDB1 as a positive control. Interestingly, only JMJD5 and DDB1, but not the other candidates, exhibited binding to HBx in yeasts (Fig. 1B). Therefore, we focused on JMJD5 for further experiments. Because HBV has eight different genotypes (A to H), we next examined whether JMJD5 is capable of binding to HBx derived from different genotypes. Although FLAG-HBx of genotypes A, B, and C were coprecipitated with HA-JMJD5 in 293T cells expressing these proteins, the interaction between JMJD5 and HBx of genotype B was weaker than that of other genotypes (Fig. 1C). To further determine the cellular localization of HBx and JMJD5, FLAG-HBx and/or HA-JMJD5 was expressed in Huh7 cells. Although HBx and JMJD5 were localized in the cytoplasm and nucleus, respectively, some fractions of JMJD5 were translocated to the cytoplasm in cells coexpressing HBx (Fig. 1D), suggesting that HBx interacts with JMJD5 in the cytoplasm. Collectively, these results suggest that JMJD5 is a novel binding partner of HBx.

**JMJD5 participates in HBV replication.** To examine the roles of JMJD5 in HBV replication, shRNA against JMJD5 was lentivirallytransduced in HepG2.2.15-7 cells, which produce HBV particles (41, 61), resulting in JMJD5-knockdown (JMJD5KD) HepG2.2.15-7 cells. We also prepared a monoclonal antibody against JMJD5 (AI6) after immunization of mice with recombinant JMJD5 proteins. Endogenous expression of JMJD5 detected by AI6 antibody was reduced in JMJD5KD HepG2.2.15-7 cells (Fig. 2A). No significant difference in cell growth was observed between control and JMJD5KD HepG2.2.15-7 cells by MTT (3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl-2H-tetrazolium bromide) assay (Fig. 2B). To examine the roles of JMJD5 on HBV replication, JMJD5KD HepG2.2.15-7 cells were incubated for 6 days. The levels of both intracellular and extracellular HBV DNAs in JMJD5KD HepG2.2.15-7 cells were lower than those of cells transfected with control shRNA (Fig. 2C). Southern blot analysis revealed a reduction of HBV DNA in JMJD5KD HepG2.2.15-7 cells (Fig. 2D). Northern blot analysis showed no significant difference in transcription levels of HBV RNA between control and JMJD5KD HepG2.2.15-7 cells (Fig. 2E). In addition, the reductions of HBV DNA by reverse transcriptase inhibitor treatment were comparable between control and JMJD5KD HepG2.2.15-7
cells (Fig. 2F), suggesting that susceptibility to treatment with the reverse transcriptase inhibitor is not affected by suppression of JMJD5 expression. To further confirm the roles of JMJD5 in HBV replication, we established JMJD5KD HepG2.2.15-7 cells expressing HA-tagged RNA interference (RNAi)-resistant JMJD5. The amounts of intracellular HBV DNA were significantly enhanced by the expression of RNAi-resistant JMJD5 in JMJD5KD HepG2.2.15-7 cells (Fig. 2G). In addition, the reduction of HBcAg expression in JMJD5KD HepG2.2.15-7 cells was rescued by the exogenous expression of RNAi-resistant JMJD5 (Fig. 2H). Recently, HepG2 cells expressing sodium taurocholate cotransporting polypeptide (NTCP) were shown to be susceptible to HBV infection (46). Next, therefore, we established the JMJD5KD cell line upon transfection with shRNA targeting LacZ (shLacZ) or shJMJD5 at 2 days postinfection, HBV DNA in JMJD5KD HepG2-hNTCP-C4 cells was significantly decreased at 10 days postinfection (Fig. 2J). The amounts of HBcAg in the culture supernatants were also decreased in the JMJD5KD HepG2-hNTCP-C4 cells (Fig. 2K). Finally, Northern blot analysis revealed that HBV RNA was significantly reduced in JMJD5KD HepG2-hNTCP-C4 cells (Fig. 2L). Together, these results suggest that JMJD5 participates in HBV replication.

