A Novel Mouse Model Harboring Hepatitis B Virus Covalently Closed Circular DNA

Zaichao Xu,1,* Li Zhao,1,* Youquan Zhong,1,* Chengliang Zhu,2,* Kaitao Zhao,1 Yan Teng,1 Xiaoming Cheng,1,3,6 Qiang Chen,4,5 and Yuchen Xia1

1State Key Laboratory of Virology and Hubei Province Key Laboratory of Allergy and Immunology, Institute of Medical Virology, School of Basic Medical Sciences, Wuhan University, Wuhan, China; 2Department of Clinical Laboratory, Renmin Hospital of Wuhan University, Wuhan, China; 3Wuhan University Center for Pathology and Molecular Diagnostics, Zhongnan Hospital of Wuhan University, Wuhan, China; 4Department of Radiation and Medical Oncology, Medical Research Institute, Zhongnan Hospital of Wuhan University, Wuhan University, Wuhan, China; 5Frontier Science Center for Immunology and Metabolism, Medical Research Institute, Wuhan University, Wuhan, China; and 6Hubei Clinical Center and Key Laboratory of Intestinal and Colorectal Diseases, Wuhan, China

SUMMARY

By using an adeno-associated virus vector carrying a replication-deficient hepatitis B virus (HBV) genome, we established a mouse model harboring covalently closed circular DNA. This model provides a unique platform for studying HBV covalently closed circular DNA and developing novel antivirals to achieve HBV cure.

BACKGROUND AND AIMS: The persistence of viral covalently closed circular DNA (cccDNA) is the major obstacle for antiviral treatment against hepatitis B virus (HBV). Basic and translational studies are largely hampered due to the lack of feasible small animal models to support HBV cccDNA formation. The aim of this study is to establish a novel mouse model harboring cccDNA.

METHODS: An adeno-associated virus (AAV) vector carrying a replication-deficient HBV1.04-fold genome (AAV-HBV1.04) was constructed. The linear HBV genome starts from nucleotide 403 and ends at 538, which results in the splitting of HBV surface and polymerase genes. Different HBV replication markers were evaluated for AAV-HBV1.04 plasmid-transfected cells, the AAV-HBV1.04 viral vector–transduced cells, and mice injected with the AAV-HBV1.04 viral vector.

RESULTS: Compared with the previously reported AAV-HBV1.2 construct, direct transfection of AAV-HBV1.04 plasmid failed to produce hepatitis B surface antigen and progeny virus. Interestingly, AAV-HBV1.04 viral vector transduction could result in the formation of cccDNA and the production of all HBV replication markers in vitro and in vivo. The formation of cccDNA could be blocked by ATR (ataxia-telangiectasia and Rad3-related protein) inhibitors but not HBV reverse transcription inhibitor or capsid inhibitors. The AAV-HBV1.04 mouse supported long-term HBV replication and responded to antiviral treatments.

CONCLUSIONS: This AAV-HBV1.04 mouse model can support HBV cccDNA formation through ATR-mediated DNA damage response. The de novo formed cccDNA but not the parental AAV vector can lead to the production of hepatitis B surface antigen and HBV progeny. This model will provide a unique platform for studying HBV cccDNA and developing novel antivirals against HBV infection. (Cell Mol Gastroenterol Hepatol 2022;13:1001–1017; https://doi.org/10.1016/j.jcmgh.2021.11.011)

Keywords: HBV; cccDNA; Adeno-Associated Virus; Immune-Competent Mouse; Antiviral.
Despite the availability of effective vaccine, hepatitis B virus (HBV) causes a major public health burden worldwide, with about 257 million people being chronically infected. Chronic hepatitis B patients are at high risk of developing liver cirrhosis and hepatocellular carcinoma. At present, 7 drugs have been approved for chronic hepatitis B treatment, including 2 forms of interferons (standard and pegylated interferon) and 5 nucleos(t)ide analogs (lamivudine, adefovir, telbivudine, entecavir [ETV], and tenofovir). All these treatments are effective to some extent but rarely lead to a cure for HBV infection.

HBV infection is initiated via virus interaction with heparan sulfate proteoglycans, which is followed by the co-transporting polypeptide (NTCP). After internalization, viral capsids are directed to the nucleus, where the HBV genomes are released. In the nucleus, relaxed circular DNA (rcDNA) is converted into covalently closed circular DNA (cccDNA), which can persist in the nucleus as a minichromosome and serve as a template for viral RNA transcription. Viral messenger RNAs are transported to the cytoplasm, where they are translated into viral proteins. Together with the viral polymerase, the pregenomic RNA is encapsidated within the newly formed capsid and reverse transcribed into progeny rcDNA. Mature nucleocapsids are then either directed to the multivesicular bodies pathway for envelopment with HBV envelope proteins or redirected to the nucleus to amplify the cccDNA pool. As the viral persistence reservoir plays a central role in HBV infection, HBV cccDNA is the key obstacle for a cure.

The lack of animal models has significantly hampered HBV basic research and drug development. Although the mouse is the best characterized and most convenient laboratory animal, it cannot be infected with HBV. Currently, several mouse models support HBV replication and chimeric mouse models harboring human hepatocytes with susceptibility to HBV infection are the only option for studying HBV using mice. Transgenic mice with 1.3-fold HBV genome were generated. Viral vector–mediated transduction of HBV DNA in mice was established with adenovirus or adeno-associated virus (AAV). In addition, hydrodynamic injection of HBV plasmid via the tail vein allows the replication of HBV in the livers of immunocompetent mice. In order to mimic the full life cycle of HBV infection, immune-deficient mice transplanted with primary human hepatocytes were used. Moreover, liver and hematopoiesis double-humanized mice models were generated to investigate the crosstalk between HBV infection and immune response. Although significant progresses have been made in establishing HBV mouse models, current models are still limited by inauthentic viral life cycle, difficulties in generation, high cost, and suboptimal immune response upon HBV infection.

As HBV cccDNA is not naturally formed in mouse hepatocytes, several cccDNA surrogate mouse models have been established. By using Cre/loxP-mediated DNA recombination or minicircle technology, mouse models with recombinant cccDNA-like molecules (rcccDNA) were generated. Although the rcccDNA supports persistent viral replication and antigen expression, the nature of rcccDNA may differ from authentic cccDNA, as additional sequences, like LoxP or attR, were inserted into the HBV genome. Interestingly, it has been reported recently that HBV cccDNA can be detected in the liver of mice transduced with AAV-HBV. However, how the cccDNA is formed and whether the cccDNA is functional in this model remain elusive. In this study, we took advantage of an AAV vector carrying a replication-deficient HBV1.04-fold genome (AAV-HBV1.04). Owing to the discontinuity of the S gene on the linear HBV genome, the AAV-HBV1.04 itself cannot express hepatitis B surface antigen (HBsAg) unless cccDNA is formed. After transduction of AAV-HBV1.04 into mouse, both HBsAg and cccDNA can be detected in the serum or liver, respectively. This novel mouse model will provide a unique platform for studying HBV cccDNA and developing novel antivirals to achieve HBV cure.

