

# Article

# Hepatitis B virus hijacks CTHRC1 to evade host immunity and maintain replication

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**Hepatitis B virus (HBV) infection causes acute and chronic liver diseases, but is not directly cytopathic. Liver injury results from repeated attempts of the cellular immune response system to control the viral infection. Here, we investigate the roles of cellular factors and signaling pathways involved in the regulation of HBV replication to reveal the mechanism underlying HBV infection and pathogenesis. We show that collagen triple helix repeat containing 1 (CTHRC1) expression is elevated in HBV-infected patients and in HBV-transfected cells through epigenetic modification and transcriptional regulation. CTHRC1 facilitates HBV replication in cultured cells and BALB/c mice by activating the PKC $\alpha$ /ERK/JNK/c-Jun cascade to repress the IFN/JAK/STAT pathway. HBV-activated CTHRC1 downregulates the activity of type I interferon (IFN), the production of IFN-stimulated genes (ISGs), and the phosphorylation of signal transducer and activator of transcription 1/2 (STAT1/2), whereas it upregulates the phosphorylation and ubiquitination of type I IFN receptors (IFNAR $\alpha/\beta$ ). Thus, our results show that HBV uses a novel mechanism to hijack cellular factors and signal cascades in order to evade host antiviral immunity and maintain persistent infection. We also demonstrate that CTHRC1 has a novel role in viral infection.**

**Keywords:** hepatitis B virus, collagen triple helix repeat containing 1 (CTHRC1), immune response, immune evasion, PKC/JNK/ERK/c-Jun cascade, IFN/JAK/STAT pathway

## Introduction

Hepatitis B virus (HBV) infection causes liver diseases, ranging from fulminant hepatitis to cirrhosis, eventually leading to hepatocellular carcinoma (HCC) (Lok and McMahon, 2009; Lin et al., 2014). The viral genome consists of an enveloped nucleocapsid containing a partially double-stranded relaxed circular DNA genome of 3.2 kb (Sato et al., 2015). The mechanisms underlying the control of HBV infection and pathogenesis are not fully understood, but it is generally accepted that host factors and molecular pathways determining fitness of virus replication contribute to the development of viral-associated diseases (Nguyen et al., 2008; Dandri and Locarnini, 2012; Nagy and Pogany, 2012).

HBV replication is strongly inhibited by interferons (IFNs) that are required for the establishment of antiviral states in infected cells. Central to the antiviral responses is the production of IFNs that induce an array of IFN-stimulated genes (ISGs) to limit virus infection (Sadler and Williams, 2008). Cytokines and chemokines are known to regulate HBV replication (Hösel et al., 2009; Keating et al., 2014). We have reported that HBV induces a novel inflammation network to regulate viral replication (Yu et al., 2011; Cao et al.,

2014). In addition, microRNA-122 and major vault protein (MVP) are involved in downregulating the replication of hepatitis viruses (Chen et al., 2011; Liu et al., 2012). Consequently, virus must develop strategies to evade host antiviral immunity to ensure persistent infection. The balance between antiviral immunity and viral replication is important for determining the course of infection and pathogenesis (Migliorini and Anders, 2012). However, much less is known about how the host antiviral response is switched off. Thus, it is of great interest to identify the host factors that attenuate IFN signaling to enhance viral replication.

In this study, we demonstrate that collagen triple helix repeat containing 1 (CTHRC1) facilitates HBV replication by stimulating the PKC/JNK/ERK/c-Jun cascade to attenuate the antiviral activity of IFN/JAK/STAT signaling. CTHRC1, a secreted protein with multiple functions, was originally discovered in carotid arteries upon balloon catheter injury (Stohn et al., 2012; Liu et al., 2013; Takeshita et al., 2013; Ikeda and Takeshita, 2014). CTHRC1 is also associated with invasion and metastasis in human cancers, including gastric carcinogenesis (Wang et al., 2012), colorectal cancer (Tan et al., 2013), ductal carcinoma (Kim et al., 2013), oral squamous cell carcinoma (Liu et al., 2013), and hepatocellular carcinoma (Zhang et al., 2015). However, the effect of CTHRC1 in regulating viral replication has not been reported. We further reveal that CTHRC1 is elevated in patients infected with HBV and

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in hepatocytes carrying the HBV genome, and HBV stimulates CTHRC1 expression by epigenetic modification and transcriptional regulation. More important, HBV-activated CTHRC1, in turn, facilitates the viral replication both *in vivo* and *in vitro*. We further reveal a novel mechanism by which HBV hijacks cellular factors to evade host antiviral immunity and maintain persistent infection.

## Results

### *CTHRC1 expression is activated in patients with chronic hepatitis B and in hepatoma cells carrying the HBV genome*

We initially revealed the effect of HBV infection on CTHRC1 expression. First, CTHRC1 protein levels were higher in the sera of patients with chronic hepatitis B ( $n = 153$ ) than in the sera of healthy individuals ( $n = 164$ ) ( $66.7 \pm 3.2$  versus  $34.8 \pm 2.5$  ng/ml,  $P < 0.0001$ ) (Figure 1A and Supplementary Table S1). Second, CTHRC1 mRNA levels were elevated in liver biopsy specimens from HBV-associated HCC tissues ( $n = 12$ ) compared with those from noncancerous tissues ( $n = 13$ ) (Figure 1B and Supplementary Table S2). Third, CTHRC1 protein levels were positively correlated with HBV DNA loads in the sera of 142 HBV-infected patients ( $r = 0.412$ ) (Figure 1C and Supplementary Table S3). Finally, CTHRC1 protein was enhanced in HepG2.2.15 cells carrying the HBV genome as compared with HepG2 cells without the HBV genome, and it was increased in HBV-transfected HepG2 cells as compared with mock cells (Figure 1D). Thus, CTHRC1 expression was activated in patients infected with HBV and in hepatoma cells carrying the HBV genome.

### *HBV stimulates CTHRC1 expression by epigenetic modification and transcription regulation*

We next aimed to elucidate the molecular mechanism by which HBV activated CTHRC1 expression. DNA methyltransferase (DNMT) activities were higher in HepG2 cells than in HepG2.2.15 cells (Figure 2A), suggesting that HBV may suppress DNMT activities. CTHRC1 promoter activity, mRNA expression, and protein production were upregulated by a DNMT inhibitor (5-Aza-dc) (Figure 2B), indicating that DNMT plays a role in CTHRC1 activation. More precise information concerning CTHRC1 epigenetic modification was also evaluated. First, DNA sequencing analyses revealed that nucleotides  $-628$  to  $-269$  of CTHRC1 promoter contain 23 CpG sites and that the percentage of methylated CpG was lower in HCC tissues (11.7%) than in noncancerous tissues (19.6%) (Figure 2C), indicating that CTHRC1 promoter DNA methylation was downregulated in HCC tissues. Second, a specificity protein 1 (Sp1)-binding element containing a CpG site ( $-581$ ) in CTHRC1 promoter was demethylated in HCC tissues, while it was methylated in noncancerous tissues (Figure 2C), suggesting that Sp1 was involved in the regulation of CTHRC1 expression. The interaction between SP1 transcription factor and CTHRC1 promoter was also demonstrated (Supplementary Figure S4). Third, DNMT3a and DNMT3b mRNA were lower in HepG2.2.15 and HBV-transfected HepG2 cells than in HepG2 and non-transfected cells, respectively (Figure 2D), indicating that HBV suppresses DNMT3a and DNMT3b expression. Fourth, CTHRC1 promoter activity, mRNA expression, and protein production were repressed by DNMT3a (Figure 2E), demonstrating that DNMT3a was responsible

for regulating CTHRC1 expression. Finally, CTHRC1 promoter activity, mRNA expression, and protein production were activated by siDNMT3a (Figure 2F), confirming the involvement of DNMT3a in CTHRC1 expression. Taken together, HBV stimulated CTHRC1 expression by epigenetic modification and transcription regulation.

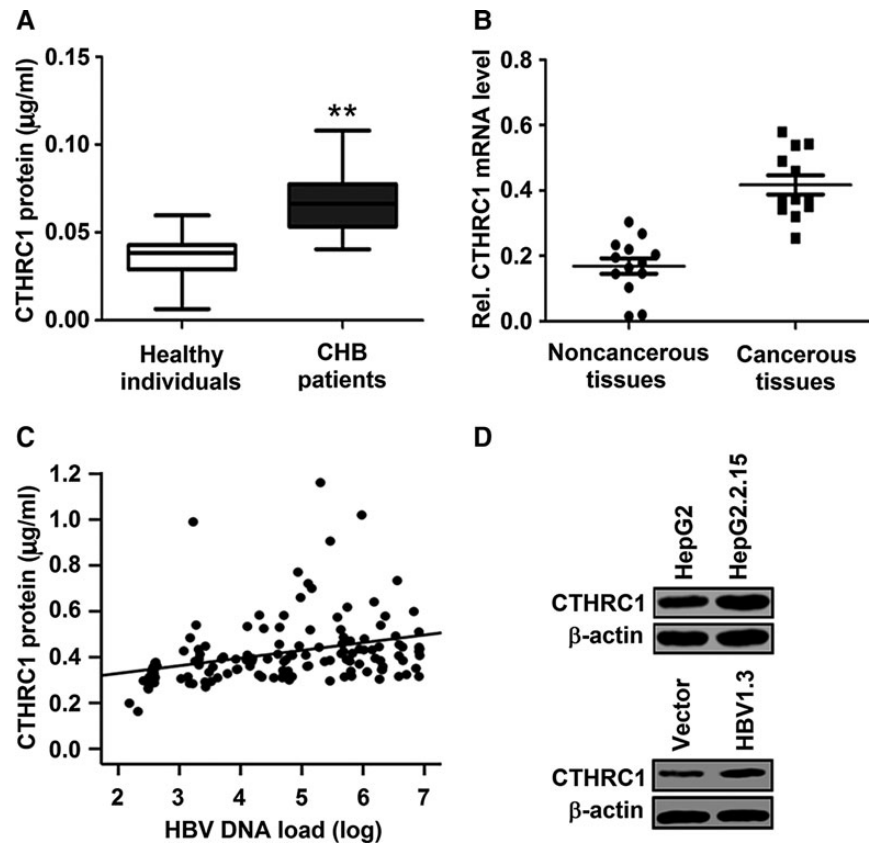
### *CTHRC1 enhances HBV replication in hepatocytes*