Establishment of JMJD5-knockout (KO) Huh7 cells by the CRISPR/Cas9 system. Next, to examine the roles of JMJD5 on viral replication in a human hepatoma cell line, Huh7, we established two JMJD5-deficient Huh7 cell lines (JMJD5KO Huh7 lines 45 and 47) and JMJD5-deficient Huh7 cell lines complemented with HA-JMJD5 (Fig. 3B). The growth of JMJD5KO Huh7 cells was similar to that of parental Huh7 cells (Fig. 3C). Although complementation with HA-JMJD5 in JMJD5-deficient Huh7 cells exhibited a slight enhancement of HSV-1 propagation, there was no effect on the propagation of JEV and HCV (Fig. 3D), suggesting that deficiency of JMJD5 in Huh7 cells has only a moderate or no effect on the propagation of HSV-1, JEV and HCV.

Deficiency of JMJD5 downregulates several host factors involved in liver homeostasis. Although JMJD5 has been shown to be a tumor suppressor against lymphoma (62), JMJD5 has also been suggested to function as an oncogene by regulating hypoxia-inducible factor 1α (HIF-1α) through its interaction with pyruvate kinase (PKM2) (63). In human hepatocellular carcinoma (HCC) cells, JMJD5 has been shown to be upregulated in tumor tissue compared to adjacent noncancerous tissue (64). Furthermore, JMJD5 has been shown to be upregulated in HCC cells compared to normal liver cells (65). Therefore, the role of JMJD5 in liver homeostasis is likely to be different depending on the cell type and the context of disease.

In conclusion, our results suggest that JMJD5 participates in both HBV and HIV replication in human hepatoma cell lines. The role of JMJD5 in liver homeostasis is likely to be different depending on the cell type and the context of disease. Further studies will be required to elucidate the mechanisms by which JMJD5 regulates the replication of these viruses and its role in liver homeostasis.
vate kinase muscle isozyme (PKM2) (40) or cyclin A1 (63) and to be involved in colon carcinogenesis (64). Therefore, the function of JMJD5, especially in hepatocytes, remains unclear. To examine the roles of JMJD5 in hepatocytes, we performed DNA microarray analysis by using parental and JMJD5KO Huh7 cells and showed that numbers of transcription factors involved in the hepatocyte development and differentiation listed by Qiagen’s Ingenuity Pathway Analysis (IPA) were downregulated in JMJD5KO Huh7
Among them, HNF4A, FOXA1, FOXA2, and FOXA3 have been shown to convert primary mouse embryonic fibroblasts into hepatocyte-like cells (65). Therefore, we next examined the roles of JMJD5 on the expression of HNF4A, CEBPA, and FOXA3 by qPCR. Deficiency of JMJD5 significantly reduced the expression of HNF4A, and exogenous expression of JMJD5 in JMJD5KO Huh7 cells recovered the expression (Fig. 4A). Similar to HNF4A expression, the expression levels of CEBPA and FOXA3 were downregulated in JMJD5KO Huh7 cells and then rescued by exogenous expression of JMJD5 (Fig. 4B and C). The jumonji C (JmjC) domain of JMJD5 has been predicted to possess activity of hydroxylase rather than demethylase (37, 38). To determine the enzymatic activity of JMJD5 on HNF4A expression, an enzymatically deficient mutant, JMJD5 H321A, that possessed a substitution of Ala for His321 in the JmjC domain, was expressed in JMJD5KO Huh7 cells and then rescued by exogenous expression of JMJD5 (Fig. 4B and C). The luciferase assay revealed that there was no significant difference in the promoter activities of the HBV nucleocapsid between JMJD5KO Huh7 cells and Huh7 cells with restored JMJD5 expression (Fig. 4E, bottom). Previous studies showed that the activity of the HBV nucleocapsid promoter was enhanced by the overexpression of HNF4A. Therefore, we examined the effect of overexpression of HNF4A on the nucleocapsid promoter. In spite of an 80-fold increase in HNF4A expression by transfection (Fig. 4F), only marginal and 2-fold increases in luciferase activity were observed in the BCP and CURS promoters, respectively (Fig. 4G). These results suggest that a 50% reduction of HNF4A expression by the knockdown of JMJD5 has no effect on the promoter activity of the HBV nucleocapsid, but JMJD5 might play some roles in the maintenance of hepatocytes. Further studies are needed to clarify the biological significance of JMJD5 in liver homeostasis.