**Results**

**Generation of a Replication-Deficient pAAV-HBV1.04 Vector**

As in the previous AAV-HBV mouse model, all HBV replication markers could derive from both AAV-HBV vector or cccDNA, whether the newly formed cccDNA is functional remains uncertain. To address this question, we generated an HBV replication-deficient pAAV-HBV1.04 vector. As shown in Figure 1A, the circular HBV genome was split from the S gene region to form a linear HBV genome. While the previously described pAAV-HBV1.2 construct contains all 4 major overlapping coding regions and expresses all HBV viral proteins, pAAV-HBV1.04 does not encode intact HBV polymerase and surface proteins. To validate its replication-deficient phenotype, we transfected Huh7 cells with pAAV-HBV1.2 or pAAV-HBV1.04 construct (Figure 1B). As expected, pAAV-HBV1.2 plasmid transfection resulted in the secretion of both hepatitis B e antigen (HBeAg) and HBsAg, whereas pAAV-HBV1.04 failed to express HBsAg (Figure 1C). In addition, HBV-DNA in the supernatant, which represents newly produced viral progeny, was not detected.
in pAAV-HBV1.04 plasmid–transfected cells (Figure 1D). These results confirmed that pAAV-HBV1.04 construct itself could not establish HBV replication.

**AAV-HBV1.04 Transduction Results in HBV cccDNA Formation In Vitro**

We next investigated whether AAV-HBV1.04 viral vector transduction was able to form cccDNA. AAV-HBV1.2 and AAV-HBV1.04 vectors were produced, titrated, and transduced into HepG2 cells or AML12 cells (Figure 2A). As shown in Figure 2B, both AAV-HBV1.2 and AAV-HBV1.04 transduction led to a significant increase of HBeAg over time in HepG2 cells. Interestingly, unlike direct plasmid transfection, HBsAg could be detected and increased gradually from AAV-HBV1.04–transduced cells (Figure 2B). Similar results could be observed on AML12 cells (Figure 2C).

To further validate the production of HBsAg, immunostaining was performed. As expected, AAV-HBV1.2–transduced HepG2 cells showed positive for both HBsAg and HBV core protein (Hbc) (Figure 2D). In accordance with the enzyme-linked immunosorbent assay (ELISA) result, HBsAg could be detected in AAV-HBV1.04–transduced HepG2 cells by immunostaining (Figure 2D). The expression of HBsAg and Hbc were further validated by Western blot (Figure 2E). Similar results were observed on AAV-HBV1.04–transduced AML12 cells (Figure 2F and G). As pAAV-HBV1.04 construct does not have an intact S gene, this result suggested that another HBV...
Figure 2. Detection of HBV markers in AAV-HBV1.04 viral vector–transduced cells. (A) Schematic representation of the experimental setting. HepG2 and AML12 cells were transduced with AAV-HBV at $5 \times 10^4$ vg per cell in 24-well plates. Secreted viral antigens in the supernatants of (B) HepG2 cells and (C) AML12 cells at different time points were determined by ELISA. (D) The levels of HBsAg and HBe protein in AAV-HBV–transduced HepG2 cells were evaluated by immunofluorescence staining. Scale bar = 75 μm. (E) The expression of HBe and HBs of AAV-HBV–transduced HepG2 cells was detected by Western blot. (F) The levels of HBsAg and HBe protein in AAV-HBV–transduced AML12 cells were evaluated by immunofluorescence staining. Scale bar = 75 μm. (G) The expression of HBe and HBs of AAV-HBV–transduced AML12 cells was detected by Western blot. (H) Cellular DNA was Hirt extracted and then subjected to Southern blot assay. Data are presented as mean ± SD.
transcription template, most likely cccDNA, was formed in this model. To test this hypothesis, cellular DNA was Hirt extracted and subjected to Southern blot analysis, the gold standard of cccDNA detection. Similar to previous report, different HBV DNA forms were detected in AAV-HBV–transduced cells.17 Although owing to the different sizes of HBV genome inserts, the pattern of bands was different for AAV-HBV1.2 and AAV-HBV1.04, cccDNA could be detected in both groups (Figure 2H). Together, these results indicated that AAV-HBV1.04 transduction could support HBV cccDNA formation in cell cultures.

**Ataxia-Telangiectasia and Rad3-Related Protein–Dependent DNA Repair Pathway Mediates cccDNA Formation**

In the life cycle of HBV infection, cccDNA is formed through the conversion of rcDNA, which is either from the genome of input virion or from recycling of the newly formed nucleocapsid. To investigate whether cccDNA in this model is derived from rcDNA, cells were treated with different antivirals, including capsid inhibitors BAY41-4109 and GLS4, and reverse transcription inhibitor ETV 1 day after AAV-HBV1.04 transduction. Both capsid inhibitor treatments resulted in decreased Hbc levels as expected (Figure 3A). Furthermore, BAY41-4109, GLS4, or ETV treatment led to suppressed HBV DNA (Figure 3B). However, HBsAg remained unchanged in all 3 groups (Figure 3C). Because HBsAg can only be generated from cccDNA in this model, these data suggested that the formation of cccDNA was not affected by these treatments. This interpretation was further confirmed by Southern blot (Figure 3D). Together, as BAY41-4109, GLS4, and ETV could block rcDNA synthesis but not cccDNA formation in this model system, our results implied that the cccDNA was formed through another mechanism.

AAV infection is known to induce host DNA damage response (DDR).19 The activation of DDR kinases may result in the formation of cccDNA through AAV-HBV template. To explore this possibility, AAV-HBV1.04–transduced cells were treated with ataxia-telangiectasia mutated (ATM) kinase inhibitor (KU-55933), ataxia-telangiectasia and Rad3-related protein (ATR) inhibitors (AZD6738, VE-821) or DNA-dependent protein kinase (DNA-PK) inhibitor (KU-57788). While ATM kinase inhibitor and DNA-PK inhibitor only slightly affected HBeAg levels, 2 ATR inhibitors significantly decreased HBsAg levels (Figure 3E). As HBsAg could serve as a marker for HBV cccDNA in this model, this result suggested that ATR inhibitors may mitigate cccDNA formation. Next, we performed Southern blot to evaluate the effect of these DDR kinase inhibitors on cccDNA formation in this model. As shown in Figure 3E, in accordance with HBsAg ELISA results, 2 ATR inhibitors mitigated HBV cccDNA formation while the AAV-HBV episome remained unaffected (Figure 3F). Together, these data indicated that AAV-HBV1.04 viral vector transduction could result in HBV cccDNA formation through ATR-mediated DDR.