CTHRC1 is a protein with multiple functions, but its role in viral infection has not been reported. We determined, for the first time, the effect of CTHRC1 on viral replication. HBV e antigen (HBeAg) and HBV s antigen (HBsAg) expression, as well as HBV core-associated DNA replication, were upregulated by CTHRC1 in HepG2, HepG2.2.15 and L02 cells in dose- and time-dependent fashions (Figure 3A and B). The HBV FP promoter, but not the S1P, CP, and XP promoters, was activated by CTHRC1 (Figure 3C). The effect of CTHRC1 on HBV replication was also evaluated by using a small interfering RNA to CTHRC1 (siCTHRC1), whose effectiveness was confirmed (Supplementary Figure S1A and B). Results showed that HBeAg and HBsAg expression and HBV DNA replication were downregulated by siCTHRC1 in HepG2, HepG2.2.15, and L02 cells (Figure 3D). In addition, HBV-specific transcripts of 4.1/3.5 and 2.4/2.1 kb were detected in HBV-transfected HepG2 cells, enhanced by CTHRC1, but reduced by siCTHRC1 (Figure 3E). Therefore, over-expression of CTHRC1 upregulated HBV replication and knock-down of CTHRC1 downregulated HBV replication.

### *PKC $\alpha$ , ERK, JNK, and c-Jun are involved in CTHRC1-activated HBV replication*

To reveal the molecular mechanism involved in CTHRC1-mediated HBV replication, we evaluated the effects of signaling components on such regulation. HBeAg and HBsAg were activated by CTHRC1, not affected by LY294002 (PI3K inhibitor), slightly reduced by PD98059 (MEK1/2 inhibitor) and SB203508 (p38 MAPK inhibitor), repressed by U0126 (ERK1/2 inhibitor), SP600125 (JNK inhibitor), and GF109203 (PKC inhibitor), and enhanced by BAY11-7039 (NK- $\kappa$ B inhibitor) (Figure 4A). These results suggested PKC, MEK, JNK, and NF- $\kappa$ B are required for CTHRC1-activated HBV replication. HBV promoter activity (Supplementary Figure S2A) and HBeAg and HBsAg expression (Figure 4B) were upregulated by CTHRC1, but downregulated by GF109203, U0126, and SP600125, confirming that PKC, ERK, and JNK played positive roles in CTHRC1-activated HBV replication.

The effects of specific PKC family members, ERK, and JNK on CTHRC1-activated HBV replication were further revealed. First, CTHRC1-activated HBeAg and HBsAg were downregulated by siPKC $\delta$ , siPKC $\beta$ , and siPKC $\epsilon$ , repressed by siPKC $\alpha$ , and not affected by siPKC $\zeta$ , siPKCD1, or siPKCD2 (Figure 4C), demonstrating a stimulatory effect of PKC $\alpha$  on CTHRC1-promoted HBV replication. Second, CTHRC1 enhanced PKC $\alpha$  translocation from cytoplasm to the membrane (Supplementary Figure S2B), indicating a correlation between PKC $\alpha$  and CTHRC1-promoted HBV replication. Third, CTHRC1-activated HBeAg and HBsAg were downregulated by the mutants of ERK and JNK (mERK1, mERK2, and mJNK) (Figure 4D), suggesting requirements of ERK and JNK for CTHRC1-activated HBV replication. Fourth, HBeAg and HBsAg expression



**Figure 1** CTHRC1 expression is correlated with HBV infection. (A) CTHRC1 proteins in the sera of HBV-infected patients ( $n = 153$ ) and healthy individuals ( $n = 164$ ) were detected by ELISA. Data represent means  $\pm$  SEM (Supplementary Table S1). Box plots illustrate medians of 25% and 75% and error bars illustrate the 5th and 95th percentiles (\*\* $P < 0.01$ ). (B) Total RNA was extracted from freshly isolated HBV-associated HCC tissues ( $n = 12$ ) and noncancerous tissues ( $n = 13$ ). CTHRC1 mRNA was detected by RT-PCR. Data represent means  $\pm$  SEM from samples tested in triplicates (Supplementary Table S2). Box plots illustrate medians of 25% and 75% and error bars illustrate the 5th and 95th percentiles (\*\* $P < 0.01$ ). (C) CTHRC1 protein levels were measured by ELISA, and HBV capsid-associated DNA loads determined by RT-PCR from the sera of HBV-infected patients ( $n = 142$ ). Data represent means  $\pm$  SEM (Supplementary Table S3). SPSS statistical software was used for statistical analysis and evaluation. (D) CTHRC1 proteins were detected by western blot in HepG2 and HepG2.2.15 cells (upper panel) and in HepG2 cells transfected with pBlue-HBV1.3 or its vector (lower panel). All samples were tested in duplicate and concentrations were determined from standard curves.

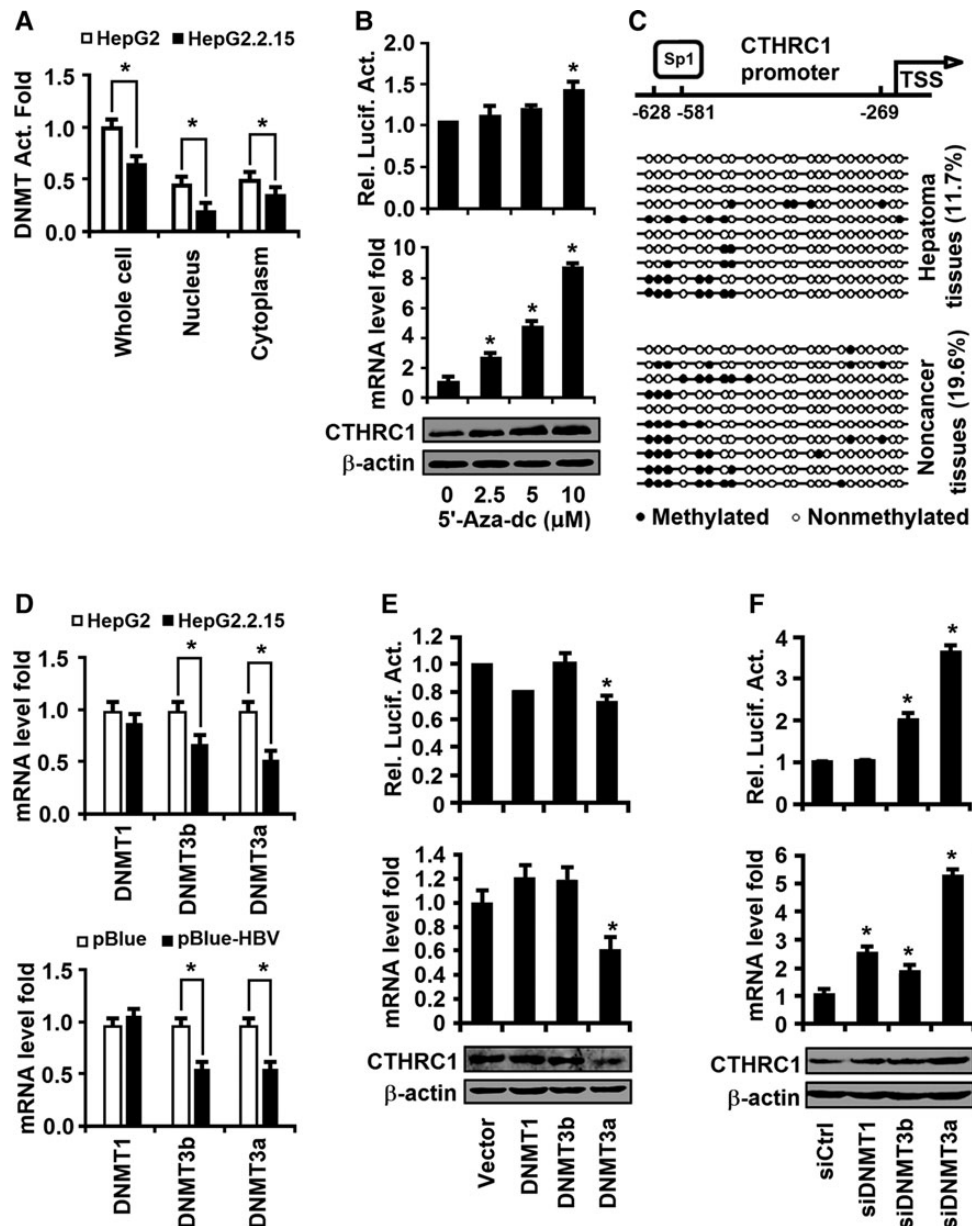
and ERK phosphorylation were upregulated by K-Ras<sup>v12</sup> (ERK activator) and downregulated by U0126 (ERK1/2 inhibitor) (Figure 4E), while HBeAg and HBsAg were repressed by siJNK (Supplementary Figure S3A). These results confirmed that ERK and JNK were involved in CTHRC1-activated HBV replication.