**JMJD5 participates in HBx-mediated HBV replication.** Next, to determine the roles of JMJD5 in HBV replication, Huh7 cells, (see Table S1 in the supplemental material). Among them, HNF4A, FOXA1, FOXA2, and FOXA3 have been shown to convert primary mouse embryonic fibroblasts into hepatocyte-like cells (65). Therefore, we next examined the roles of JMJD5 on the expression of HNF4A, CEBPA, and FOXA3 by qPCR. Deficiency of JMJD5 significantly reduced the expression of HNF4A, and exogenous expression of JMJD5 in JMJD5KO Huh7 cells recovered the expression (Fig. 4A). Similar to HNF4A expression, the expression levels of CEBPA and FOXA3 were downregulated in JMJD5KO Huh7 cells and then rescued by exogenous expression of JMJD5 (Fig. 4B and C). The jumonji C (JmjC) domain of JMJD5 has been predicted to possess activity of hydroxylase rather than demethylase (37, 38). To determine the enzymatic activity of JMJD5 on HNF4A expression, an enzymatically deficient mutant, JMJD5 H321A, that possessed a substitution of Ala for His321 in the JmjC domain, was expressed in JMJD5KO Huh7 cells and then rescued by exogenous expression of JMJD5 (Fig. 4B and C). The luciferase assay revealed that there was no significant difference in the promoter activities of the HBV nucleocapsid between JMJD5KO Huh7 cells and Huh7 cells with restored JMJD5 expression (Fig. 4E, bottom). Previous studies showed that the activity of the HBV nucleocapsid promoter was enhanced by the overexpression of HNF4A. Therefore, we examined the effect of overexpression of HNF4A on the nucleocapsid promoter. In spite of an 80-fold increase in HNF4A expression by transfection (Fig. 4F), only marginal and 2-fold increases in luciferase activity were observed in the BCP and CURS promoters, respectively (Fig. 4G). These results suggest that a 50% reduction of HNF4A expression by the knockdown of JMJD5 has no effect on the promoter activity of the HBV nucleocapsid, but JMJD5 might play some roles in the maintenance of hepatocytes. Further studies are needed to clarify the biological significance of JMJD5 in liver homeostasis.

**JMJD5 participates in HBx-mediated HBV replication.** Next, to determine the roles of JMJD5 in HBV replication, Huh7 cells,
JMJD5KO Huh7 cells, and Huh7 cells with restored JMJD5 expression were transfected with an HBV expression plasmid (pHBV C_JPNAT), and the levels of intracellular HBV DNA were determined by qPCR and expressed as the relative expression after normalization with expression of GAPDH. Data represent the means ± standard deviations of two independent analyses. Significant differences are indicated by asterisks (*, P < 0.05). (D) Expression levels of HNF4A in Huh7 cells, JMJD5KO Huh7 cells (line 45), and JMJD5KO Huh7 cells complemented with either the wild-type or the H321A mutant of HA-JMJD5 were determined by qPCR and expressed as the relative expression after normalization with expression of GAPDH. Data represent the means ± standard deviations of two independent analyses. Significant differences are indicated by asterisks (*, P < 0.05). (E) Reporter plasmids carrying a firefly luciferase gene under either the basic core promoter (BCP-Luc) or the core upstream regulatory sequence and BCP (CURS BCP-Luc) of the HBV nucleocapsid were transfected with pRL-SV40 into JMJD5KO Huh7 cells (line 45) and JMJD5KO Huh7 cells complemented with HA-JMJD5. Relative luciferase activities were determined by using a dual-luciferase kit (Promega) at 24 h posttransfection. Data represent the means ± standard deviations of two independent analyses. (F) Levels of mRNA of HNF4A were determined by qPCR upon overexpression of HNF4A. Data are shown as relative expression levels normalized by the expression of GAPDH. (G) The reporter plasmids (BCP-Luc and CURS BCP-Luc) were transfected with pRL-SV40 into Huh7 cells and those overexpressing HNF4A. Relative luciferase activities were determined by using a dual-luciferase kit (Promega) at 24 h posttransfection. Data represent the means ± standard deviations of two independent analyses. Significant differences are indicated by asterisks (*, P < 0.05).
Huh7 cells were transfected with pHBV plasmids (genotype C, C_JPNAT strain), and intracellular core-incorporated HBV DNA was examined by Southern blotting. (E) HA-JMJD5 were transfected with pCMV HBV pgRNA plasmids, and intracellular core-incorporated HBV DNA was quantified by qPCR. Data represent the means ± standard deviations of two independent analyses. (D) JMJD5KO Huh7 cells (line 45) and JMJD5KO Huh7 cells complemented with HA-JMJD5 were transfected with pCMV HBV pgRNA plasmids, and intracellular core-incorporated HBV DNA was examined by Southern blotting. (B) Huh7 cells, JMJD5KO Huh7 cells (lines 45 and 47), and JMJD5KO Huh7 cells complemented with HBx were transfected with either the wild-type JMJD5 or G135E JMJD5 together with HBV expression plasmids. Although the expression of G135E JMJD5 restored the expression of HNF4A to the same level as in the wild-type JMJD5 (Fig. 6D), HBV replication was not rescued by the expression of G135E JMJD5 in JMJD5KO Huh7 cells (Fig. 6E), suggesting that the interaction between JMJD5 and HBx is crucial for HBV replication. In addition, to examine the role of the hydroxylase activity of JMJD5 in HBV replication, we complemented JMJD5KO Huh7 cells with either the wild-type JMJD5 or a hydroxylase-deficient mutant of JMJD5 H321A. H321A JMJD5 exhibited binding to HBx (Fig. 6F) but did not rescue the replication of HBV in JMJD5KO Huh7 cells (Fig. 6G), suggesting that hydroxylase activity is also essential for HBV replication. Taken together, our data suggest that the interaction of JMJD5 with HBx and the enzymatic activity of the JmjC domain in JMJD5 play crucial roles in HBV replication.