**AAV-HBV1.04 Transduction Results in HBV cccDNA Formation and Long-Term Persistence In Vivo**

We next tested if this AAV-HBV1.04 viral vector can be used to establish an HBV cccDNA mouse model. We injected mice with the recombinant AAV-HBV1.04 through tail vein and collected different samples at the indicated time points (Figure 4A). Results showed that serum HBeAg became positive right after injection and peaked after 8 weeks in both high-dose (5 × 10^11 viral genome [vg]) and low-dose (5 × 10^10 vg) groups (Figure 4B). HBsAg of the low-dose group showed similar kinetics as HBeAg, while HBsAg of the high-dose group reached the peak at 4 weeks after injection (Figure 4C). Serum HBV DNA could also be detected after injection (Figure 4D). The expressions of Hbc and HBsAg were further determined by immunostaining, as shown in Figure 4E; more than 50% of the hepatocytes were positive for Hbc and HBsAg 1 week after transduction. By Western blot, the expression of Hbc could be detected 44 weeks after transduction (Figure 4F). The expression of Hbc could only be observed in the liver, but not in other organs or tissues, suggesting that the AAV-HBV1.04 only targeted the mouse liver (Figure 4G). No obvious infiltrating lymphocytes or liver injury was observed 1 week after injection (Figure 4H).

We next evaluated the formation of HBV cccDNA in this mouse model. Southern blot revealed varied HBV DNA forms including HBV rcDNA, double-stranded linear DNA (dsDNA), AAV-HBV episome, and HBV cccDNA (Figure 5A). To further distinguish different HBV DNA forms in this mouse model, a previously described strategy combined with different enzymes digestion was applied.21 Briefly, T5 exonuclease could digest linear and partial double-stranded forms of DNA; restriction endonuclease SacII could cut the AAV vector backbone thus linearize the AAV-HBV episome; the combination of both T5 exonuclease and SacII could cut all HBV DNA forms except cccDNA (Figure 5B). As shown in Figure 5D, Hirt-extracted DNA of HepAD38 cells showed 3 distinct bands including cccDNA, which is resistant to T5 exonuclease treatment. Similar to AAV-HBV1.2 transduction, a single band, which has the same size as cccDNA extracted from HepAD38 cells and could not be detected by the AAV-specific probe, was uncovered with the AAV-HBV1.04–transduced sample after T5 exonuclease and SacII treatments (Figure 5D). In addition, the presence of cccDNA was further verified by heating the sample to 85°C, which denatures rcDNA and dsDNA, but not cccDNA, into single-stranded DNA. As shown in Figure 5E, cccDNA from HepAD38, AAV-HBV1.2, or AAV-HBV1.04–transduced mouse liver is resistance to 85°C denaturation. Further treatment with EcoRI converted cccDNA into dsDNA (Figure 5E). Furthermore, Hirt-extracted AAV-HBV1.04–transduced samples were treated with T5 exonuclease and SacII, and the intact HBV cccDNA was confirmed by sequencing (Figure 6A and B).

To further examine whether HBV cccDNA generated in this model could resemble the cccDNA formed by natural HBV infection, the association of cccDNA with several
known cccDNA binding proteins was evaluated. Chromatin immunoprecipitation (ChIP) assays revealed that histone H3, HBC, and polymerase II were associated with cccDNA (Figure 6C). Together, these results suggested that the chromatization and transcriptional activation of cccDNA formed in this model does not differ from wild-type cccDNA formed through infection.

We next evaluated whether cccDNA could persist in the mouse liver. Southern blot of Hirt-extracted hepatic DNA revealed that cccDNA could be detected 44 weeks after AAV transduction in a dose-dependent manner (Figure 6D). Interestingly, HBsAg, HBeAg (Figure 7A), and Hbc (Figure 7B) could be detected even 66 weeks after transduction, suggesting long-term persistence of HBV cccDNA in this model. Further, it was confirmed by Southern blot (Figure 7C).

AAV-HBV1.04 Mouse Model Is Suitable for Antiviral Evaluation

Next, we evaluated whether this AAV-HBV1.04 mouse model was suitable for studying antivirals against HBV. One week after AAV-HBV1.04 injection, mice were orally dosed with ETV every day by gavage or injected intravenously with polyinosinic-polycytidylic acid [Poly(I:C)]. Mice treated in parallel with phosphate-buffered saline (PBS) served as control animals. Two weeks after treatments, different HBV replication markers were determined (Figure 8A). ETV, as a reverse-transcription inhibitor, is known to efficiently block HBV DNA synthesis but has little effect on cccDNA or viral proteins. As expected, ETV treatment clearly suppressed circulating HBV DNA but not HBsAg and HBeAg in the mice serum (Figure 8B). Inside the liver, ETV had no impact on HBV cccDNA, as demonstrated by Southern blot (Figure 8C). It did not change small HBs, HBC, and capsid as shown by Western blot (Figure 8D) but strongly suppressed capsid HBV DNA, as demonstrated by Southern blot (Figure 8D). When the mice were treated with Poly(I:C), all the tested HBV markers were strongly reduced, including HBsAg and HBeAg in the serum (Figure 8E), and small HBs, HBC, capsid, and capsid HBV DNA in the liver (Figure 8F). These results suggested that AAV-HBV1.04 mouse model is suitable for the evaluation of antivirals.

Taken together, we established a novel HBV mouse model that harbors cccDNA. This AAV-HBV1.04 mouse model could support HBV cccDNA formation through ATM-mediated DDR. While the AAV-HBV1.04 vector does not encode HBsAg and HBV polymerase, the HBsAg and HBV virion could only be generated from cccDNA. This model will provide a unique platform for studying HBV cccDNA and developing novel antivirals against HBV infection.

Discussion

While vaccine is available to prevent HBV infection, lifelong treatment is needed for chronically infected individuals. Complete sterilizing cure of hepatitis B, which is defined as undetectable HBsAg in serum and elimination of all forms of HBV DNA including cccDNA, can hardly be achieved. The current aim of chronic hepatitis B treatment is a "functional cure." It is defined as seroclearance of HBsAg, undetectable serum HBV DNA, and normal liver enzymes and histology after stopping treatment, which requires complete blockade of HBV cccDNA. As the key obstacle for HBV cure, cccDNA is a crucial target for antiviral treatment. However, the narrow species tropism of HBV has greatly limited the development of animal models harboring cccDNA and hindered the progress of drug development. Chimpanzees are the only nonhuman primate fully susceptible to HBV infection. Additionally, tree shrews can be experimentally infected with HBV. Although the mouse is the best characterized and most convenient laboratory animal, it cannot be infected with HBV.

The identification of NTCP as an HBV receptor offers the opportunity to establish novel HBV infection models, including fully permissive immunocompetent HBV mouse models. Unfortunately, mouse NTCP cannot facilitate HBV entry. Furthermore, mouse hepatocytes overexpressing human NTCP only support the infection of hepatitis D virus, while HBV infection, specifically cccDNA formation, remains restricted. Additionally, cccDNA could not be established in HBV transgenic mice with high-level HBV replication. These results indicated that HBV cccDNA cannot be formed in mouse hepatocytes through the conventional pathway as in human cells.