The downstream targets of PKC $\alpha$ /ERK/JNK signaling involved in CTHRC1-activated HBV replication were then investigated. It is known that HBV enhancer I contains a binding site for activator protein 1 (AP-1), a protein consisting two subunits, c-Fos and c-Jun, which is a downstream target of the ERK/JNK pathway (Cai et al., 2011; Jin et al., 2011; Wang et al., 2014). We speculated that AP-1 is a potential activator for HBV replication. Results revealed that HBeAg and HBsAg were upregulated by CTHRC1, slightly downregulated by si-c-Fos, and significantly repressed by si-c-Jun (Figure 4F). In addition, p-c-Jun could bind to the AP-1-binding sequence of HBV genome (Supplementary Figure S3B). p-ERK, p-JNK, and p-c-Jun were activated by CTHRC1 and repressed by GF109203 (Supplementary Figure S3C). Thus, PKC $\alpha$ , ERK, JNK, and c-Jun were involved in CTHRC1-activated HBV replication.

#### *CTHRC1 facilitates HBV replication by activating the PKC $\alpha$ /JNK/c-Jun cascade*

The effect of CTHRC1 on PKC $\alpha$ /ERK/JNK/c-Jun signaling was evaluated. p-PKC $\alpha$ , p-ERK, and p-JNK (but not PKC $\alpha$ , ERK, JNK, and GAPDH) were upregulated by CTHRC1 and downregulated by siCTHRC1 (Figure 5A). p-PKC $\alpha$ , p-ERK, p-JNK, and p-c-Jun were enhanced by CTHRC1 in HepG2 and LO2 cells in dose-dependent fashions (Figure 5B). The results demonstrated that CTHRC1 activated the phosphorylation of PKC $\alpha$ , JNK, ERK, and c-Jun.

The correlation between PKC $\alpha$ /ERK/JNK and c-Jun was then analyzed. In whole-cell lysates, p-c-Jun was upregulated by CTHRC1, repressed by GF109203, downregulated by SP600125, but relatively unaffected by U0126. In the cytosol, p-c-Jun was downregulated by CTHRC1 and slightly upregulated by SP600125, GF109203, and U0126. In the nucleus, p-c-Jun was enhanced by CTHRC1, reduced by SP600125 and GF109203, and relatively unaffected by U0126 (Figure 5C). These results indicated that CTHRC1, PKC, and JNK facilitated p-c-Jun translocation from cytosol to nucleus. Moreover, p-c-Jun was mainly distributed in the cytosol in the absence of

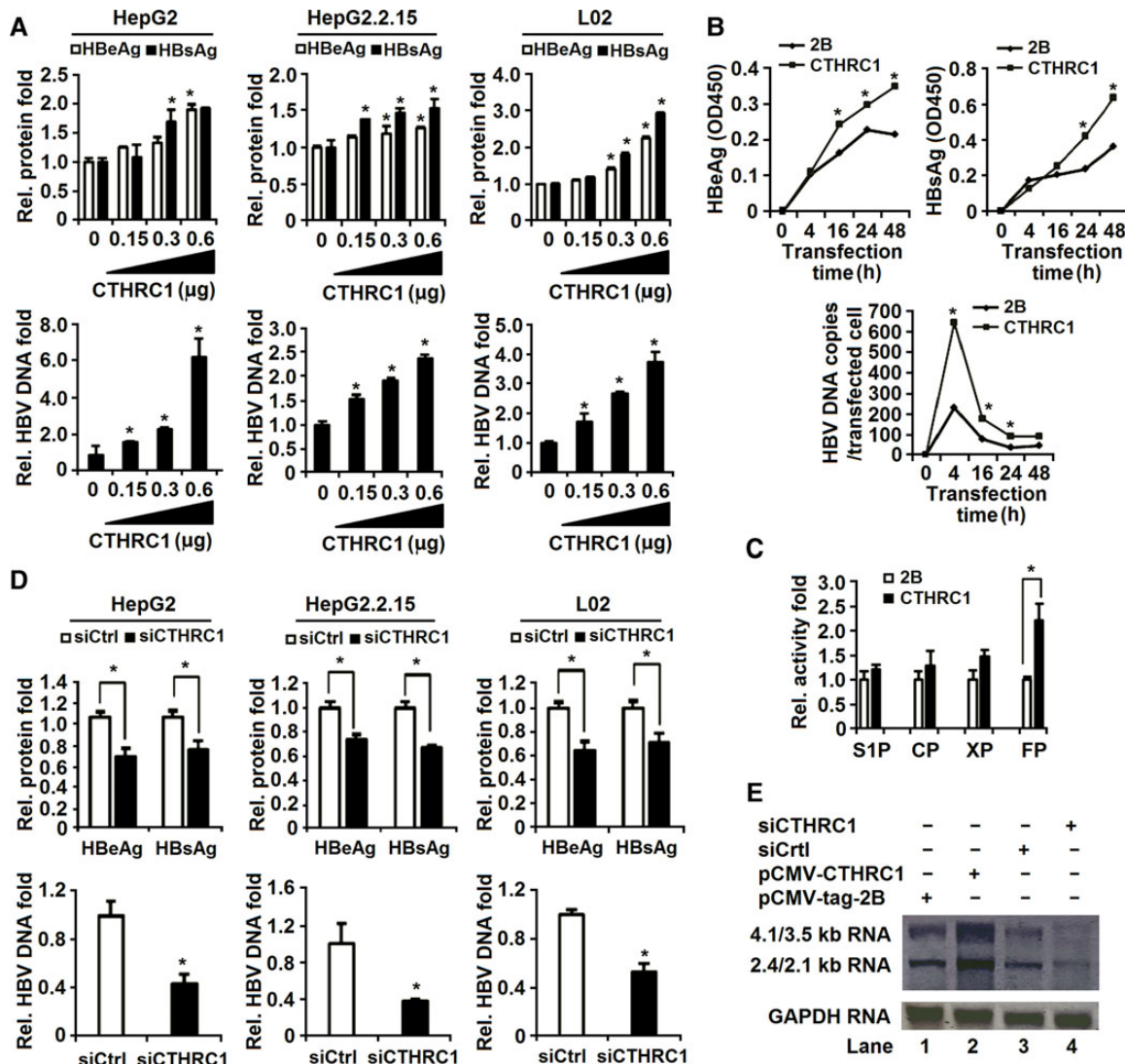


**Figure 2** DNA methyltransferase plays a role in HBV-activated CTHRC1 expression. (A) DNMT activities of whole-cell lysates, nuclear extracts, and cytoplasmic extracts of HepG2 and HepG2.2.15 cells, respectively, were measured using the EpiQuik DNA Methyltransferase Activity Assay Kit ( $*P < 0.05$ ). (B) HepG2 cells were transfected with pCTHRC1-Luc and treated with DNMT inhibitor (5'-aza-2'-deoxycytidine, 5'-aza-dc) to a final concentration of 2.5, 5, and 10  $\mu$ M at 6 h post-transfection. Cells were allowed to grow for an additional 24 h for RNA detection by RT-PCR and an additional 48 h for protein detection by luciferase assay and western blot. Luciferase activity is presented as mean  $\pm$  SD,  $n = 3$  ( $*P < 0.05$ ). (C) Genomic DNAs isolated from HBV-associated HCC tissue and noncancerous tissue were treated with sodium bisulfite, and the CTHRC1 promoter was amplified by PCR, cloned, and subjected to sequencing analysis. Each row of circles represents a single sequenced clone, the solid circle indicates methylated cytosine, and the open circle indicates non-methylated cytosine. (D) DNMT1, DNMT3a, and DNMT3b mRNA levels in HepG2 and HepG2.2.15 cells (upper panel) and in HepG2 cells transfected with or without pBlue-HBV-1.3 (lower panel) were detected by RT-PCR. (E) HepG2 cells were co-transfected with pCTHRC1-Luc and pCDNA-DNMT1, pCDNA-DNMT3a, pCDNA-DNMT3b, or control vector. (F) HepG2 cells were co-transfected with pCTHRC1-Luc and siDNMT1, siDNMT3a, siDNMT3b, or siCtrl. (E and F) CTHRC1 promoter activity was determined by luciferase assay (upper panels), CTHRC1 mRNA was determined by RT-PCR (middle panel), and CTHRC1 protein was detected by western blot (bottom panels).

CTHRC1, while a proportion of p-c-Jun was located to the nucleus in the presence of CTHRC1, but this distribution was reversed by GF109203 and SP600125 (Figure 5D), further confirming the positive

roles of CTHRC1, PKC, and JNK in regulating p-c-Jun. Thus, CTHRC1 facilitates HBV replication by activating the PKC $\alpha$ /JNK/c-Jun cascade.





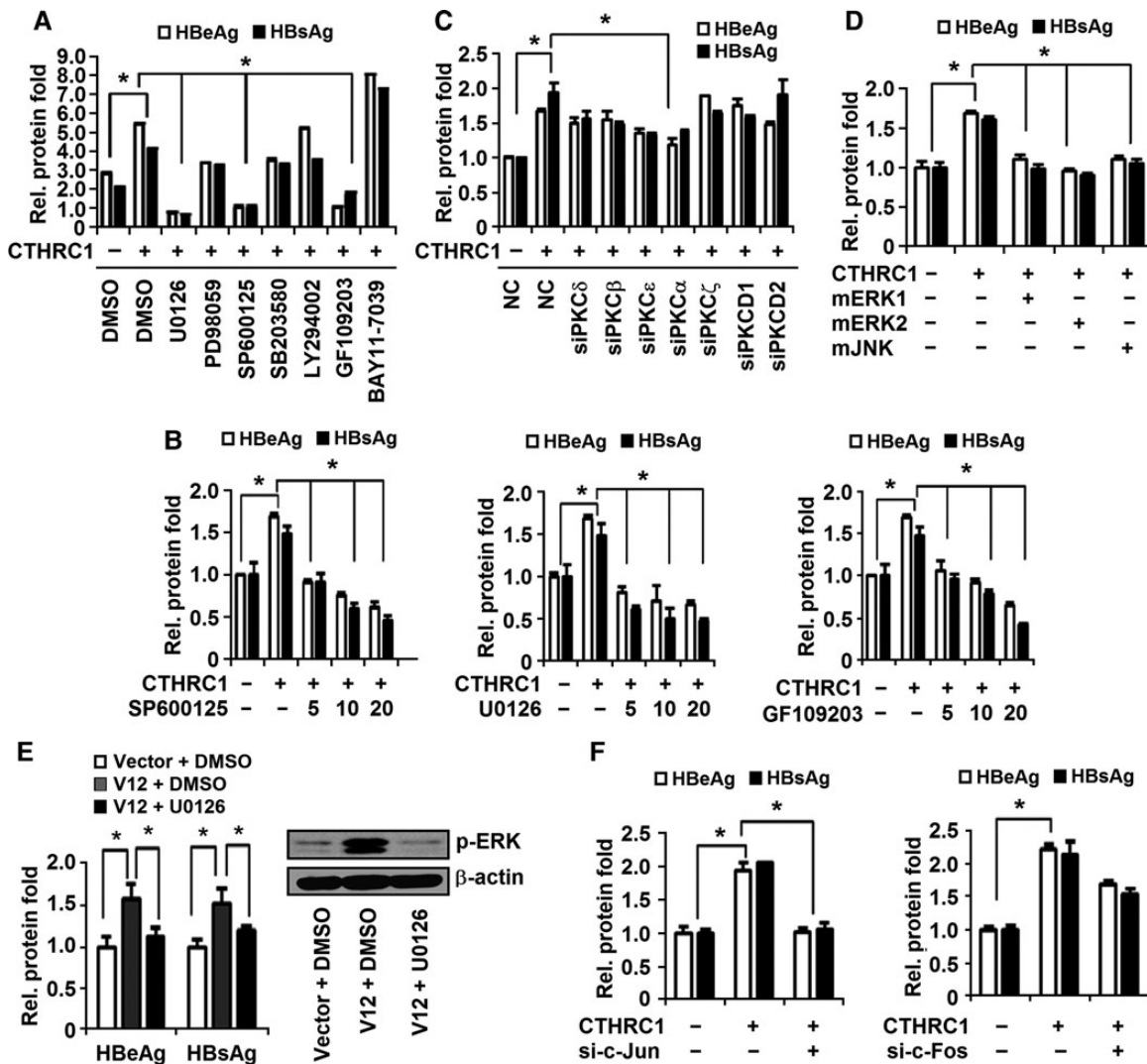
**Figure 3** CTHRC1 enhances HBV replication in hepatocytes. (A) L02 cells and HepG2 cells were co-transfected with pBlue-HBV1.3 and pCMV-CTHRC1, and HepG2.2.15 cells were transfected with pCMV-CTHRC1 at different concentrations, as indicated. HBeAg and HBsAg proteins in culture supernatants were detected by ELISA (upper panels), and HBV core-associated DNAs in cell lysates were determined by RT-PCR (lower panels). (B) HepG2.2.15 cells were transfected with pCMV-CTHRC1 for different times, as indicated. HBeAg and HBsAg were detected by ELISA and HBV core-associated DNAs were determined by RT-PCR. (C) HepG2 cells were co-transfected with pCMV-CTHRC1 and plasmids carrying each of the four pGL3-HBV promoters: S1P, CP, XP, and FP. HBV promoter activities were measured by luciferase activity assay at 48 h post-transfection. Data shown are mean  $\pm$  SD,  $n = 3$  (\* $P < 0.05$ ). (D) L02 cells and HepG2 cells were co-transfected with pBlue-HBV1.3 and siCTHRC1, and HepG2.2.15 cells were transfected with siCTHRC1. HBeAg and HBsAg were detected by ELISA (upper panels), and HBV core-associated DNAs were determined by RT-PCR (lower panels). (E) HepG2 cells were co-transfected with pHBV-1.3 and pCMV-CTHRC1 (or pCMV-tag-2C) or siCtrl (or siCTHRC1). HBV mRNAs were isolated and detected by northern blot using GAPDH mRNA as the loading control.

#### CTHRC1 suppresses antiviral activity of IFN/JAK/STAT signaling

The downstream events of the PKC $\alpha$ /ERK/JNK/c-Jun cascade involved in CTHRC1-activated HBV replication were evaluated. HBV replication is inhibited by IFNs, which induces an array of ISGs to limit virus infection (Lütgehetmann et al., 2011; Belloni et al., 2012; Chen et al., 2013; Hao et al., 2013). Thus, we investigated the role of CTHRC1 in regulating the activities of IFNs. HBeAg and HBsAg expression (Figure 6A) and HBV core-associated DNA replication (Figure 6B) were downregulated by IFN- $\alpha$  (type I IFN), IFN- $\gamma$  (type II IFN), and IFN- $\lambda$ 1 (type III IFN), respectively, but

the inhibitory effects were repressed by CTHRC1, suggesting that CTHRC1 downregulated anti-HBV activities of IFN- $\alpha$ , IFN- $\gamma$ , and IFN- $\lambda$ 1.

The mechanism by which CTHRC1 regulates IFN anti-HBV activities was then elucidated. The expression of IFN- $\alpha$ / $\beta$  receptors (IFNAR $\alpha$  and IFNAR $\beta$ ) and ISGs (PKR, Mx1, and OAS1) and the phosphorylation of STAT1/2 were upregulated by IFN- $\alpha$  and downregulated by CTHRC1 (Figure 6C). IFNAR $\alpha$  and IFNAR $\beta$  expression on the membranes was enhanced by IFN- $\alpha$  and reduced by CTHRC1 (Figure 6D). In addition, HBeAg and HBsAg were upregulated by



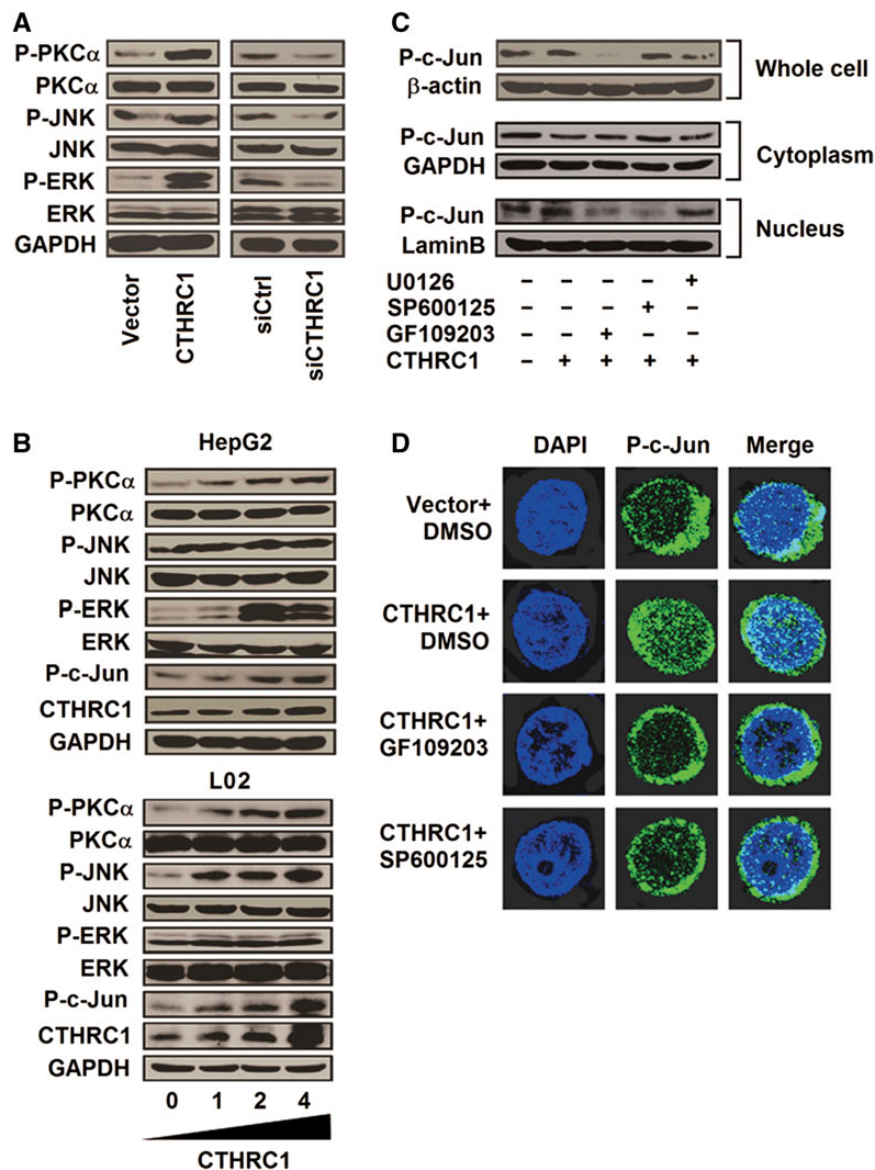
**Figure 4** Signaling components are involved in CTHRC1 regulated HBV replication. (A) HepG2 cells were co-transfected with pCMV-CTHRC1 and pBlue-HBV1.3 and treated with PD98059 (ERK1/2 inhibitor), SB203580 (p38 inhibitor), U0126 (MEK1/2 inhibitor), SP600125 (JNK inhibitor), GF109203 (PKC inhibitor), or BAY11-7039 (NK-κB inhibitor). (B) HepG2 cells were co-transfected with pCMV-CTHRC1 and pBlue-HBV1.3 and treated with U0126, SP600125, or GF109203 at concentrations of 5, 10, and 20 μM. (C) HepG2 cells were co-transfected with pCMV-CTHRC1 and pBlue-HBV1.3, along with plasmids expressing siPKCδ, siPKCα, siPKCβ, siPKCε, siPKCζ, siPKCD1, siPKCD2, or siCtrl. (D) HepG2 cells were co-transfected with pCMV-CTHRC1 and pBlue-HBV1.3, along with each of the three dominant negative mutants (mERK1, mERK2, and mJNK) of ERK or JNK. (E) HepG2 cells were transfected with or without V12 plasmid expressing Ha-Ras<sup>v12</sup> (ERK activator) and treated with or without U0126 (MEK1/2 inhibitor). Phosphorylated ERK and β-actin in the cell lysates were detected by western blot. (F) HepG2 cells were co-transfected with pCMV-CTHRC1 and pBlue-HBV1.3, along with si-c-Jun, si-c-Fos, or siCtrl. (A–F) HBeAg and HBsAg in the culture supernatants were measured by ELISA. Data shown are mean ± SD, *n* = 3 (\**P* < 0.05).

CTHRC1, and this stimulatory effect of CTHRC1 was not affected by JAK/STAT inhibitor, RUXO (Figure 6E). These results demonstrated that CTHRC1 facilitated HBV replication by suppressing antiviral activities of IFN/JAK/STAT pathway. The correlation between the PKCα/ERK/JNK/c-Jun cascade and the IFN/JAK/STAT pathway regulated by CTHRC1 was further evaluated. IFNARα and IFNARβ were upregulated by IFN-α, downregulated by CTHRC1, and reversed by GF109203 and U0126 (Figure 6F). In addition, both phosphorylation and ubiquitination of IFNARα were enhanced by CTHRC1, but repressed by GF109203 and U0126 (Figure 6G).

These results revealed that CTHRC1 activated HBV replication by activating PKCα/ERK/JNK/c-Jun signaling to suppress IFN/JAK/STAT activities.

#### *CTHRC1 facilitates HBV replication by repressing IFN-α antiviral activity in BALB/c mice*

The effect of CTHRC1 on HBV replication was further investigated *in vivo*. BALB/c mice were injected with Ad-siCTHRC1-infected or Ad-siCtrl-infected HepG2.2.15 cells. CTHRC1 mRNA and protein were detected in the tumors of mice carrying Ad-siCtrl, but

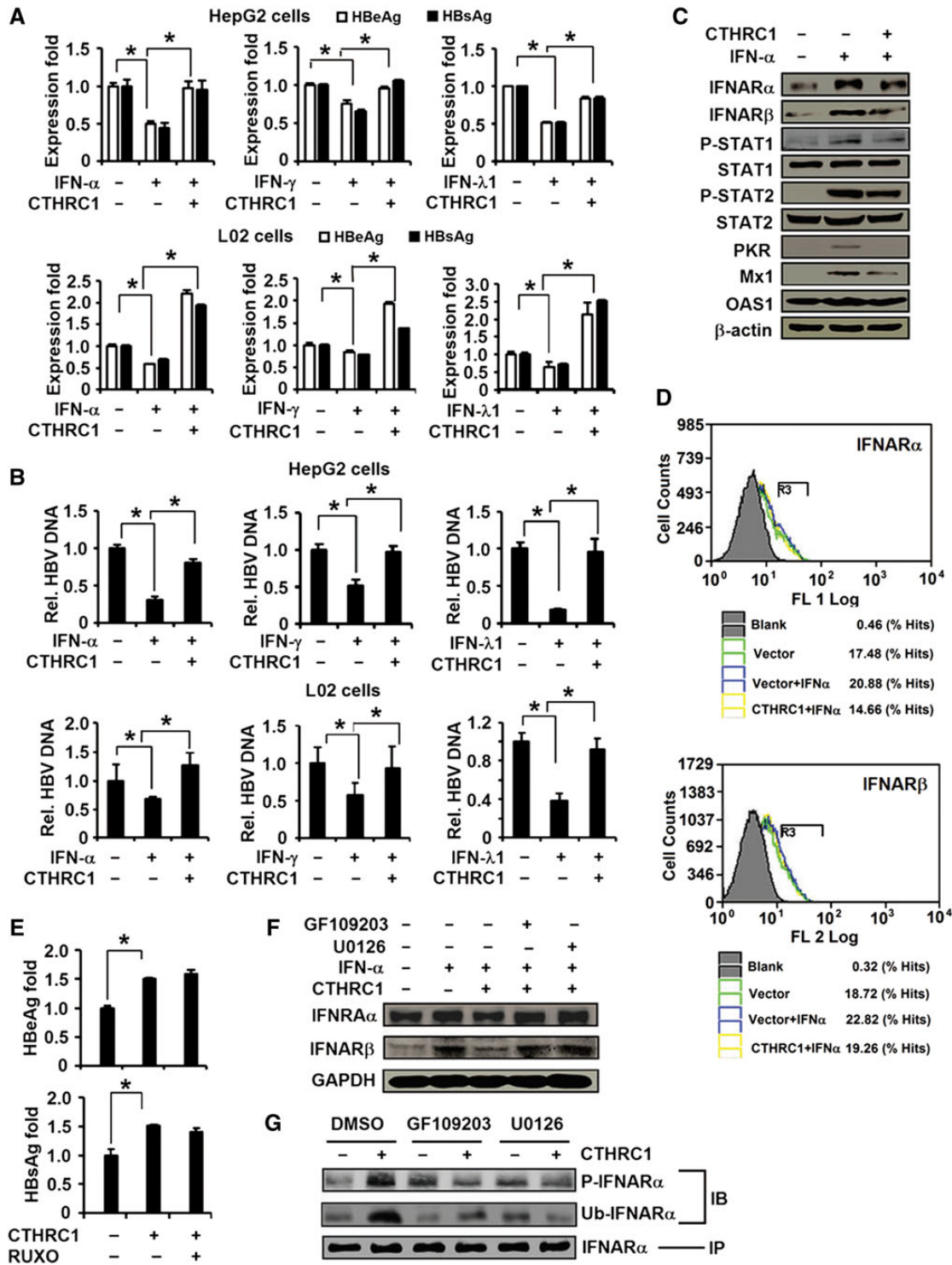


**Figure 5** The PKCα-ERK-JNK-c-Jun cascade is required for CTHRC1 regulated HBV replication. **(A)** HepG2 cells were transfected with pCMV-CTHRC1 or control (left panel) and siCTHRC1 or siCtrl (right panel). Cell lysates were prepared and proteins determined by western blot. **(B)** HepG2 (upper panel) or L02 cells (lower panel) were transfected with pCMV-CTHRC1 at concentrations of 1, 2, and 4 μg. Cell lysates were prepared and proteins determined by western blot using corresponding antibodies. **(C)** HepG2 cells were transfected with pCMV-CTHRC1 and treated with MEK, JNK, or PKC inhibitor (U0126, SP600125, or GF109203). Whole-cell lysates, nuclear extracts, and cytoplasm extracts were prepared, and phosphorylated c-Jun (p-c-Jun) and β-actin proteins were detected by western blot. **(D)** HepG2 cells were transfected with pCMV-CTHRC1 and treated with SP600125 or GF109203. After fixation, the cells were immunostained with anti-p-c-Jun antibody and the nucleus stained by DAPI and visualized by confocal laser microscopy.

repressed in the tumors of mice carrying Ad-siCTHRC1 (Figure 7A), indicating that siCTHRC1 was effective. In correlation with CTHRC1 protein, HBV proteins (HBeAg and HBsAg) were also reduced in the sera of mice injected with Ad-siCTHRC1-infected HepG2.2.15 cells (Figure 7B), suggesting that knock-down of CTHRC1 downregulates HBV protein expression. In addition, HBcAg (an indicator of HBV replication) and CTHRC1 were significantly reduced in the tumors of mice in the presence of siCTHRC1 (Figure 7C), indicating that knock-down of CTHRC1 downregulated HBV replication *in vivo*.

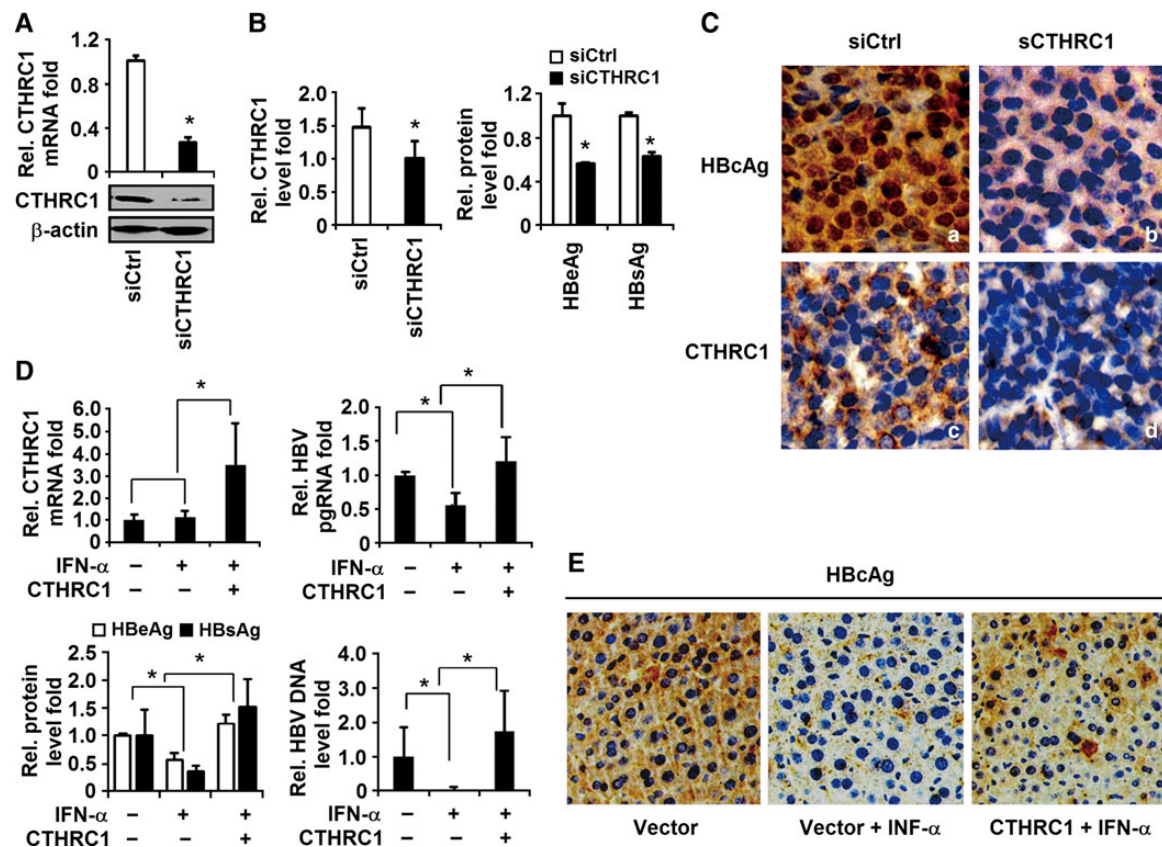
The mechanism by which CTHRC1 regulates HBV replication was also revealed *in vivo*. Recombinant adeno-associated virus (AAV) carrying the HBV genome (AAV-HBV1.3) and pCMV-CTHRC1 were hydrodynamically injected into the tail veins of mice and then treated with or without rhIFN-α. In the mice sera, CTHRC1 mRNA, HBV pre-genomic RNA (pgRNA), HBsAg and HBeAg proteins, and HBV DNA were repressed by rhIFN-α and enhanced by CTHRC1 (Figure 7D), indicating a negative effect of CTHRC1 on anti-HBV activity of IFN-α in mice. In the mice livers, HBcAg was detected and significantly





**Figure 6** CTHRC1 activates the PKC $\alpha$ -ERK-JNK-c-Jun cascade to repress anti-HBV activity of the IFN-JAK-STAT pathway. **(A and B)** HepG2 cells and L02 cells were co-transfected with pCMV-CTHRC1 and pBlue-HBV1.3, and then treated with rhIFN- $\alpha$  (300 IU/ml), rhIFN- $\gamma$  (10 ng/ml), or rhIFN- $\lambda$  (100 ng/ml). HBeAg and HBsAg in culture supernatants were detected by ELISA. HBV core-associated DNAs in cell lysates were determined by RT-PCR. **(C)** HepG2 cells were transfected with pCMV-CTHRC1 and then treated with or without rhIFN- $\alpha$  (300 IU/ml). Proteins in cell lysates were detected by western blot. **(D)** HepG2 cells were transfected with pCMV-CTHRC1 or vector and treated with rhIFN- $\alpha$  (300 IU/ml) for 48 h. Cells were stained with anti-IFNAR $\alpha$  antibody (upper panel) or anti-IFNAR $\beta$  antibody (upper panel), and then analyzed by FACS analysis. **(E)** HepG2 cells were seeded at a density of  $4.0 \times 10^5$  cells per well in six-well plates, grown to  $\sim 80\%$  confluence, treated with JAK inhibitor (RUXO), and then co-transfected with pBlue-HBV1.3 and pCMV-CTHRC1. HBeAg and HBsAg in culture supernatants were detected by ELISA.





**Figure 7** CTHRC1 facilitates HBV replication in BALB/c mice. (A–C) HepG2.2.15 cells were infected with recombinant adenovirus carrying siCTHRC1 (Ad-siCTHRC1) or its control (Ad-siCtrl), and then injected into the subcutaneous tissues of BALB/c nude mice (6 weeks old, six males for each group). Sera and tumors were collected at 3 weeks post-injection. (A) CTHRC1 mRNA was determined by RT–PCR and CTHRC1 protein detected by western blot in the mice tumors (NC,  $n = 6$ ; siCTHRC1,  $n = 6$ ). (B) CTHRC1 (left panel), HBeAg, and HBsAg (right panel) were detected by ELISA in the mice sera (NC,  $n = 6$ ; siCTHRC1,  $n = 6$ ). (C) Mice tumors were immunohistochemically stained with anti-HBeAg antibody (a and b) or anti-CTHRC1 antibody (c and d). Original magnification, 200×. (D and E) Ten micrograms of recombinant adeno-associated virus carrying the HBV genome (pAAV-HBV1.3) and 20 μg of pCMV-CTHRC1 in a volume equivalent to 0.1 ml/g of mouse body weight were hydrodynamically injected into the tail veins of BALB/c mice (6 weeks old, six females for each group) within 5–8 sec. rhIFN-α (1350 U/g of mouse body weight) was then injected into the tail vein of mice every day for 7 days and the mice sacrificed. (D) HBeAg and HBsAg in the mice sera were detected by ELISA. CTHRC1 mRNA, HBV pgRNA, and HBV core-associated DNA in mice liver tissues were determined by RT–PCR. (E) Mice liver tissues were immunohistochemically stained with anti-HBeAg antibody. Original magnification, 200×.

reduced by IFN-α, but such repression was largely recovered by CTHRC1 (Figure 7E), confirming the negative role of CTHRC1 in anti-HBV activity of IFN-α in mice. Thus, CTHRC1 enhanced HBV replication *in vivo* by repressing the antiviral activity of IFN-α.

## Discussion

Chronic HBV infection is one of the major causes of liver disease, but it is not directly cytopathic, and liver injury appears to be caused by repeated attempts of the cellular immune responses to control the infection (Liang, 2009). It is important for HBV to adapt unique replication strategy to ensure survival within the infected cells, while complex virus-host interplay ensures the virus fulfilling

its replication requirements, yet still evading host immunity. Understanding of cellular and viral interactions during HBV replication is necessary for the development of more effective therapeutic strategies to improve the management of HBV-infected patients. Here, we reveal a novel mechanism by which HBV activates a multiple function protein, CTHRC1, which, in turn, facilitates viral replication by regulating two important signaling cascades and several cellular factors.

We initially show that CTHRC1 levels are elevated in HBV-infected patients and highly correlated with the viral load and that HBV activates CTHRC1 in cultured cells. We thus provide the first evidence that HBV activates CTHRC1 *in vivo* and *in vitro*. In revealing the

(F) HepG2 cells were transfected with pCMV-CTHRC1, treated with inhibitors, and added with or without rhIFN-α (300 IU/ml). INNAα, IFNARβ, and GAPDH proteins in the cell lysates were detected by western blot. (G) HepG2 cells were transfected with pCMV-CTHRC1 and then treated with GF109203, U0126, or DMSO. Cells were lysed and then subjected to immunoprecipitation (IP) using anti-IFNAR1 antibody, followed by immunoblotting (IB) with anti-p-IFNARα antibody or anti-ubiquitin antibody, respectively.

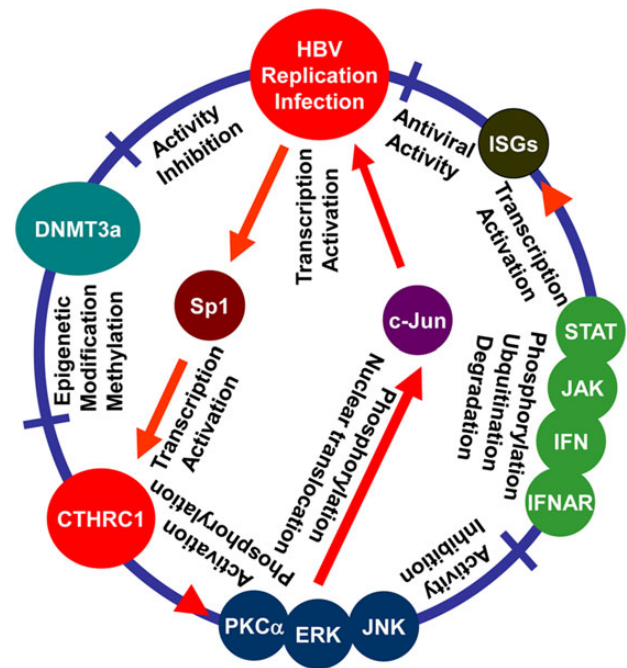
mechanism involved in such regulation, we demonstrate that HBV represses DNMT3a activity, reduces CTHRC1 DNA methylation, and allows Sp1 binding to the CTHRC1 promoter to activate gene expression. Epigenetic modification has been found to be associated with HBV-induced HCC (Su et al., 2007). We previously reported that viral infection activates cellular gene expression through demethylation-associated recruitment of transcription factors to their promoters (Yue et al., 2011; Fang et al., 2012). Thus, we suggest that aberrant epigenetic modification and transcriptional regulation play important roles in regulating gene expression during viral infection.

Since CTHRC1 expression is highly correlated with HBV infection, we speculate that CTHRC1 may functionally associate with viral replication. CTHRC1 has circulating hormone characteristics with broad implications for cellular metabolism and physiology (Stohn et al., 2012). Here, we demonstrate for the first time that CTHRC1 facilitates HBV replication in cultured cells and in nude mice, providing strong evidence for the new role of CTHRC1 in viral replication both *in vitro* and *in vivo*. The mechanism by which CTHRC1 enhances HBV replication is also elucidated: CTHRC1 activates HBV replication by stimulating PKC $\alpha$ /ERK/JNK/c-Jun cascades. PKC, ERK, and JNK have been implicated in relaying extracellular signals to the nucleus and in mediating proliferation, differentiation, apoptosis and stress by regulating transcription factors (Hill and Treisman, 1995). Our results provide insights into a new role of the PKC $\alpha$ /ERK/JNK/c-Jun cascade in regulating HBV replication.

Moreover, we also determine the downstream events of the PKC $\alpha$ /ERK/JNK/c-Jun pathway mediated by CTHRC1. It is well-known that viral replication is inhibited by IFNs that establish multifaceted antiviral responses (Schoggins et al., 2011). Central to the antiviral response is the production of an array of ISGs to limit virus infection (Sadler and Williams, 2008). Here, we demonstrate that HBV replication is downregulated by IFN- $\alpha$  and upregulated by CTHRC1 in cultured cells and nude mice, suggesting that CTHRC1 facilitates HBV replication by attenuating IFN antiviral activity *in vivo* and *in vitro*.

We further elucidate the mechanism involved in such regulation. First, IFNAR $\alpha/\beta$  and ISGs are activated by IFN- $\alpha$  and repressed by CTHRC1, confirming that CTHRC1 suppresses IFN antiviral activity. Second, STAT1/2 phosphorylation is upregulated by IFN- $\alpha$  and downregulated by CTHRC1, suggesting that CTHRC1 suppresses the JAK/STAT cascade. Third, IFNAR $\alpha$  is activated by IFN- $\alpha$  and repressed by CTHRC1, but reversed by inhibitors of PKC and MEK, demonstrating that CTHRC1 downregulates IFN/STAT/JAK activity through upregulating PKC/ERK. Fourth, IFNAR $\alpha$  phosphorylation and ubiquitination are enhanced by CTHRC1 but reduced by inhibitors of PKC and MEK, revealing that PKC and ERK are required for the phosphorylation and ubiquitination of IFNAR $\alpha$  mediated by CTHRC1. It is reported that IFNAR $\alpha$  is degraded after phosphorylation mediated by the Ras/Raf/MEK/ERK pathway (Zhang et al., 2012) and that IFNAR $\alpha$  degradation is initiated by co-internalization with IFNAR $\beta$  and subjected to ubiquitination (Kumar et al., 2003). Therefore, we believe that CTHRC1 facilitates HBV replication through degradation of IFNAR $\alpha/\beta$ .

In conclusion, HBV activates CTHRC1 expression both *in vivo* and *in vitro* by epigenetic modification and transcriptional regulation.



**Figure 8** A proposed mechanism by which HBV hijacks cellular factors to evade host antiviral immunity. During HBV infection, the virus activates CTHRC1 expression both *in vivo* and *in vitro* by epigenetic modification and transcriptional regulation. HBV initially represses DNMT3a activity, resulting in the demethylation of CTHRC1 promoter DNA and the enhancement of the binding of Sp1 to the CTHRC1 promoter to activate gene expression. HBV-activated CTHRC1, in turn, facilitates viral replication through activation of the PKC $\alpha$ /ERK/JNK cascade by enhancing the phosphorylation and activity of signaling components. Interestingly, this cascade activates AP-1 by stimulating the phosphorylation and nuclear translocation of its subunit c-Jun, enhancing the binding of c-Jun to the HBV promoter to facilitate viral replication. Importantly, the PKC $\alpha$ /ERK/JNK cascade attenuates the antiviral activity of the IFN/JAK/STAT pathway by repressing the activity of JAK and the phosphorylation of STAT1/2, enhancing the phosphorylation, ubiquitination, and degradation of type I IFN receptors (IFNAR $\alpha/\beta$ ), and inhibiting the expression of ISGs, including PKR, Mx1, and OAS1.

More important, HBV-activated CTHRC1 facilitates the viral replication by stimulating the PKC $\alpha$ /ERK/JNK cascade through activating phosphorylation and activity of the signaling components. This cascade then activates AP-1 by stimulating phosphorylation and nuclear translocation of its subunit c-Jun and by enhancing the binding of c-Jun to HBV promoter to facilitate viral replication. In addition, the PKC $\alpha$ /ERK/JNK cascade attenuates the antiviral activity of IFN/JAK/STAT signaling through repressing JAK activity and STAT1/2 phosphorylation, enhancing IFNAR $\alpha/\beta$  phosphorylation, ubiquitination, and degradation, and inhibiting ISG expression, which results in the evasion of host antiviral immunity and facilitation of HBV replication (Figure 8). Thus, we reveal a novel circular promotion mechanism by which HBV hijacks cellular factors and signaling cascades to evade host antiviral immunity. In addition, we discover a new role for CTHRC1 in HBV replication and persistent infection.

## Materials and methods

### Clinical samples

Peripheral blood samples were obtained from 153 patients (94 males and 59 females, mean age 39 years) with CHB admitted to RenMin Hospital of Wuhan University, China (Supplementary Table S1). All patients were confirmed to be HBV positive (but were negative for HCV, HDV, and HIV-1), were not suffering from any concomitant illness, and did not show any serological markers suggestive of autoimmune disease. Peripheral blood samples were also obtained from 164 healthy individuals (98 males and 66 females, mean age 40 years) with no history of liver disease who were randomly selected as controls from the local blood donation center (Supplementary Table S1). Twelve hepatoma tissues were obtained from HCC patients infected with HBV admitted to RenMin Hospital of Wuhan University, China and 13 noncancerous tissues were obtained from healthy individuals who died from traffic accidents (Supplementary Table S2). Blood samples for detection of gradient HBV DNA load were obtained from 142 patients with CHB (Supplementary Table S3). Informed consent in writing was obtained from each patient. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval by the review committee of Wuhan University.

### Cells and cultures

HepG2 and HepG2.2.15 cells were cultured in Dulbecco's modified Eagle's medium (Gibco BRL) and LO2 cells were cultured in RPMI-1640 medium (Gibco BRL) supplemented with 10% fetal calf serum (Gibco BRL), 100 U/ml penicillin, and 100 µg/ml streptomycin sulfate. The cells were maintained at 37°C in a 5% CO<sub>2</sub> incubator.

### Plasmids, small interfering RNAs, antibodies, and inhibitors

The CTHRC1 expressing plasmid was constructed as follows. Its coding region was amplified from cDNA isolated from HepG2 cells by using the primers CTHRC1-F (5'-AATGATATCATGCGACCCAGG GCCCGCC-3') and CTHRC1-R (5'-AATCTCGAGTTATTTGGTAGTTCT TCAAT-3'), and then inserted into the *EcoRV* and *XhoI* sites of the vector pCMV-Tag2B. To generate pBlue-HBV1.3, we amplified the DNA fragment carrying the HBV genome (ayw subtype) from HepG2.2.15 cells and inserted it into pBluescript II (Invitrogen). pCTHRC1-Luc was constructed by inserting the DNA fragment of CTHRC1 promoter into the *MluI* and *HindIII* sites of pGL3-basic (Invitrogen) by using the sense primer 5'-ACTACGCGTGAGATCG AGACCATCTGG-3' and the anti-sense primer 5'-TATAAGCTTGGCTC CCGGCTGCCGGCA-3'. The V12 encoding activated Ha-Ras, prepared by a former colleague in our laboratory, was sub-cloned into the *BamHI* and *EcoRI* sites of the vector pCMV-Tag2A to generate pHa-Ras.

Plasmids expressing siRNAs against PKC family members were constructed by ligating corresponding pairs of oligonucleotides to pSilencer2.1-U6 neo (Ambion, Inc.). The siRNAs against c-Jun, c-Fos, CTHRC1, JNK, and siRNA-Ctrl were synthesized and purchased from the RiboBio Company. The sequences of these siRNAs were as follows: siCTHRC1 sense: 5'-CGUUGGUUUU CACAUUCAdTdT-3' and anti-sense: 5'-UGAAUCUCAAUACCAAC GTdTdT-3'; siJNK sense: 5'-GCCAGUAAUAGUAGAAdTdT-3' and anti-sense: 3'-dTdTCCGGUCAUUUAUACAUCAU-5'; siC-Jun sense:

5'-GGCACAGCUUAAACAGAAAdTdT-3' and anti-sense: 3'-dTdTCCG UGUCGAAUUUGUCUUU-5'; siC-Fos sense: 5'-CTTCATCCCCACGGT CACTdTdT-3' and anti-sense: 3'-dTdTGAAGUAAGGGUGCCAGUGA-5'.

ERK1 and ERK2 mutants (mERK1 and mERK2) were gifts from Dr Cobb of the University of Texas Southwestern Medical Center, and JNK mutant (mJNK) was a gift from Dr Karin of the University of California at San Diego. The human DNMT1 full-length cDNA clone (pcDNA 3.1-DNMT1) was a gift from Dr Moshe Szyf of the McGill University. Human DNMT3a and DNMT3b full-length cDNA clones (pcDNA3-DNMT 3a, pcDNA3-DNMT3b) were gifts from Dr Arthur D. Riggs of Beckman Research Institute of the City of Hope. siRNAs, including siDNMT1, siDNMT3a, and siDNMT3b, were designed and inserted into the pSilencer2.1-U6 (Ambion). Sequences for the sense oligonucleotides for the most effective knockdown constructs were as follows: siDNMT1: 5'-GATCCAAT GGCAGATGCCAACAGCTTCAAGAGAGCTGTTGGCATCTGCCATTCCTTT TTTGGAAA-3'; siDNMT3a: 5'-GATCCACTACATCAGCAAGCGCAATTC AAGAGATTGCGCTTGCTGATGTAGTAGTTTTTGGAAA-3'; siDNMT3b: 5'-GATCCATGACGGATGCCTAGAGTTCTCAAGAAACTCTAGGCATCC GTCATCTTTTTTTGGAAA-3'.

Antibodies against phosphorylated STAT1 (p-STAT1) (sc-7988), ERK (sc-153), OAS (sc-98424), PKR (sc-100378), STAT2 (sc-476), IFNAR1 (sc-7391), IFNAR2 (sc-30014), p-IFNAR1 (sc-135700), p-c-Jun (sc-16311X), SP1 (sc-59X), JNK (sc-7345), and CTHRC1 (sc-98747) were purchased from Santa Cruz technology company (Santa Cruz). Antibodies against p-ERK (#4370s), p-STAT2 (#4441S), STAT1 (#9172), p-JNK (#9255), p-PKCα/β II (#9375), PKCα (#2056), and p-c-Jun (#3270) were purchased from Cell Signaling Technology (Beverly); anti-β-actin antibody and anti-GAPDH antibody were purchased from CWBio (Beijing CoWin Biotech); and anti-Mx1(13750-1-AP) antibody and anti-LaminB1 (660951-1-Ig) antibody were purchased from Proteintech Group.

The inhibitors GF109203, PD98059, SP600125, SB203580, LY294002, BAY11-7039, and U0126 were purchased from Tocris Bioscience (Bristol), and ruxolitinib (Ruxo) was purchased from Axon Medchem (Groningen). 5-Aza-2'-deoxycytidine was purchased from Sigma. All of the inhibitors were dissolved in dimethylsulfoxide (Sigma).

### Bisulfite modification of DNA and bisulfite genomic sequencing, cell transfection and luciferase activity assay, western blot analysis, and northern blot analysis

Details are described in Supplementary materials and methods.

### RNA extraction and real-time PCR

Total RNA was extracted from cells by using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Total RNA extract was treated with DNase I (Promega) at 37°C for 30 min, and 1 µg of total RNA was used as a template for reverse transcription by M-MLV Reverse Transcriptase (Promega) with random primers at 37°C for 60 min. Real-time PCR was performed in a Light Cycler 480 thermal cycler (Roche) under the following conditions: heat activation of the polymerase at 95°C for 5 min, followed by 40 cycles of 95°C for 15 sec, 55°C for 15 sec, and 72°C for 20 sec. Fluorescence was then measured, with a final melting curve step from 50°C to 95°C to examine the quality of the detection primers. Primers for CTHRC1

detection were forward 5'-ACAAAGATGCGTTCAAATAG-3' and reverse 5'-CCACAGAAGAAGTGCGATG-3'. Primers for internal reference GAPDH detection were forward 5'-AAGGCTGTGGGCAAGG-3' and reverse 5'-TGGAGGAGTGGGTGTGCG-3'. Real-time PCR was carried out with Bestar real-time PCR master mix (DBI Bioscience).

#### *Nuclear extraction*

After being serum-cultured for 24 h, cells were washed twice with cold phosphate-buffered saline (PBS), harvested, and incubated in two volumes of buffer A (10 mM HEPES, pH8.0, 0.5% Nonidet P-40, 1.5 mM MgCl<sub>2</sub>, 10 mM KCl, 0.5 mM dithiothreitol [DTT], and 200 mM sucrose) for 5 min at 4°C with tube flipping. The crude nuclei were collected by centrifugation for 30 sec; pellets were rinsed with buffer A, resuspended in one volume of buffer B (20 mM HEPES, pH7.9, 1.5 mM MgCl<sub>2</sub>, 420 mM NaCl, 0.2 mM ethylenediaminetetraacetic acid [EDTA], and 1.0 mM DTT), and incubated on a shaking platform for 30 min at 4°C. Nuclei preparations were centrifuged for 5 min and supernatants collected. Cocktail protease inhibitor tablets were added to each type of buffer. Nuclear extracts were stored at -70°C until use.

#### *Chromatin immunoprecipitation assay*

Chromatin immunoprecipitation (ChIP) assays were performed according to the X-ChIP Protocol (Abcam). Briefly, formaldehyde was added to the culture medium to a final concentration of 1%. The cells were then washed twice with PBS, scraped and lysed in lysis buffer (1% SDS, 10 mM Tris-HCl, pH8.0, 1 mM PMSF, 50 mg/ml of both aprotinin and leupeptin) for 10 min on ice. Lysates were sonicated on ice and debris were removed by centrifugation at 15000 *g* for 15 min at 4°C. One-fourth of the supernatant was used as DNA input control. The remaining supernatant was diluted 10-fold with buffer (0.01% SDS, 1% Triton X-100, 1 mM EDTA, 10 mM Tris-HCl, pH8.0, and 150 mM NaCl) and incubated with antibody against p-c-Jun overnight at 4°C. Immunoprecipitated complexes were collected using protein A/G agarose beads. The pellets were washed with dialysis buffer (2 mM EDTA, 50 mM Tris-HCl, pH8.0) and incubated at 67°C for 5 h to reverse the formaldehyde cross-link. DNA was precipitated with ethanol and extracted three times with phenol/chloroform. Finally, pellets were resuspended in TE buffer and subjected to PCR amplification using primers as follows: AP-1 sense: 5'-ACCTTACCCCGTTGCCCGCAACGGC-3' and AP-1 anti-sense: 5'-GAGGAGCCGAAAAGGTTCCACGCACGC-3'; SP1 sense: 5'-CCCAGCTGCTCTGGAGGCTGAGGCAGG-3' and SP1 anti-sense: 5'-TTGTTTGTGTTTGA GACAGAGTCTCGC-3'.

#### *Immunoprecipitation*

Cells were washed with PBS at 48 h after transfection, collected by centrifugation at 3000 *g*, resuspended with 500 µl pre-cooled lysis buffer for each sample, and subjected to ultrasonication. After centrifugation, the supernatants were collected and transferred to 1.5 ml microcentrifuge tubes. Twenty microliters of protein A/G agarose (Santa Cruz) and 10 µl IFNAR antibodies were added to each tube, and the tubes were gently vortexed at 4°C for 3 h. The agarose was collected by centrifugation at 3000 *g*, washed three

times with lysis buffer, resuspended with 20 µl 2× SDS sample buffer, and boiled for 5 min. The supernatant was collected after centrifugation at 10000 *g* and subjected to electrophoresis and western blotting. p-IFNAR was probed with the appropriate antibody, and IFNAR1 was re-probed in a second-round western blot following washing of the membrane with stripping buffer.

#### *Immunofluorescence*

HepG2 cells grown on sterile cover slips were transfected with pCMV-CTHRC1 or control vector at 40% confluence for 48 h. Cells were fixed with 4% paraformaldehyde for 15 min, washed three times with PBS, permeabilized with PBS containing 0.5% Triton X-100 for 5 min, washed three times with PBS, and blocked with PBS containing 4% bovine serum albumin for 1 h at room temperature. The cells were then incubated with the primary antibody p-c-Jun overnight at 4°C, followed by incubation with fluorescein isothiocyanate-conjugated goat anti-rabbit secondary antibodies (Proteintech Group) for 1 h. Nuclei were stained with DAPI (Roche), and the cells were visualized by confocal laser microscopy (Fluoview FV1000; Olympus).

#### *HBV protein assay and capsid-associated DNA analysis*

At 48 h post-transfection, levels of HBsAg and HBeAg proteins in cell culture media were determined by ELISA using HBV HBsAg antigen and HBeAg antigen diagnostic kits (Shanghai KeHua Biotech), respectively.

HepG2.2.15 cells were homogenized in 1 ml lysis buffer (50 mM Tris-HCl, pH7.5, 0.5% Nonidet P-40, 1 mM EDTA, and 100 mM NaCl) and mixed gently at 4°C for 1 h, and then 10 ml MgCl<sub>2</sub> (1 M) and 10 ml DNase (10 mg/ml) were added and incubated for 2 h at 37°C. Viral cores were precipitated by adding 35 ml EDTA (0.5 M) and 225 ml 35% polyethylene glycol and incubated at 4°C for at least 30 min. The cores were concentrated by centrifugation, and the pellets were resuspended in 10 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA, 1% SDS, and 20 ml proteinase K (25 mg/ml) and incubated overnight. HBV capsid-associated DNA released from lysed cores was extracted with phenol and chloroform, precipitated with isopropanol, and resuspended in Tris-EDTA. HBV capsid-associated DNAs were quantified by real-time PCR according to the procedures provided by the manufacturer (PG Biotech). Primers used in real-time PCR were as follows: P1: 5'-ATCTCG-CTGCTATGCCTCATCTT-3', P2: 5'-ACAGTGGG GAAAGCCC-TACGAA-3', and probe: 5'-TGGCTAGTTTACTAGTGCCATT TG-3'. PCR was performed and analyzed using a Roche LC480.

#### *Animal studies*

HepG2.2.15 cells were infected with siCTHRC1-recombinant adenovirus (Ad-siCTHRC1) or its control (siCtrl-recombinant adenovirus, Ad-siCtrl), and then injected into the subcutaneous tissues of BALB/c nude mice (6 weeks old, six males for each group). Sera and tumors were collected at 3 weeks post-treatment. CTHRC1 mRNA was determined by real-time PCR and CTHRC1 protein was detected by western blot in the lysates of mice tumors. CTHRC1, HBeAg, and HBsAg proteins were detected by ELISA in the sera of mice. Immunohistochemical staining of mice tumor tissues was done by the Wuhan Biotechnology Company. For hydrodynamic injection experiments: 10 µg of recombinant adeno-associated virus



integrated with the HBV genome (pAAV-HBV1.3) and 20 µg of pCMV-CTHRC1 in a 0.9% NaCl solution equivalent to 0.1 ml/g of mice body weight were hydrodynamically injected into the tail veins of BALB/c mice (6 weeks old, six females for each group) within 5–8 sec using the method described previously (Yang et al., 2002). Recombinant human IFN-α (rhIFN-α) (1350 U/g of mouse body weight) was injected into the tail vein of treated mice every day for 7 days, and then the mice were sacrificed. HBcAg and HBsAg proteins in the mice sera were detected by ELISA, and CTHRC1 mRNA, HBV pgRNA and HBV core-associated DNA in the mice liver tissues were determined by RT-PCR. HBcAg protein was immunohistochemically stained with anti-HBcAg antibody. All animals received human care according to the criteria outlined in the 'Guide for the Care and Use of Laboratory Animals' prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH publication 86-23 revised 1985) and the Guidelines of Wuhan University.

### Statistics

All experiments were reproducible, and each set was repeated at least three times with similar results. Parallel samples were analyzed for normal distribution by the Kolmogorov–Smirnov test. Abnormal values were eliminated according to a follow-up Grubbs' test. Levene's test for equality of variances was performed, which provided information for the Student's *t*-test to distinguish the equality of means. Means were illustrated using a histogram with error bars representing  $\pm$  SD, and  $P < 0.1$  was considered statistically significant.

### Supplementary material

Supplementary material is available at *Journal of Molecular Cell Biology* online.

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**Conflicts of interest:** none declared.

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