**DISCUSSION**

HBx is a multifunctional protein, and HBx transgenic mice generated by several groups develop HCC at around 1 year of age (17). HBx had been also shown to integrate into several host genes such as LINE1 and TERT, and these chimeric transcriptional products stimulate liver cancer incidents (20, 70), suggesting that HBx directly or indirectly participates in liver carcinogenesis. Numerous molecular mechanisms of HBx-induced HCC have already been reported. For example, HBx has been shown to directly interact with the tumor suppressor gene p53 and to inactivate p53-mediated transcription (25). Although HBx has been shown to activate RAS (7), the incidence of HBx-induced HCC in mice was accelerated by the knockdown of p53 (71), suggesting that inhibition of p53 by HBx is not sufficient to induce liver cancer in mice. In addition, cooperation with NRAS was not needed for HBx-induced liver cancer in mice (71). Further studies are needed to clarify the molecular mechanisms by which HBx promotes the development of HCC and the infection with HBV. The production of HBV particles in culture supernatants upon transfection with pHBVΔX was significantly lower than that with the wild-type HBV plasmid (21–23). Moreover, HBVΔX failed to induce viremia in chimeric mice transplanted with human hepatocytes (24), suggesting that HBx is required for HBV propagation.

In this study, we identified JMJD5 as a novel HBx-binding protein regulating HBV replication. Although HBx has been shown to localize in both the cytoplasm and nucleus (72), HBx was mainly detected in the cytoplasm in this study, while JMJD5 was clearly localized in the nucleus. Coexpression of JMJD5 with HBx resulted in the translocation of some fraction of JMJD5 from the cytoplasm into the nucleus (Fig. 6A), suggesting that JMJD5 interacts with HBx and regulates HBV replication in both the cytoplasm and nucleus. These results suggest that JMJD5 participates in HBx-mediated HBV replication.
the nucleus to cytoplasm. The expression level of HBx is low in the liver of hepatitis B patients, and studies based on overexpression of HBx have not employed physiological conditions (3). Indeed, our data suggested that HBx specifically interacts with JMJD5 although further studies will be needed to investigate the interaction between JMJD5 and HBx under physiological conditions.