In the HBV life cycle, the formation of cccDNA requires capsid nuclear transportation, uncoating of rcDNA, and additional DNA repair processes. The detailed molecular events underlying the conversion of rcDNA to cccDNA remain elusive. Many evidences suggest that multiple steps have occurred, including release of the covalently bound viral polymerase and RNA primer from the respective negative- and positive-strand rcDNA templates, cleavage of terminal redundant sequence from the negative strand, repair of the incomplete positive strand, and ligation of both DNA strands. The process may involve a bunch of host factors like flap structure-specific endonuclease 1, DNA polymerases, proliferating cell nuclear antigen, replication...
Figure 4. AAV-HBV1.04 transduction in vivo. (A) Female C57BL/6 mice (6–8 weeks old) were infected with AAV-HBV1.04 at $5 \times 10^{10}$ or $5 \times 10^{11}$ vg equivalents through tail vein injection. Samples were collected at the indicated time points. The kinetics of (B) HBeAg, (C) HBsAg, and (D) HBV DNA in mouse serum were evaluated. (E) Liver sections at week 1 posttransduction were collected for immunostaining of HBc and HBsAg. Scale bar = 75 μm. (F) Liver samples at week 44 posttransduction were collected to examine HBc by Western blot. (G) Different tissues from AAV-HBV1.04–transduced C57BL/6 mice at week 1 posttransduction were collected and HBc was tested by Western blot. (H) Liver sections from AAV-HBV1.04–transduced C57BL/6 mice 1 week after injection were collected and stained with hematoxylin and eosin. Scale bar = 50 μm.
factor C complex, and DNA ligases.\textsuperscript{27–30} In our AAV-HBV1.04 mouse model, treatment of ETV did not interfere with the formation of cccDNA. As ETV blocks reverse transcription it thus restricts synthesis of HBV rcDNA, and this result suggested that cccDNA in this system is originated from AAV-HBV1.04 episome recombination other than rcDNA conversion. In agreement with our conclusion, a recent study showed that HBc-deficient AAV-HBV1.3 still supported cccDNA formation demonstrating that the genesis of cccDNA was independent of HBV replication.\textsuperscript{18} By using different DNA damage repair signaling inhibitors, we identified that cccDNA in this model was formed through the ATR pathway. Several kinase family proteins are essential to the DDR. Among them, the ATR protein is responsible primarily for the repair of single-stranded breaks.\textsuperscript{31} In particular, ATR plays a vital role in responding to replication stress, which occurs if DNA damage accumulates and impedes the fundamental process of DNA replication.\textsuperscript{31} Previous studies demonstrated that AAV replication was able to trigger DDR.\textsuperscript{32–34} According to our data, we could speculate that the cccDNA formed in this model was generated by DNA damage signaling kinase ATR-mediated DDR.

In one recent study on an AAV-HBV transduction model, the authors observed that the disappearance of AAV-HBV episome did not result in a significant decline of HBV viral load, which suggests the existence of another HBV replication template.\textsuperscript{35} Lucifora et al\textsuperscript{17} and our study demonstrated that the cccDNA formed from AAV-HBV model is another source of HBV replication.\textsuperscript{18} Although it is not converted from rcDNA, the cccDNA in this model is intact and functional. The sequencing data showed that the cccDNA from AAV-HBV1.04 transduction shared identical sequence as wild-type HBV cccDNA. ChIP assays further confirmed the interaction of histone, HBC, and polymerase II with cccDNA, suggesting that the chromatinization and transcriptional regulation of this cccDNA is similar to the cccDNA formed via natural infection.

In previous AAV-HBV1.2 or AAV-HBV1.3 mouse models, all viral proteins can generate from both AAV-HBV episome and cccDNA.\textsuperscript{17,18} Thus, the persistence of the AAV-HBV episome will interfere with the analysis of HBV replication markers initiated by cccDNA. As our AAV-HBV1.04 construct itself is HBV replication deficient, the production of HBsAg, viral polymerase, and HBV progeny could only be generated from cccDNA. This model provides a unique platform for studying and developing novel antivirals against HBV cccDNA.

As HBV cccDNA is the key reason for viral persistence, attacking this molecule represents the holy grail to HBV cure. Cell division or direct killing of infected cells by T cells attacking this molecule represents the holy grail to HBV cure. Cell division or direct killing of infected cells by T cells attacking this molecule.

Materials And Methods

Construction of Plasmids

The HBV1.04 (3318 bp) and HBV1.2 (3718 bp) DNA fragments were amplified and inserted into AAV vector by ABlonal MultiF Seamless Assembly Mix (RK21020; Abclonal, Wuhan, China). The recombinant plasmids (pAAV-HBV1.04 and pAAV-HBV1.2) contain the 1.04 and 1.2 HBV fragments spanning nucleotides flanked by inverted terminal repeats of AAV, WPRE (woodchuck hepatitis virus posttranscriptional regulatory element), and human growth hormone polyadenylation signal. Primer sequences are listed in Table 1.

Cell Cultures and Transfection

Huh7, HepG2, AML12, HepAD38, and HEK293T cells were maintained in Dulbecco’s modified Eagle medium supplemented with 10% fetal bovine serum (Lonsera, Montevideo, Uruguay) and 1% penicillin/streptomycin (Gibco, Waltham, MA). The cell cultures were maintained in a 5% CO\textsubscript{2} atmosphere at 37°C. PEI MAX 40K (PolySciences, Warrington, PA) was used for DNA transfection according to the manufacturer’s instructions.

AAV Production and Transduction

HEK293T cells were seeded in 150-mm plate, plasmids (pAAV-HBV1.04 or pAAV-HBV1.2, pAAV2/8-RC, and pHelper in 1:1:1 molar ratio) were cotransfected by using PEI MAX 40K (PolySciences, Warrington, PA). At 72 hours posttransfection, the cell culture supernatants and cells were harvested and filtered with 0.22-μm filter, then concentrated with Amicon Ultra-15 centrifugal filter unit (MilliporeSigma, Burlington, MA). The titer of recombinant AAV was determined by quantitative polymerase chain reaction (qPCR) as described previously.\textsuperscript{14} Primer sequences are listed in Table 1.

For AAV-HBV transduction experiments, HepG2 and AML12 cells were seeded in 24-well plates and transduced with 5 × 10\textsuperscript{4} vg per cell diluted in Dulbecco’s modified...
Eagle medium supplemented with 10% fetal bovine serum (Lonsera), 1% penicillin/streptomycin (Gibco), and 4% PEG8000. One day posttransduction, cells were washed 3 times with PBS and treated further as indicated in the following experiment.

**Drugs and Inhibitors**
Bay 41-4109 racemate (HY-100029A), GLS4 (HY-108917), and ETV (HY-13623) were purchased from MedChemExpress (Monmouth Junction, NJ) and Poly(I:C) (tlr-picw) was purchased from InvivoGen (San Diego, CA). ATM kinase inhibitor (KU-55933), ATR inhibitors (AZD6738, VE-821), and DNA PK inhibitor (KU-57788) were purchased from Selleck Chemicals (Houston, TX).

**Mouse Experiments**
C57BL/6 mice were purchased from China Three Gorges University Laboratory Animal Center (Yichang, China). Six-
to 8-week-old female mice were used in all experiments. Mice were injected with the recombinant AAV-HBV1.04 (5 × 10^10 or 5 × 10^11 vg diluted in 200 μL PBS) through tail vein injection as described previously. Blood samples were collected at indicated time points, and serum samples were obtained after centrifugation at 1200 g for 15 minutes at 4°C to examine HBV DNA, HBeAg, and HBsAg. The levels of DNA and protein in the liver were determined. The protocol was approved by the Ethic Committee of Animal Facility, Wuhan University.

**Analysis of HBV Replication Markers**

Secreted HBeAg and HBsAg in culture supernatants were determined by ELISA (Kehua Bio-Engineering, Shanghai, China). Serum HBsAg and HBeAg levels were measured at Renmin Hospital of Wuhan University (Wuhan, China) by electrochemiluminescence immunoassay using the Elecsys HBsAg II and Elecsys HBeAg kits (Roche Diagnostics, Penzberg, Germany) according to the manufacturer's direction. The sample results are presented in the form of a relative cutoff index (signal to cutoff ratio, S/CO). Extracellular HBV DNA was quantified by qPCR using a HBV viral DNA quantitative fluorescence diagnostic kit (Sansure Biotech, Changsha, China), and serum HBV DNA was measured with HBV-DNA real-time qPCR kit (Fosun Diagnostics, Shanghai, China).

**Western Blot**

Protein samples from cells and mouse liver tissues were obtained by different treatments. The cells were lysed by using lysing buffer (50 mM Tris-HCl [pH 8.0], 5 mM EDTA, 150 mM NaCl, 1% NP-40, 0.1% sodium dodecyl sulfate [SDS]) with protease inhibitor, and 5 mg homogenized liver tissues by Tissue Cell-Destroyer (DS1000; Novastar, Wuhan, China) were suspended in RIPA lysis buffer (Sigma-Aldrich, St Louis, MO) with protease inhibitor, and both were lysed on ice for 30 minutes. The supernatant of cell lysates was collected after centrifugation (4°C, 12,000 g for 10 minutes). Then, the protein concentration of the cells and liver tissues was determined using a Bradford Assay Kit (Bio-Rad Laboratories, Hercules, CA) and a BCA Assay Kit (Biosharp, Hefei, China), respectively. In general, 30-μg protein samples were separated by SDS polyacrylamide gel electrophoresis using Mini-PROTEAN Tetra (Bio-Rad Laboratories, Hercules, CA) and transferred into a PVDF membrane (Millipore, Burlington, MA) using a Mini Trans-Blot system (Bio-Rad Laboratories). Following blocked with 5% nonfat milk for 1 hour at room temperature, the membrane was incubated with the primary antibody overnight at 4°C, washed 3 times with Tris-Buffered Saline with Tween, then incubated with secondary antibody. Finally, the signals were detected using Immobilon Western chemiluminescent horseradish peroxidase substrate (Millipore) by a Luminescent Image Analyzer (Syngene, Cambridge, United Kingdom).
Immuno-Fluorescence Staining

Immuno-fluorescence staining of HBc and HBsAg were performed as described. Briefly, resected mouse liver tissue samples were immediately fixed in 4% paraformaldehyde (Invitrogen, Waltham, MA) for 24 hours, then dehydrated in 40% sucrose solution for 24 hours and embedded in NEG-50 (Thermo Fisher Scientific, Waltham, MA) at −80°C. Liver sections (8-μm thick) were permeabilized with 0.1% Triton X-100 (Sigma-Aldrich) for 10 minutes, 3 times. Slices were then blocked with 10% goat serum (Boster Biological Technology, Wuhan, China) for 1 hour at room temperature, and stained with rabbit anti-HBc (Gene Technology, Shanghai, China) or mouse anti-HBs (S1) overnight at 4°C. Subsequently, samples were incubated with secondary antibody Alexa Fluor 568 goat anti-mouse IgG (Invitrogen) or Alexa Fluor 488 goat anti-rabbit IgG (Invitrogen) for 1 hour at room temperature. The nucleus was stained with Hoechst33258 (Invitrogen) for 5 minutes at room temperature. The images were captured with a confocal laser scanning microscope (Leica, Wetzlar, Germany). More information about the antibodies are listed in Table 2.

Hematoxylin and Eosin Staining

Hematoxylin and eosin staining of mouse liver tissues was by Wuhan Powerful Biology Technology (Wuhan, China) according to the standardized treatment. Briefly, resected mouse liver tissue samples were immediately fixed in 4% paraformaldehyde (Invitrogen) for 24 hours. The tissues were sliced into 5-μm-thick sections, deparaffinized, and stained with hematoxylin and eosin. The staining results were observed under the light microscopy.

Southern Blot

DNA was extracted from cells or 50-mg homogenized liver tissues by Hirt procedure as described previously. DNA samples (cells, 20 μg; liver tissues, 30 μg) were
separated by agarose gels, transferred to nylon membrane by capillary siphon method. Then, the nylon membrane was fixed by UV crosslinking and followed by prehybridization, hybridized with probe. Probes were prepared by Random Primer DNA Labeling Kit Ver. 2 (Takara Bio, Shiga, Japan). Finally, the hybridization signal was collected by Typhoon FLA 9500 imager (GE Healthcare Life Sciences, Piscataway, NJ).

**Detection of HBV Nucleocapsid and Encapsidated HBV DNA**

Detection of HBV nucleocapsid and encapsidated HBV DNA in liver tissue was performed as described previously.45 Briefly, 20-mg homogenized liver tissues by Tissue Cell-homogenizer (DS1000; Novastar) were suspended in 150 μL lysis buffer (10 mM Tris-HCl [pH 7.5], 50 mM NaCl, 1 mM EDTA, 8% sucrose, and 1% NP-40) and incubated on ice for 10 minutes. The lysate was then centrifuged at 15,800 g for 10 minutes at 4°C. Then 1.2 μL 1 M MgCl2, 4 μL 10 mg/mL DNase I, and 3 μL 100 mg/mL RNase A were added into the supernatant and incubated at 37°C for 20 minutes. The lysate was then centrifuged at 15,800 g for 10 minutes at 4°C. The supernatants were collected and separated by electrophoresis in 1.6% agarose gel. In order to test viral capsid, the agarose gel was directly blotted onto PVDF membranes by capillary blotting. The membranes were blocked with 5% nonfat milk for 1 hour at room temperature then incubated with anti-HBc antibody (Gene Technology) overnight at 4°C. The signals were detected using Immobilon Western chemiluminescent horseradish peroxidase substrate (Millipore) by a Luminescent Image Analyzer (Syngene). In order to detect capsid DNA, the membrane was denatured in denaturation buffer (0.5 M NaOH and 1.5

### Table 1. Primer Sequences for PCR and qPCR in This Study

| Oligo Name     | Sequence Order (5'→3') |
|----------------|-----------------------|
| HBV1.04-fwd1   | G GCCGACGCAGTGTCTAGATTCCTCTCCATCCTGCTGC |
| HBV1.04-rev1   | GGTGGAAGGTTGGAATCCCCTGACTGAGCAGGAGATG |
| HBV1.04-fwd2   | GAAATCCACAACCCTCCACC |
| HBV1.04-rev2   | TCGAATAACCGGTTCAGTCATGTCAGCTGAGGAGATG |
| HBV1.2-fwd1    | GGCCGCACGCAGTTGTCTAGATCCCTGAGCAGGAGATG |
| HBV1.2-rev1    | GGCAAAAGATTCTGCCCCCAT |
| HBV1.2-fwd2    | ATGGAAGGAGGAGTTCG |
| HBV1.2-rev2    | TCGAATAACCGGTTCAGTCATGTCAGCTGAGGAGATG |
| AAV-HBV-fwd    | CGGGTTGGTTAATT |
| AAV-HBV-rev    | TAAGCCTGGAGATGGAGAT |
| cccDNA 92-fwd | GCCTATGATGAGAGAGATG |
| cccDNA 2251-rev | AGCTGGAGGCTGATCCTA |
| HBV1.04-HBx-fwd | ATGGGCTGTAGCTGCTG |
| HBV1.04-EcoRI-rev | CCATGGTAGGCTGAGG |
| HBV1.04-EcoRI-fwd | CCTCAGGCCATGCGAAGGAGAGATG |
| HBV1.04-HBx-rev | GGAAGCAGTATGATAT |

### Table 2. Antibodies Used for Western Blot and Immunostaining

| Antibody          | Company/Reference | Cat#       | Host          | Dilution                      |
|-------------------|-------------------|------------|---------------|------------------------------|
| HBc               | Gene Technology   | GB058629   | Rabbit        | 1:1000 (Western blot), 1:50 (immunostaining) |
| HBc               | Self-made         |            | Rabbit        | 1:1000 (Western blot)        |
| β-actin           | Cell Signaling Technology | #4970 | Rabbit | 1:5000 (Western blot)        |
| GAPDH             | Proteintech       | 60004-1-lg | Rabbit        | 1:1000 (Western blot)        |
| HBs (S1)          | Kindly provided by Prof. Xinwen Chen |   | Mouse         | 1:50 (immunostaining)        |
| HBsAg             | Novus Biologicals | NB100-62652 | Rabbit    | 1:2000 (Western blot)        |
| Histone H3        | ABclonal Technology | A2348 | Rabbit | 1:50 (cccDNA ChIP), 1:1000 (Western blot) |
| IgG               | Cell Signaling Technology | #2729 | Rabbit | 1:100 (cccDNA ChIP)          |
| RNA polymerase II | Kindly provided by Prof. Kaiwei Liang |   | Rabbit | 1:20 (cccDNA ChIP)           |
| RPB1/POLR2A       | Beyotime Biotechnology | AF7788 | Rabbit | 1:1000 (Western blot)        |

HBC, hepatitis B virus core protein; HBs, hepatitis B surface; Ig, immunoglobulin;
M NaCl), and neutralized in neutralization buffer (1.5 M NaCl and 1 M Tris-HCl [pH 7.4]). Then, the membrane was fixed by UV crosslinking, prehybridized, and hybridized with DNA probe. Finally, the hybridization signal was collected by Typhoon FLA 9500 imager (GE Healthcare Life Sciences).

ChIP Assay

ChIP assay was performed as described previously. Briefly, 50-mg liver tissues were minced by Tissue Cell-Destroyer (DS1000; Novastar) and homogenized in PBS and fixed in 1% formaldehyde (Invitrogen) for 10 minutes at room temperature, and 1/20 volume of 2.5 M glycine (Sigma-Aldrich) was added to quench formaldehyde. Then, ChIP lysis buffer (50 mM Tris-HCl [pH 8.0], 5 mM EDTA, 150 mM NaCl, 1% NP-40, 0.1% SDS, protease inhibitor) was added and samples were kept on ice for 30 minutes. The lysates were sheared by sonication using a Bioruptor Plus (Diagenode, Seraing, Belgium). Crosslinked chromatin samples were incubated with indicated antibody or normal rabbit immunoglobulin G in a rotator overnight at 4°C. Subsequently, protein A/G-conjugated agarose beads (Smart-Lifesciences, Changzhou, China) were added and incubated in a rotator overnight at 4°C, then the beads were collected and washed 3 times. To elute DNA fragments, immunocomplexes were incubated with elution buffer (50 mM Tris-HCl [pH 8.0], 20 mM EDTA, 1% SDS, protease inhibitor) and neutralized in neutralization buffer (1.5 M NaCl), and neutralized in neutralization buffer (1.5 M NaCl and 1 M Tris-HCl [pH 7.4]). Then, the membrane was fixed by UV crosslinking, prehybridized, and hybridized with DNA probe. Finally, the hybridization signal was collected by Typhoon FLA 9500 imager (GE Healthcare Life Sciences).

Statistical Analysis

Statistical analysis was performed by GraphPad Prism 7.0 software (GraphPad Software, San Diego, CA). The data presented as the mean ± SD (n ≥ 3). The statistical analyses were carried out using Student’s unpaired 2-tailed t test. P values <.05 were considered significant. *P < .05, **P < .01, and ***P < .001.

References

1. Revill PA, Chisari FV, Block JM, Dandri M, Gehring AJ, Guo H, Hu J, Kramvis A, Lampertico P, Janssen HLA, Leverro M, Li W, Liang TJ, Lim SG, Lu F, Penicaud MC, Tavis JE, Thimme R; Members of the ICEHBWGCChairs I-HSG, Advisors I-HS, Zoulim F. A global scientific strategy to cure hepatitis B. Lancet Gastroenterol Hepatol 2019;4:545–558.
2. Polaris Observatory C. Global prevalence, treatment, and prevention of hepatitis B virus infection in 2016: a modelling study. Lancet Gastroenterol Hepatol 2018;3:383–403.
3. Zhao K, Liu A, Xia Y. Insights into hepatitis B virus DNA integration-55 years after virus discovery. Innovation 2020;1:100034.
4. Xia Y, Liang TJ. Development of direct-acting antiviral and host-targeting agents for treatment of hepatitis B virus infection. Gastroenterology 2019;156:311–324.
5. Xia Y, Cheng X, Blossey CK, Wisskirchen K, Esser K, Protzer U. Secreted interferon-inducible factors restrict hepatitis B and C virus entry in vitro. J Immunol Res 2017;2017:4828936.
6. Yan H, Zhong G, Xu G, He W, Jing Z, Gao Z, Huang Y, Qi Y, Peng B, Wang H, Fu L, Song M, Chen P, Gao W, Ren B, Sun Y, Cai T, Feng X, Sui J, Li W. Sodium taurocholate cotransporting polypeptide is a functional receptor for human hepatitis B and D virus. Elife 2012;1:e00049.
7. Guidotti LG, Matzke B, Schaller H, Chisari FV. High-level hepatitis B virus replication in transgenic mice. J Virol 1995;69:6156–6169.
8. Sprinzl MF, Oberwinkler H, Schaller H, Protzer U. Transfer of hepatitis B virus genome by adenovirus vectors into cultured cells and mice: crossing the species barrier. J Virol 2001;75:5108–5118.
9. Djon S, Bourgine M, Godon O, Levillayer F, Michel ML. Adeno-associated virus-mediated gene transfer leads to persistent hepatitis B virus replication in mice expressing HLA-A2 and HLA-DR1 molecules. J Virol 2013;87:5554–5563.
10. Yang PL, Althage A, Chung J, Chisari FV. Hydrodynamic injection of viral DNA: a mouse model of acute hepatitis B virus infection. Proc Natl Acad Sci U S A 2002;99:13825–13830.
11. Dandri M, Burda MR, Torok E, Pollokr JM, Iwanska A, Sommer G, Rogiers X, Rogler GE, Gupta S, Will H, Greten H, Petersen J. Repopulation of mouse liver with human hepatocytes and in vivo infection with hepatitis B virus. Hepatology 2001;33:981–988.
12. Yuan L, Jiang J, Liu X, Zhang Y, Zhang L, Xiu J, Wu K, Li X, Cao J, Guo X, Shi D, Li J, Jiang L, Sun S, Wang T, Hou W, Zhang T, Zhu H, Zhang J, Yuan Q, Cheng T, Li J, Xia N. HBV infection-induced liver cirrhosis development in dual-humanised mice with human bone mesenchymal stem cell transplantation. Gut 2019;68:2044–2056.
13. Qi Z, Li G, Hu H, Yang C, Zhang X, Leng Q, Xie Y, Yu D, Zhang X, Gao Y, Lan K, Deng Q. Recombinant covalently closed circular hepatitis B virus DNA induces prolonged viral persistence in immunocompetent mice. J Virol 2014;88:8045–8056.
14. Li G, Zhu Y, Shao D, Chang H, Zhang X, Zhou D, Gao Y, Lan K, Deng Q. Recombinant covalently closed circular DNA of hepatitis B virus induces long-term viral persistence with chronic hepatitis in a mouse model. Hepatology 2018;67:56–70.
15. Wu M, Wang C, Shi B, Fang Z, Qin B, Zhou X, Zhang X, Yuan Z. A novel recombinant cccDNA-based mouse model with long term maintenance of rcccDNA and antigenemia. Antiviral Res 2020;180:104826.
16. Yan Z, Zeng J, Yu Y, Xiang K, Hu H, Zhou X, Gu L, Wang L, Zhao J, Young JAT, Gao L. HBVcircle: a novel tool to investigate hepatitis B virus covalently closed circular DNA. J Hepatol 2017;66:1149–1157.
17. Lucifora J, Salveti A, Marniquet X, Mailly L, Testoni B, Fusil F, Inchauspe A, Michelet M, Michel ML, Leverro M,
18. Ko C, Su J, Festag J, Bester R, Kosinska AD, Protzer U. Intramolecular recombination enables the formation of hepatitis B virus (HBV) cccDNA in mice infected with a recombinant AAV-HBV vector. Antiviral Res 2017;145:14–19.

19. Collaco RF, Bevington JM, Bhrigu V, Kalman-Maltese V, Trempe JP. Adeno-associated virus and adenovirus co-infection induces a cellular DNA damage and repair response via redundant phosphatidylinsitol 3-like kinase pathways. Virology 2009;392:24–33.

20. Lok AS, Zoulim F, Dusheiko G, Ghany MG. Hepatitis B infection. PLoS Pathog 2016;12:e1005893.

21. Laras A, Koskinas J, Dimou E, Kostamena A, Wieland SF. The chimpanzee model for hepatitis B virus infection. Cold Spring Harb Perspect Med 2015;5:a021469.

22. Walter E, Keist R, Niederöst B, Pult I, Blum HE. Hepatitis B virus infection of tupaia hepatocytes in vitro and in vivo. Hepatology 1996;24:1–5.

23. Yan H, Peng B, He W, Zhong G, Qi Y, Ren B, Gao Z, Jing Z, Song M, Xu G, Sui J, Li W. Molecular determinants of hepatitis B and D virus entry restriction in mouse sodium taurocholate cotransporting polypeptide. J Virol 2013;87:7977–7991.

24. Yen H, Peng B, He W, Zhong G, Qi Y, Ren B, Gao Z, Jing Z, Song M, Xu G, Sui J, Li W. Molecular determinants of hepatitis B and D virus entry restriction in mouse sodium taurocholate cotransporting polypeptide. J Virol 2013;87:7977–7991.

25. Lempp FA, Mutz P, Lipps C, Wirth D, Bartenschlager R, Urban S. Evidence that hepatitis B virus replication in mouse cells is limited by the lack of a host cell dependency factor. J Hepatol 2016;64:556–564.

26. Xia Y, Guo H. Hepatitis B virus cccDNA: Formation, regulation and therapeutic potential. Antiviral Res 2020;180:104824.

27. Kitanuma K, Que L, Shimadu M, Koura M, Ishihara Y, Wakae K, Nakamura T, Watashi K, Wakita T, Muramatsu M. Flap endonuclease 1 is involved in cccDNA formation in the hepatitis B virus. PLoS Pathog 2018;14:e1007124.

28. Qi Y, Gao Z, Xu G, Peng B, Liu C, Yan H, Yao Q, Sun G, Liu Y, Tang D, Song Z, He W, Sun Y, Guo J, Li W. DNA Polymerase kappa is a key cellular factor for the formation of covalently closed circular DNA of hepatitis B virus. PLoS Pathog 2016;12:e1005893.

29. Long Q, Yan R, Hu J, Cai D, Mitra B, Kim ES, Marchetti A, Zhang H, Wang S, Liu Y, Huang A, Guo H. The role of host DNA ligases in hepatadnavirus covalently closed circular DNA formation. PLoS Pathog 2017;13:e1006784.

30. Wei L, Ploss A. Core components of DNA lagging strand synthesis machinery are essential for hepatitis B virus cccDNA formation. Nat Microbiol 2020;5:715–726.

31. Liu S, Opiyo SO, Manthey K, Glanzer JG, Ashley AK, Amerin C, Troksa K, Shrivastav M, Nickoloff JA, Oakley GG. Distinct roles for DNA-PK, ATM and ATR in RPA phosphorylation and checkpoint activation in response to replication stress. Nucleic Acids Res 2012;40:10780–10794.

32. Schwartz RA, Carson CT, Schubert C, Weitzman MD. Adeno-associated virus replication induces a DNA damage response coordinated by DNA-dependent protein kinase. J Virol 2009;83:6269–6278.

33. Cataldi MP, McCarty DM. Differential effects of DNA double-strand break repair pathways on single-strand and self-complementary adeno-associated virus vector genomes. J Virol 2010;84:8673–8682.

34. Jurvansuu J, Fragkos M, Ingemarsdotter C, Beard P. Chk1 instability is coupled to mitotic cell death of p53-deficient cells in response to virus-induced DNA damage signaling. J Mol Biol 2007;372:397–406.

35. Suarez-Amaran L, Usai C, Di Scala M, Godoy C, Ni Y, Hommel M, Palomo L, Segura V, Olague C, Vales A, Ruiz-Ripa A, Buti M, Salido E, Prieto J, Urban S, Rodriguez-Frias F, Aldabe R, Gonzalez-Aseguinolaza G. A new HDV mouse model identifies mitochondrial antiviral signaling protein (MAVS) as a key player in IFN-beta induction. J Hepatol 2017;67:669–679.

36. Allweiss L, Volz T, Giersch K, Jah R, Raffa G, Petersen J, Lohse AW, Beninati C, Pollicino T, Urban S, Lutgheetman M, Dandi M. Proliferation of primary human hepatocytes and prevention of hepatitis B virus infection efficiently deplete nuclear cccDNA in vivo. Gut 2018;67:542–552.

37. Bohne F, Chmielewski M, Ebert G, Wiegmann K, Kirschner T, Schulze A, Urban S, Kronke M, Abken H, Protzer U. T cells redirected against hepatitis B virus surface proteins eliminate infected hepatocytes. Gastroenterology 2008;134:239–247.

38. Xia Y, Stadler D, Lucifora J, Reisinger F, Webb D, Hösel M, Michler T, Wisskirchen K, Cheng X, Zhang K, Chou WM, Wetten gel JM, Malo A, Bohne F, Hoffmann D, Eyer F, Thimme R, Falk CS, Thasler WE, Heikenwalder M, Protzer U. Interferon-γ and tumor necrosis factor-α produced by T cells reduce the HBV persistence form, cccDNA, without cytolysis. Gastroenterology 2016;150:194–205.

39. Lucifora J, Xia Y, Reisinger F, Zhang K, Stadler D, Cheng X, Sprinzl MF, Koppensteiner H, Makowska Z, Volz T, Remouchamps C, Chou WM, Thasler WE, Huser N, Durante l D, Liang T, Munk C, Heim MH, Browning JL, Dejardin E, Dandri M, Schindler M, Heikenwalder M, Protzer U. Specific and nonhepatotoxic degradation of nuclear hepatitis B virus cccDNA. Science 2014;343:1221–1228.

40. Koh S, Kah J, Tham CYL, Yang N, Ceccarello E, Chia A, Chen M, Khakpoor A, Pavesi A, Tan AT, Dandi M, Bertoletti A. Nonlytic lymphocytes engineered to express virus-specific T-cell receptors limit HBV infection by activating APOBEC3. Gastroenterology 2018;155:180–193.

41. Gilmore S, Tam D, Dick R, Appleby T, Birkus G, Willkom M, Delaney WE, Notte GT, Feierbach B. Antiviral activity of GS-5801, a liver-targeted prodrug of a lysine demethylase 5 inhibitor, in a hepatitis B virus primary human hepatocyte infection model. J Hepatol 2017;66:2.
42. Teng Y, Xu Z, Zhao K, Zhong Y, Wang J, Zhao L, Zheng Z, Hou W, Zhu C, Chen X, Protzer U, Li Y, Xia Y. Novel function of SART1 in HNF4alpha transcriptional regulation contributes to its antiviral role during HBV infection. J Hepatol 2021;75:1072–1082.

43. Cao L, Wu C, Shi H, Gong Z, Zhang E, Wang H, Zhao K, Liu S, Li S, Gao X, Wang Y, Pei R, Lu M, Chen X. Coexistence of hepatitis B virus quasispecies enhances viral replication and the ability to induce host antibody and cellular immune responses. J Virol 2014;88:8656–8666.

44. Liang K, Woodfin AR, Slaughter BD, Unruh JR, Box AC, Rickels RA, Gao X, Haug JS, Jaspersen SL, Shilatifard A. Mitotic transcriptional activation: clearance of actively engaged Pol II via transcriptional elongation control in mitosis. Mol Cell 2015;60:435–445.

45. Zhang S, Guo JT, Wu JZ, Yang G. Identification and characterization of multiple TRIM proteins that inhibit hepatitis B virus transcription. PLoS One 2013;8:e70001.

46. Lee TI, Johnstone SE, Young RA. Chromatin immunoprecipitation and microarray-based analysis of protein location. Nat Protoc 2006;1:729–748.

Received July 14, 2021. Accepted November 30, 2021.

Correspondence
Address correspondence to: Yuchen Xia, PhD, Institute of Medical Virology, School of Basic Medical Sciences, Wuhan University, Wuhan, Hubei 430071, China. e-mail: yuchenx@whu.edu.cn; fax: 0086-27-68759222.

Acknowledgments
The authors thank Mr. Cong Li and the staffs at the Research Center for Medicine and Structural Biology of Wuhan University for technical assistance.

CRediT Authorship Contributions
Zaichao Xu, MSc (Investigation: Equal; Methodology: Equal; Validation: Lead; Writing – original draft: Supporting; Writing – review & editing: Equal)
Li Zhao, MSc (Investigation: Equal; Methodology: Equal; Validation: Equal; Writing – review & editing: Supporting)
Youquan Zhong, Mr (Investigation: Equal; Methodology: Equal; Writing – review & editing: Supporting)
Chengliang Zhu, PhD (Investigation: Equal; Writing – review & editing: Supporting)
Kaitao Zhao, PhD (Investigation: Supporting; Writing – review & editing: Supporting)
Yan Teng, MSc (Investigation: Supporting; Writing – review & editing: Supporting)
Xiaoming Cheng (Formal analysis: Supporting; Validation: Supporting; Writing – review & editing: Supporting)
Yuchen Xia, PhD (Formal analysis: Supporting; Resources: Supporting; Writing – review & editing: Supporting)

Conflicts of interest
The authors disclose no conflicts.

Funding
This work was supported by the National Natural Science Foundation of China (project no. 81971936), the Fundamental Research Funds for the Central Universities, Hubei Province’s Outstanding Medical Academic Leader Program, the Gilead Sciences Research Scholars Program in Liver Disease Asia, the Foundation for Innovative Research Groups of the Natural Science Foundation of Hubei (project no. 2020CF015), and the Research and Innovation Team Project of Hubei Health Commission (project no. WJ2021C002).