Knockdown of JMJD5 in HepG2.2.15-7 cells resulted in significant reductions of intracellular and extracellular HBV DNA, and expression of an RNAi-resistant JMJD5 in the knockdown cells restored the replication of viral DNA and expression of core protein, suggesting that the expression of JMJD5 protein participates in the replication of HBV. To further confirm the role of the interaction between JMJD5 and HBx in the replication of HBV, we generated JMJD5-knockout Huh7 cells and found that replication of HBV was severely impaired in the knockout cells. Although amounts of pgRNA were no different in control and JMJD5KD HepG2.2.15-7 cells (Fig. 2E), intracellular HBV DNA levels were lower in JMJD5KO Huh7 cells even with the transcription of pgRNA by the CMV promoter (Fig. 5C and D), suggesting that interaction of JMJD5 and HBx participates in posttranscriptional steps such as stability and multimerization of HBV capsids. In addition, rescue of the replication of HBV/ΔX in the knockout cells was required for exogenous expression of both HBx and JMJD5. Although our loss-of-function and gain-of-function data suggest that JMJD5 participates in the HBx-mediated HBV replication, further studies are needed to clarify the role of JMJD5 in the replication of HBV in more detail.

JMJD5 was originally identified as a tumor suppressor gene by gene trap screening (62); however, JMJD5 has also been reported as an oncogene by other groups (40, 63). DNA microarray analysis of JMJD5-knockout Huh7 cells revealed that deficiency of JMJD5 suppresses the expression of liver factors, as reported previously (65, 73, 74). Exogenous expression of JMJD5, particularly the JmjC domain of JMJD5, in the JMJD5-knockdown cells restored the expression of the liver factors, including HNF4A, CEBPA, and FOXA3. To confirm the role of JMJD5 on hepatocyte differentiation through the regulation of liver factors, investigation of the liver-specific JMJD5-knockout mice is needed.

Random mutagenesis and functional screening using yeasts identified Gly135 of JMJD5 as a critical amino acid residue for interaction with HBx, and replacing Gly135 with Glu abrogated the rescue of HBV replication by the expression of JMJD5 in the JMJD5-knockout cells. Furthermore, the expression of an enzymatically deficient JMJD5 mutant in which His321 was replaced...
with Ala in the JmjC domain was also incapable of restoring HBV replication in the JMJD5-knockout cells. These results suggest that both the association of JMJD5 with HBx and the hydroxylase activity in JMJD5 are required for efficient replication of HBV. JMJD6, which has a JmjC domain structurally similar to that of JMJD5 (36, 38), possesses l-lysyl-hydroxylase activity and catalyzes the U2 small nuclear ribonucleoprotein auxiliary factor 65-kDa subunit (U2AF65) to regulate RNA splicing (75) and hydroxylation of lysine residues in histone (76, 77). Further studies are needed to identify the molecular targets of JMJD5 in hepatocytes.

Structural modeling and disorder predictions in silico suggested the presence of an alpha helix domain in the N terminus of JMJD5 (Fig. 7A) and the possibility of direct interaction with partner proteins through the exposed Gly315 on the surface of JMJD5. In addition, the molecular surface around Gly315 in JMJD5 was deduced to be highly hydrophobic (Fig. 7B), and substitution of Glu for Gly315 drastically altered the electrostatic potential from positive to negative (Fig. 7C), consistent with a scenario in which substitution of Glu for Gly315 in JMJD5 leads to abrogation of the interaction with HBx.

In conclusion, we have identified JMJD5 as a novel HBx-binding partner and suggested that both the hydroxylase activity of JMJD5 and the direct interaction of JMJD5 with HBx through Gly315 facilitate HBV replication. Currently, reverse transcriptase inhibitors are available only for the treatment of hepatitis B patients. Intervention with the interaction between JMJD5 and HBx might be a novel target for the development of anti-HBV agents.

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and independent prognostic effect of HBx protein on the outcome of HCC patients.

The expression of HBx protein in HCC is associated with increased cell proliferation and survival, and it is important for the growth and progression of HCC. The protein interacts with various cellular proteins, including p53, p21, and other signaling pathways, which contributes to the development of HCC.

The role of HBx protein in HCC suggests that it is a potential target for therapeutic intervention. The development of specific inhibitors or therapeutic agents targeting the HBx protein could be a promising therapeutic strategy for the treatment of HCC.

In conclusion, HBx protein plays a crucial role in the pathogenesis of HCC, and its expression is associated with a poor prognosis. Further studies are needed to better understand the molecular mechanisms and functional significance of HBx protein in HCC, which may lead to the development of effective therapeutic strategies.

References:


