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A functional variant in *UBE2L3* contributes to HBV infection and maintains cccDNA stability by inducing degradation of APOBEC3A protein

Li Zhou[1,2#], Ji-Hua Ren[1#], Sheng-Tao Cheng[1#], Hong-Mei Xu[3], Wei-Xian Chen[4], Da-Peng Chen[5], Vincent Kam Wai Wong[6], Betty Yuen Kwan Law[6], Yi Liu[1], Xue-Fei Cai[1], Hua Tang[1], Hai-Bo Yu[1], Jie-Li Hu[1], Yuan Hu[1], Hong-Zhong Zhou[1], Fang Ren[1], Lin He[1], Zhong-Wen Hu[1], Hui Jiang[1], Hong-Yan Xu[1], Ai-Long Huang[1*], Juan Chen[1*]

[1]The Key Laboratory of Molecular Biology of Infectious Diseases designated by the Chinese Ministry of Education, Institute for Viral Hepatitis, Department of Infectious Diseases, The Second Affiliated Hospital, Chongqing Medical University, Chongqing, China; [2] Department of Epidemiology, School of Public Health and Management, Chongqing Medical University, Chongqing, China; [3]Department of Infectious Diseases, The Children's Hospital of Chongqing Medical University, Chongqing, China; [4]Department of Clinical Laboratory, The Second Affiliated Hospital of Chongqing Medical University, Chongqing, China; [5]Department of Clinical Laboratory, The Children's Hospital of Chongqing Medical

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University, Chongqing, China;[6]State Key Laboratory of Quality Research in Chinese Medicine, Macau University of Science and Technology, Macau, China;

[#]These authors contributed equally to this work

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Email:

Li Zhou, zhouli_tj@163.com

Ji-Hua Ren, renjihua2016@cqmu.edu.cn

Sheng-Tao Cheng, 18908320046@163.com

Hong-Mei Xu, xuhongm0095@sina.com

Wei-Xian Chen, chenweixian75@163.com

Da-Peng Chen, 476538577@qq.com

Vincent Kam Wai Wong, bowaiwong@gmail.com

Betty Yuen Kwan Law, yklaw@must.edu.mo

Yi Liu, liuyicq@gmail.com

Xue-Fei Cai, xfcaiii@163.com

Hua Tang, tanghua86162003@cqmu.edu.cn

Hai-Bo Yu, yubobo1993@163.com

Jie-Li Hu, hujieli1977@163.com

Yuan Hu, tottyhy@163.com

Hong-Zhong Zhou, zhouhongzhong888@163.com

Fang Ren, 18380456067@163.com

Lin He, 18380456080@163.com

Zhong-Wen Hu, zhong940528@163.com

Hui Jiang, kareena1995@qq.com

Hong-Yan Xu, 837490711@qq.com

Ai-Long Huang, ahuang1964@163.com

Juan Chen, chenjuan2014@cqmu.edu.cn

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Corresponding Authors

Contact Information:

Juan Chen, Prof., Ph.D, Room 617, College of Life Sciences Building, 1 YiXueYuan Road, YuZhong District, Chongqing, 400016, China, Telephone Number: +86-23-68486780, Fax: +86-23-68486780, Email: chenjuan2014@cqmu.edu.cn.

Ai-Long Huang, Prof., MS, Room 617, College of Life Sciences Building, 1 YiXueYuan Road, YuZhong District, Chongqing, 400016, China, Telephone Number: +86-23-68486780, Fax: +86-23-68486780, Email: ahuang1964@163.com.

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List of Abbreviations: HBV, hepatitis B virus; GWAS, genome-wide association study; UBE2L3, ubiquitin conjugating enzyme E2 L3; CHB, chronic hepatitis B; ALT, alanine aminotransferase; AST, aspartate aminotransferase; cccDNA, covalently closed circular DNA; NTCP, Na+/taurocholate cotransporting polypeptide; PHH, primary human hepatocytes; APOBEC3A, apolipoprotein B mRNA editing enzyme catalytic subunit 3A; IFN-α, interferon-α; SNP, single nucleotide polymorphism; HBsAg, Hepatitis B surface antigen; HLA, human leukocyte antigen; TCF19, transcription factor 19; EHMT2,

euchromatic histone-lysine-methyltransferase 2; UPS, ubiquitin-proteasome system; E1s, ubiquitin-activating enzymes; E2s, ubiquitin-conjugating enzymes; E3s, ubiquitin-protein ligases; 53BP1, tumor protein p53 binding protein 1; NF-κB, nuclear factor kappa B; SLE, systemic lupus erythematosus; PIs, persistently HBV infected subjects; SRs, spontaneously recovered subjects; NF-E2, nuclear factor, erythroid 2; NRF2, nuclear factor, erythroid 2 like 2; ELK, ETS transcription factor; HLF, hepatic leukemia factor; PBMCs, peripheral blood mononuclear cells; SLE, systemic lupus erythematosus; LUBAC, linear ubiquitin chain assembly complex; HIV-1, human immunodeficiency virus 1; HPV, human papillomavirus; HBeAg, Hepatitis B e Antigen.

Abstract

Hepatitis B virus (HBV) infection is a common infectious disease, in which nuclear cccDNA plays a key role in viral persistence, viral reactivation after treatment withdrawal and drug resistance. Recent genome-wide association study (GWAS) has identified *UBE2L3* gene is associated with increased susceptibility to chronic HBV infection (CHB) in adult. However, the association between UBE2L3 and children with CHB and the underlying mechanism remains unclear. In this study, we performed two stages case-control studies including adults and independent children in Chinese Han population. The rs59391722 in promoter of *UBE2L3* gene was significantly associated with HBV infection in both adults and children groups and the rs59391722 G allele increased the promoter activity of *UBE2L3*. Serum UBE2L3 protein levels were positively correlated with HBV viral load and HBeAg levels in children with CHB. In HBV infection cell model, *UBE2L3* knockdown significantly reduced total HBV RNAs, 3.5-kb RNA as well as covalently closed circular DNA (cccDNA) in HBV-infected HepG2-NTCP and human primary hepatocytes (PHH). Mechanistic study found UBE2L3 maintained cccDNA stability by inducing proteasome-dependent degradation

of APOBEC3A, which is responsible for the degradation of HBV cccDNA. Moreover, IFN-α treatment markedly decreased UBE2L3 expression while *UBE2L3* silencing reinforces the antiviral activity of IFN-α on HBV RNAs, cccDNA and DNA. rs59391722 in *UBE2L3* was correlated with HBV DNA suppression and HBeAg loss in response to IFN-α treatment of children with CHB. **Conclusion** These findings highlight a novel host gene *UBE2L3* contributing to the susceptibility to persistent HBV infection. *UBE2L3* may involve to IFN-mediated viral suppression and serve as potential target in the prevention and treatment of HBV infection.

Introduction

Hepatitis B virus (HBV) infection is a worldwide health problem that can lead to fibrosis, cirrhosis and hepatocellular carcinoma (1, 2). The recent studies estimated that the global prevalence of HBV surface antigen (HBsAg) in 2016 was 3.9% and 291 million individuals with HBV infection (3). Most chronic hepatitis B patients acquired their infections around the time of birth or during early childhood because the risk of chronic infection is 90% when infection occurs in infancy (4-6). Mother-to-child transmission is the main transmission routes in the recent decade. Early childhood transmission can be prevented with a highly effective infant vaccine. In China, three birth-dose vaccination before age 1 year has achieved 99% efficacy (3). However, China has the greatest number of HBsAg-positive patients. The recent study estimated that more than 80 million adults and 37,000 children were chronic carriers in China (3). There is an urgent health care need to understand and control chronic HBV infection.

HBV infection is generally attributed to immunological factors, viral factors, environmental factors, host genetic factors, and the interactions between them. Recently, several genome-wide association studies (GWASs) have identified single nucleotide polymorphisms (SNPs) at eight loci are associated with chronic HBV infection in Asian population (7-14). Most of the reported loci are located in human leukocyte antigen (HLA) regions, such as *HLA-DP*, *HLA-DQ*, *HLA-C*, *TCF19* and *EHMT2*. Only one locus, *UBE2L3*,

is in a non-HLA region. Hu et al. has firstly reported the rs4821116 in the intron of *UBE2L3* as a new locus for susceptibility to HBV infection in Chinese population (11). The A allele of rs4821116 associated with higher expression of *UBE2L3* which suggesting the potential function of *UBE2L3* in HBV infection. However, some studies have not replicated this association between the *UBE2L3* and HBV infection. GWASs conducted in another two case control studies in China have not found association between rs4821116 at *UBE2L3* and chronic HBV infection (7, 12). Additionally, data in children are scarce because most studies only included adults.

The ubiquitin-proteasome system (UPS) ensures regulation of the protein pool in eukaryotic cells by ubiquitination and proteasome-mediated degradation. It plays an important role in a variety of basic cellular processes as well as many viral infection (15, 16). Ubiquitination involves at least three classes of enzymes: ubiquitin-activating enzymes (E1s), ubiquitin-conjugating enzymes (E2s) and ubiquitin-protein ligases (E3s)(17). UBE2L3 functions as an E2 ubiquitin-conjugating enzyme and has been demonstrated to participate in ubiquitination of several proteins, including p53(18), 53BP1(19), c-fos and NF-κB precursor p105(20) in vitro. UBE2L3 has also been reported to be associated with several autoimmune disorders, such as Crohn's disease (21), rheumatoid arthritis (22) and systemic lupus erythematosus (SLE) (23). However, the molecular function of UBE2L3 in HBV infection has not been elucidated.

To identify whether the host gene *UBE2L3* conferred susceptibility to chronic HBV infection, we conduct a population-based study to examine the genetic determinants of HBV infection in *UBE2L3* in adults and independent children, coupled with experimental studies to characterize the function of *UBE2L3* in HBV replication. Mechanistic study found that high UBE2L3 maintained HBV cccDNA stability by inducing degradation of APOBEC3A protein. Therefore, our work established UBE2L3 as potential target in the prevention and treatment of HBV infection.

Materials and methods

Study populations

We performed two independent case control studies. The first stage consisted of 519 cases and 531 sex- and age- frequency matched controls derived from Chongqing City (in Southwest China). The subjects in the first stage are adults, as defined the age>18 years. Subjects who had been positive for both HBV surface antigen (HBsAg) and hepatitis B core antibody (HBcAb, anti-HBc) for at least 6 months were defined as persistently HBV infected subjects (PIs, cases). Those who were negative for HBsAg and positive for both anti-HBs and anti-HBc were defined as spontaneously recovered subjects (SRs, controls). The second stage, which was used to confirm the results obtained from the first stage, consisted of 274 cases and 353 controls. The study design has been described elsewhere (24). Briefly, the subjects in the second stage were children aged from 6 months to 12 years. Of these children, cases were defined as both HBsAg and anti-HBc positive; controls were defined as HBsAg negative and hepatitis B surface antibody (HBsAb, anti-HBs) positive.

The diagnosis of chronic hepatitis B infection (CHB) was based on HBsAg-seropositivity for more than six months and HBV DNA detectable according to the guideline for diagnosis and treatment of chronic hepatitis. The diagnosis of inactive carriers was HBsAg-seropositivity for more than six months, HBeAg negative, HBV DNA less than 2000 IU/mL and normal ALT levels. The subjects were recruited from the Second Affiliated Hospital and the Children's Hospital of Chongqing Medical University in Chongqing between January 2014 and December 2017. All participants or their parents provided written informed consent. This research project was approved by the ethical committees at Chongqing Medical University.

Tagging SNP selection and genotyping

We explored the LD structure around the SNP rs4821116 at *UBE2L3,* as reported by GWAS (11), using the HapMap Project database (phase Ⅱ+Ⅲ Feb 09, on NCBI B36

assembly, db SNP126). A total of 17 SNPs in *UBE2L3* were genotyped in our study. These SNPs cover a 70-kb genomic region on chromosome 22 (National Centre for Biotechnology Information build GRCH37 from 21560000 to 21630000), continuously from the 5' flanking to 3' flanking genomic region (including all exons, relevant exon-intron bound aries, and ~2 kilobase promoter region).

Blood samples were collected from each participant in ethylenediamine tetraacetic tubes, and the genomic DNA was extracted using a Tiangen DP319-02 kit (Tiangen Company, Beijing, China). Genotyping was performed according to the Mass Array time-of-flight mass spectrometer (Sequenom Company, USA). Polymerase chain reaction and extension primers were designed using Mass ARRAY Assay Design 3.1 software (Sequenom Company, USA). The genotyping procedures were performed according to the manufacturer's iPLEX Application Guide (Sequenom Company, USA). The PCR amplification conditions included an initial precycle incubation of 94°C for 10min, followed by 45 cycles of denaturation at 94°C for 20s, annealing at 56°C for 30s, and extension at 72°C for 60s. All the genotyping reactions were performed in 384-well plates. Each plate included four randomly selected duplicates and six negative controls using double distilled water. The average concordance rate for the genotypes was 99.5%.

Cell culture

HepAD38 and HepG2-NTCP cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 400μg/ml G418. Primary human hepatocytes (PHH) were cultured in hepatocyte medium (Sciencell, USA). Huh-7 cells were cultured in DMEM supplemented with 10% FBS, 100 IU/ml penicillin, and 100 μg/ml streptomycin. All cells were maintained at 37°C in humidified CO2 (5%) incubators. Cells were authenticated by short tandem repeat (STR) fingerprinting by Beijing Microread Genetics Company Limited recently.

Statistical analysis

The associations between the proportion of subjects with HBV infection and genotypes of the SNPs were estimated by computing the odds ratios (ORs) and 95% confidence intervals (CIs) from the multivariate logistic regression with adjustments for age and sex. The comparison of mean between two groups was conducted by using Student-*t* test while the comparison for more than two groups was conducted using one-way ANOVA. Pearson's test was used to evaluate the correlation coefficiency (*r*) of the serum UBE2L3 and clinical biomarkers (HBV viral load, HBeAg, ALT and AST levels). If measured values did not meet the assumptions of normality and homogeneity of variances, log-transformation was used before *t*-tests were performed. Log-transformation of HBV viral load, HBeAg, ALT and AST levels was used before Pearson's test. Correlation between the two proteins was determined using Spearman correlation test. For cell model data, results are expressed as mean±SD from three independent experiments. Statistics were performed with the non-parametric Mann-Whitney U test. A value of $p<0.05$ was considered significant (* $p<0.05$; ** $p<0.01$). All of the data analyses were carried out using the statistical analysis software package SPSS 19.0 (SPSS Inc., Chicago, IL, USA).

Results

rs59391722 is associated with HBV infection and risk allele increases *UBE2L3* expression

We performed fine mapping of a 70-kb region of *UBE2L3* by genotyping 17 SNPs in a total of 519 persistently HBV infected subjects (HBV carriers; cases) and 531 spontaneously recovered subjects (naturally cleared HBV infection, controls) in adults, followed by validation of top candidate SNPs in 274 HBV infected children and 353 children who were never infected HBV (Supplementary Table 1). The genotype frequencies of all SNPs were in Hardy-Weinberg equilibrium ($p>0.05$) in controls. We identified that rs59391722 located at -1128 bp of the promoter region of *UBE2L3* was the most strongly SNP associated to susceptibility of HBV infection. Compared with the rs59391722 GG genotype, the CC

genotype had a lower HBV infection risk, and the adjusted per-allele OR for HBV infection was 0.82 (95%CI: 0.68-0.95, *p*=0.011) (Fig.1A and Supplementary Table 2). We next replicated the association in additional case control study of independent children. Genotypic association analysis also demonstrated that rs59391722 was significantly associated with HBV infection in children, after adjusting for age and sex (*p*=0.004, OR=0.59, 95%CI: 0.41-0.84) (Supplementary Table 2). All SNPs in *UBE2L3* were in high LD with rs59391722 (D'=0.87-0.94, r^2=0.39-0.82).

The association of rs59391722 genotype with UBE2L3 mRNA and protein levels was further analyzed. Our results showed significantly increased expression of *UBE2L3* mRNA levels in GG subjects compared with CC subjects in 60 healthy individuals *(p*=0.009) (Fig.1B). Similar results were obtained from data in the Genotype-Tissue Expression Project (GTEx) database, showing significantly higher UBE2L3 expression of GG subjects than CC subjects in whole blood (Supplementary Fig.1). Consistently, a strong linear relationship was noted between genotypes of rs59391722 and UBE2L3 protein level in serum and liver tissues in normal controls (Fig.1C-D). Because rs59391722 is located within the *UBE2L3* promoter region, we examined whether this SNP could affect the promoter activity of *UBE2L3* by luciferase reporter assay. Construct pGL3-(-1128G) showed higher luciferase activity compared with constructs pGL3-(-1128C) in liver cancer cells (Fig.1E). To further investigate whether the C>G transition at rs59391722 changes the binding pattern of transcription factors, we sequenced the rs59391722 in several human liver cancer cell lines and identified Huh-7 cell line carrying the rs59391722 CC genotype and HepG2 carrying the rs59391722 GG genotype. The bioinformatics analysis predicted the affinity of NF-E2 was increased while NRF2, ELK, HLF binding sites were abolished after rs59391722 C>G transition. ChIP assay confirmed that the binding of NF-E2 to the promoter of *UBE2L3* was increased in HepG2 cells compared with Huh-7 cells (Fig.1F). However, no significant difference of the binding of NRF2, ELK, HLF to rs59391722 contained region was observed between Huh-7 and HepG2 cells (Fig.1F).

***UBE2L3* correlates with HBV infection in adults and children**

To further validate the role of *UBE2L3* in HBV infection, the expression of *UBE2L3* was examined in PBMCs or serum from persistently infected HBV (PIs) adults and spontaneously recovered from HBV infection (SRs) adults. Our results showed that the *UBE2L3* mRNA levels were significantly higher in PIs group than those in SRs group ($p<0.0001$) (Supplementary Fig.2A). Consistently, increased UBE2L3 protein levels were observed in the serum of PIs ($p<0.0001$) (Supplementary Fig.2B). Furthermore, we found significantly positive correlation between the serum UBE2L3 protein levels and HBV viral load and HBsAg levels in the adults PIs with positive HBeAg (Pearson correlation coefficient $r=0.39$, $p=6\times10^{-4}$ and $r=0.36$, $p=9\times10^{-4}$, respectively) (Supplementary Fig.2C-D). Importantly, the *UBE2L3* mRNA levels in liver biopsy were higher in CHB patients compared with inactive carriers ($p=0.013$, Supplementary Fig.2E). A significantly positive correlation between *UBE2L3* mRNA level and cccDNA in liver biopsy was observed in CHB patients (Pearson correlation coefficient $r=0.53$, $p=0.04$, Supplementary Fig.2F).

Most patients with chronic HBV infection acquired their infections around the time of birth or during early childhood, and HBV infected children progress more rapidly than adults. We further investigated the relationship between *UBE2L3* and chronic HBV infection in the children. The children with CHB had significantly higher *UBE2L3* mRNA levels in PBMCs and protein levels in the serum compared to the healthy children (Fig.2A-B). In children with CHB, we also observed that serum UBE2L3 protein levels were positively correlated with the HBV viral load (Pearson correlation coefficient $r=0.58$, $p=0.01$, Fig.2C) and HBeAg levels (Pearson correlation coefficient $r=0.48$, $p=0.03$, Fig.2D). Interestingly, both of the two main types of serum transaminases ALT and AST levels had strongly positive correlation with serum UBE2L3 protein children with CHB (Pearson correlation coefficient $r=0.56$, $p=0.01$ and $r=0.54$, $p=0.01$, respectively, Fig. 2E-F). However, we observed there was no correlation between the serum transaminases and UBE2L3 protein in CHB adults.

UBE2L3 facilitates HBV replication and transcription

To examine whether UBE2L3 plays a role in HBV replication, HepG2-NTCP cells or PHH were infected with normalized amount of HBV before transduction with lentivirus expressing shRNA targeting UBE2L3 (shUBE2L3-1 and shUBE2L3-2) (Fig.3A). Gene silencing of *UBE2L3* resulted in decreased HBV core DNA level as evidenced by real-time PCR and southern blotting analysis (Fig.3B). Importantly, *UBE2L3* knockdown inhibited both HBV total RNAs and 3.5-kb RNA levels by using real-time PCR (Fig.3C-D). Northern blotting analysis indicated the decreased levels of HBV 3.5-kb, 2.4-kb and 2.1-kb RNA in UBE2L3-depleted HepG2-NTCP cells (Fig.3E). No cytotoxic effects were observed both in HepG2-NTCP cells and PHHs 5 days after lentiviral transduction (Supplementary Fig.3A-B). In order to determine whether UBE2L3 modulated HBV RNA stability or rather acts at the transcription level, HepG2-NTCP cells were treated with RNA polymerase II inhibitor actinomycin D to block RNA synthesis. The decay kinetics of HBV RNAs was measured in the presence or absence of *UBE2L3* knockdown. As shown in Fig.3F, *UBE2L3* knockdown did not significantly change the velocity of total HBV RNAs and 3.5-kb RNA degradation, suggesting *UBE2L3* silencing negatively modulates viral RNA synthesis.

The role of UBE2L3 in HBV replication was confirmed in HepG2-NTCP cells or PHH infected with lentivirus expressing wild type UBE2L3 or a catalytically dead (C86S) mutant ($UBE2L3^{C86S}$) (Supplementary Fig.4A). Ectopic expression of UBE2L3 led to increased HBV core DNA level as evidenced by real-time PCR and southern blotting analysis, whereas $UBE2L3^{C86S}$ had no effect (Supplementary Fig.4B-C). Importantly, UBE2L3 overexpression promoted total HBV RNAs and 3.5-kb RNA levels both in HepG2-NTCP cells and PHHs (Supplementary Fig.4D-E). Northern blotting analysis confirmed increased HBV 3.5-kb, 2.4-kb and 2.1-kb RNA levels in HepG2-NTCP cells overexpressing wild type UBE2L3 but not C86S mutant (Supplementary Fig.4F). Collectively, these findings indicate that endogenous UBE2L3 promotes HBV transcription and replication.

UBE2L3 reduces APOBEC3A protein to maintain cccDNA stability

Since cccDNA is the template for the transcription of all HBV RNAs, we next investigate the role of UBE2L3 on cccDNA level. HBV cccDNA in Hirt extracts of HBV-infected cells was measured by both real-time PCR and southern blotting analysis. *UBE2L3* overexpression enhanced cccDNA level (Fig. 4A-B), whereas *UBE2L3* knockdown resulted in a significant reduction of cccDNA both in HepG2-NTCP cells and PHH (Fig. 4C-D). The effect of UBE2L3 was also examined in minicircle HBV cccDNA with a Gaussia Luciferase reporter (mcHBV-GLuc cccDNA), which providing a very sensitive and quick method to trace amount of cccDNA (25). As illustrated in Fig. 4E, when the mcHBV-GLuc cccDNA was transfected into HepG2 or Huh-7 cells, *UBE2L3* knockdown inhibited GLuc activity. Furthermore, 3D-PCR showed that *UBE2L3* knockdown in HBV-infected HepG2-NTCP cells resulted in a lower temperature of cccDNA denaturation compared with control cells. The sequencing data further revealed that markedly increase of G to A mutations was accumulated in UBE2L3-depleted cells, indicating deamination of cytidines to uridine in the HBV cccDNA (Fig. 4F). After cytidine deamination, DNA glycosylases recognize and remove damaged bases from DNA by cleaving the base-sugar (N -glycosylic) bond to create an apurinic/apyrimidinic site (AP site) (26). To investigate whether HBV cccDNA contains AP sites in UBE2L3-depleted cells, we quantified cccDNA after digestion of Hirt-extracted DNA with an AP endonuclease (APE1) which can cleave the phosphodiester backbone within an AP site. APE1 treatment further decreased cccDNA level in UBE2L3 knock-down cells, however APE1 had no effect on cccDNA levle in control cells (Supplementary Fig.5). These data suggest UBE2L3 is involved in the cytidine deamination and AP sites formation in the HBV cccDNA, which may lead to its degradation.

The APOBEC3 protein family is comprised of seven DNA cytidine deaminases (APOBEC3A, B, C, D, F, G and H), and has been reported to mediate the cytidine deamination, apurinic/apyrimidinic site formation, and finally cccDNA degradation(26-28). These finding prompted us to hypothesize that APOBEC family members might be involved in the cccDNA modulation mediated by UBE2L3. To test this hypothesis, we first examined expression of seven APOBEC3 members in UBE2L3-overexpressing cells. Ectopic

expression of UBE2L3 decreased exogenous and endogenous expression of APOBEC3A, whereas it had no obvious effect on other members (Fig. 5A and Supplementary Fig.6). Consequently, UBE2L3 overexpression led to decreased recruitment of APOBEC3A to cccDNA (Fig. 5B). The protein levels of UBE2L3 and APOBEC3A were examined in liver biopsies from five CHB patients and five inactive carriers (Fig. 5C). Interestingly, correlative analysis indicated that UBE2L3 protein showed a significant negative correlation with APOBEC3A expression (Pearson correlation coefficient r=0.75, *p*=0.01) (Fig. 5C). Next, we confirmed whether APOBEC3A is involved in UBE2L3-mediated cccDNA modulation. UBE2L3 overexpression resulted in increased level of HBV 3.5-kb, 2.4-kb and 2.1-kb RNA levels as well as cccDNA level (Fig. 5D-F). However, this effect of UBE2L3 could be blocked by introduction of APOBEC3A in HBV-infected HepG2-NTCP cells (Fig.5D-F). Moreover, 3D-PCR assays showed that APOBEC3A could induce the deamination of HBV cccDNA. However, UBE2L3 overexpression markedly antagonized the effect of APOBEC3A on cccDNA (Supplementary Fig.7). Collectively, these data suggest that UBE2L3 regulates cccDNA stability through promoting APOBEC3A protein degradation.

UBE2L3 promotes ubiquitination and proteasome-dependent degradation of APOBEC3A

To explore whether ubiquitin-proteasome machinery is involved in regulation of APOBEC3A by UBE2L3, HBV-infected HepG2-NTCP cells ectopically expressing UBE2L3 and APOBEC3A were then treated with cycloheximide to block *de novo* protein synthesis. The half-life of APOBEC3A protein was significantly decreased in UBE2L3-overexpressing cells relative to control cells (Fig. 6A). UBE2L3-mediated degradation of APOBEC3A was blocked in the presence of proteasome inhibitor MG132, suggesting UBE2L3 decreased APOBEC3A protein via proteasome-mediated degradation (Fig. 6B). Moreover, the coimmunoprecipitation assays found that ectopic expression of UBE2L3 was coprecipitated with APOBEC3A (Fig. 6C). To assess if UBE2L3 binding to APOBEC3A account for its ubiquitination-mediated proteasomal degradation, we measured ubiquitinated-APOBEC3A in

cells overexpressing UBE2L3. Overexpression of UBE2L3 led to increased ubiquitination of APOBEC3A while MG132 treatment significantly induced higher levels of ubiquitination (Fig. 6D). To identify the potential sites of ubiquitylation, the on-line tool PhosphoSite Plus was used and predicted the lysine 137 in APOBEC3A might be ubiquitylated. The mutation of Lys 137 to arginine blocked the UBE2L3-mediated ubiquitylation and degradation of APOBEC3A (Fig. 6E-F), suggesting that Lys 137 of APOBEC3A is a key ubiquitylation site targeted by UBE2L3.

Expression of UBE2L3 correlates with response to interferon treatment

It has been reported that the expression of APOBEC3A positively correlates with interferon-α (IFN-α) responses in CHB patients(26, 29). To test whether UBE2L3-mediated APOBEC3A modulation was involved in response to interferon treatment, the expression of UBE2L3 and APOBEC3A were examined in HBV-infected cells in response to IFN-α. As shown in Fig.7A, IFN-α treatment markedly decreased UBE2L3 and increased APOBEC3A expression in both HBV-infected HepG2-NTCP cells and PHH. Importantly, *UBE2L3* silencing reinforces the antiviral activity of IFN-α on HBV RNAs, cccDNA and core DNA in HBV-infected PHH (Fig. 7B-E). In contrast, *UBE2L3* overexpression antagonized the antiviral effect of IFN-α (Supplementary Fig. 8A-D).

The correlation between UBE2L3 expression and IFN-α response was further examined in clinical sample of children with CHB. Consistently, serum UBE2L3 level was markedly decreased in children with CHB after IFN treatment (Fig. 7F). Interestingly, the children with rs59391722 GG genotype had a limited HBV DNA suppression and HBeAg loss in response to IFN-α treatment than those with rs59391722 CC or CG genotype (Fig. 7F). The percentage of HBeAg seroconversion and HBsAg clearance in the patients with rs59391722 CC/CG genotype was higher than rs59391722 GG genotype (16.7% *vs.* 0%) in CHB children. However, no significant differences were detected between the two groups of rs59391722 genotypes due to the small sample size. Together, these data from both cell model and clinical setting of patients suggest that higher expression of *UBE2L3* indicate a

worse response to IFN-α treatment. UBE2L3 rs59391722 is a predictor for the response to IFN-α treatment for CHB patients.

Discussion

It is well known that perinatal transmission is the most important mode of HBV infection in endemic areas, including China. The risk of HBV chronic infection is 90% when infection occurs in infancy, but <5% when infection occurs in adults(30). When HBV infection is acquired perinatally during the first few years of life, children usually become HBeAg-positive with high levels of viral replication. Spontaneous HBeAg seroconversion rates vary by age and are <2% per year in children younger than 3 years. Although universal immunization against HBV, mother-to-child transmission still accounts for the majority of cases of CHB (31). Recently, eight GWASs identified several variants linking genetic susceptibility to persistent HBV infection in Asia population(7-9, 11-14, 32) These studies identified that most of SNPs associated with the HBV infection were located in the HLA region and few of them was located in non-HLA loci, such as UBE2L3. However, no GWASs focused on the association between HBV infection and children patients.

In the present study, we demonstrated that allele rs59391722 GG in *UBE2L3* promoter was not only strongly associated with persistence of HBV infection in Chinese adults, but also significantly associated with the CHB susceptibility in the children. The luciferase reporter assay found that C>G change at rs59391722 enhanced *UBE2L3* promoter activity. The children with CHB have significantly higher UBE2L3 mRNA level in PBMCs and protein level in the serum compared to the healthy children. Unexpectedly, although the number of clinical samples in our study is small due to limited availability of clinical sample in children with CHB, *UBE2L3* was positively correlated with HBV viral load, HBeAg levels, ALT and AST levels. To the best of our knowledge, this is the first study of genetic variants associated with HBV infection in both adults and children. The *UBE2L3* was identified as the susceptibility gene for persistence of HBV infection in children with CHB with small sample size. This phenomenon might be due to children with HBV infection

having fewer background co-factors, such as drug use, co-infections and other disease, which may made effects of host factors on HBV infection more clearly identified.

SNP rs59391722 is located in the promoter region of *UBE2L3* gene, which encodes E2 ubiquitin-conjugating enzyme, also known as UbcH7. *UBE2L3* is associated with increased susceptibility to numerous autoimmune diseases, including Crohn's disease (21), rheumatoid arthritis (22), systemic lupus erythematosus(SLE)(23, 33) and Hashimoto's thyroiditis(34), in genome-wide association studies and other genetic studies. Functional study further found that *UBE2L3* is essential for LUBAC-mediated activation of NF-κB $CD19^+$ cells and $CD14^+$ monocytes (35). However, the specific role of *UBE2L3* in persistent HBV infection has never been reported. Consistent with its high expression in CHB patients, functional study found that wild type UBE2L3 overexpression, not a catalytically dead (C86S) mutant, promoted HBV transcription and replication in both HBV-infected HepG2-NTCP cell line and PHH. Both real-time PCR and Northern blot revealed that UBE2L3 overexpression facilitated HBV transcripts. 7-aminoactinomycin D treatment further revealed that UBE2L3 directly enhanced HBV RNAs transcription from cccDNA, rather than modulating HBV RNAs stability. HBV establishes a stable nuclear cccDNA which serves as the template for transcription of all viral mRNAs and secures HBV persistence. Importantly, the cccDNA level was elevated in UBE2L3-overexpression cells. In contrast, *UBE2L3* silencing inhibited HBV RNAs transcription from cccDNA. 3D-PCR further revealed that *UBE2L3* knockdown led a lower temperature of cccDNA. Taken together, these findings link the E2 ubiquitin-conjugating enzymatic activity of UBE2L3 with cccDNA degradation.

The apolipoprotein B mRNA-editing catalytic polypeptide 3 (APOBEC3) protein family comprising of seven DNA cytidine deaminases (APOBEC3A, B, C, D, F, G and H) plays an important role in the innate immune response to viral infections by editing viral genomes (26, 36-38). A broad range of viruses has been reported to be restricted by APOBEC3A and 3B, including human immunodeficiency virus 1 (HIV-1)(39-41), parvovirus(42-44), herpesvirus (45, 46), human papillomavirus (HPV)(47, 48) and hepatitis B virus (HBV)(26, 49). During HBV infection, APOBEC3A or 3B activity is essential to induce deamination and AP-site formation in HBV cccDNA leading to its degradation (26). Consistently, we identified

APOBEC3A, but not APOBEC3B, as the downstream target of UBE2L3 by screening the seven APOBEC3 members. APOBEC3A could increase cccDNA deamination in PHH. Moreover, UBE2L3 overexpression reduced APOBEC3A protein level via ubiquitin-dependent proteasomal degradation. Importantly, ectopically expression of APOBEC3A reverted UBE2L3-induced enhancement of cccDNA level and RNA transcription. Our data suggest high UBE2L3 promotes cccDNA stability by inducing APOBEC3A proteasomal degradation in hepatocyte.

Recently, it has been reported that induction of APOBEC3A or 3B by IFN-α or T-cell cytokines is essential for cccDNA degradation(26). APOBEC3A and APOBEC3B mRNA levels are up-regulated in liver biopsies from CHB patients treated with IFN-α compared to a treatment-naïve group (50). As an E2 ubiquitin-conjugating enzyme for APOBEC3A protein degradation, we therefore investigated whether UBE2L3 was involved to interferon-mediated viral suppression. In HBV infection cell models, IFN-α treatment resulted in decreased UBE2L3 level and increased APOBEC3A level. Moreover, UBE2L3 overexpression antagonized the anti-viral effect of IFN-α, which suggesting UBE2L3/APOBEC3A axis plays important role in IFN-treatment response. Importantly, the correlation between UBE2L3 and IFN-α response was further confirmed in children with CHB. The mRNA level of *UBE2L3* was markedly decreased children with CHB after IFN treatment. The children with rs59391722 GG exhibited limited HBV DNA suppression and HBeAg loss in response to IFN-α treatment than those with rs59391722 CC or CG genotype. In this scenario, it suggested that *UBE2L3* plays an important role in the response to IFN-α. However, large sample of children with CHB under IFN-α treatment are needed for further analysis. The liver biopsies of children with CHB would be ideal for analyzing the correlation between UBE2L3, APOBEC3A and cccDNA under IFN-α treatment.

In summary, this study indicates that the high *UBE2L3* expression not only contribute to the viral persistence both in adults and children, but also influence the response to IFN-α treatment. We further identify high UBE2L3 is important for HBV cccDNA stability by inducing degradation of APOBEC3A. It may shed light on the prevention and clinical implications of *UBE2L3* in control of HBV infection.

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Figure Legends

Fig.1 Functional variant rs59391722 in *UBE2L3* gene is significantly associated with chronic HBV infection and gene expression. (A) Regional plots of SNPs around *UBE2L3* in HBV infection case control study in adults. (B) rs59391722 GG genotype was associated with increased *UBE2L3* mRNA expression in PBMC of 60 healthy individuals. *UBE2L3* mRNA levels were determined by quantitative RT-PCR. (C) rs59391722 GG genotype was associated with increased UBE2L3 protein levels in serum of healthy individuals. The serum UBE2L3 levels were measured by enzyme-linked immunosorbent assays (ELISA). (D) rs59391722 GG genotype was associated with increased UBE2L3 protein levels in normal liver tissues. The UBE2L3 protein level in liver was determined by western blotting analysis and β–actin was used as a loading control. (E) Luciferase reporter assay with constructs containing the rs59391722 G or rs59391722 C allele in *UBE2L3* promoter in Huh-7 and HepG2 cells. (F) ChIP assay for several transcription factors associated with *UBE2L3* promoter, including NF-E2, ELK, HLF and NRF2 in Huh-7 cell line carrying the rs59391722 CC genotype and HepG2 cells carrying the rs59391722 GG genotype. Western blotting analysis were performed to confirm that transcription factors (NF-E2, ELK, HLF, NRF2) were immunoprecipitated in the ChIP experiments.

Fig2. UBE2L3 correlates with HBV infection in children. (A) The children with CHB had significantly higher levels of *UBE2L3* mRNA expression in PBMCs. (B) The children with CHB had significantly higher levels of UBE2L3 protein in serum. The serum UBE2L3 levels were measured by enzyme-linked immunosorbent assays (ELISA). (C-D) Correlation between the serum UBE2L3 levels and HBV viral load and HBeAg levels in children with CHB. (E-F) Correlation between the serum UBE2L3 levels and transaminases ALT and AST levels in children with CHB. The HBV viral load, HBeAg levels, ALT and AST levels were log10 transformed. The correlation coefficiency (*r*) and the two-tailed *P* values were evaluated by Pearson's test.

Fig.3 Gene silencing of *UBE2L3* inhibits HBV transcription. HepG2-NTCP cells or PHH were infected with 2×10^3 genome equivalents/cells of HBV for 24 h and then transduced with lentivirus expressing shCont or shRNA targeting UBE2L3 (shUBE2L3-1 or shUBE2L3-2). (A) Western blotting analysis for UBE2L3 in HBV-infected HepG2-NTCP and PHH 4 days after lentiviral transduction. (B) *UBE2L3* knockdown inhibited HBV core DNA in HBV-infected PHH and HepG2-NTCP cells. Intracellular viral core DNA were subjected to real-time PCR and Southern blotting analysis. For southern blot analysis, the position of intracellular HBV relax circle (RC), double strand DNA (DS) and single strand DNA (SS) were indicated. The level of β-actin in the lysates (the st1 step of core DNA extraction) was used as controls for cell numbers. (C-D) *UBE2L3* knockdown inhibited HBV RNAs in HBV-infected PHH and HepG2-NTCP cells. HBV RNAs were extracted and analyzed by real-time PCR. (E) The effect of *UBE2L3* silencing on HBV RNAs in HepG2-NTCP cells was analyzed by Northern blotting analysis. Each panel was loaded with equally total RNA and probed with a length of 1000bp (HBV genome 126-1225bp), plus strand specific HBV probe. Ribosomal RNAs (28s and 18s) were used as loading control. (F) *UBE2L3* silencing did not affect HBV RNA stability. HBV-infected HepG2-NTCP cells were transduced with indicated lentivirus and incubated with 7-aminoactinomycin D (5μg/ml). Total HBV RNAs and 3.5-kb RNA were quantified by real-time PCR at indicated times and the amount of RNA at time zero was set at 100%. For B-D, quantitative data are presented as mean±SD from three independent experiments. Three biological replicates were performed for every independent experiment.

Fig.4 UBE2L3 affects cccDNA level in HepG2-NTCP and PHH. (A-B) Overexpression of wild type *UBE2L3* increased cccDNA level. Hirt-extracted DNA from HepG2-NTCP and PHH overexpressing *UBE2L3* or vector was digested with T5 exonuclease, followed by real-time PCR analysis using cccDNA selective primers (A) and southern blotting analysis (B). Mitochondrial gene Cox1 was hybridized as the loading control for cccDNA. (C-D) *UBE2L3* knockdown reduced cccDNA in HBV-infected HepG2-NTCP cells and PHH. (E) *UBE2L3* knockdown reduced cccDNA in the mcHBV-GLuc cccDNA model.

UBE2L3-depleted HepG2 or Huh-7 cells was transfected with mcHBV-GLuc cccDNA. The Relative GLuc levels were calculated by Renilla Luciferase Assay System after 3 days post-transfection. (F) *UBE2L3* knockdown induced cccDNA degradation. The cccDNA stability was analyzed by 3D-PCR using denaturation temperature gradient of 88 °C-82 °C followed by agarose gel electrophoresis. For analyzing the mutation matrices of cccDNA in each group, cccDNA fragments amplified at 94 °C or 87.4°C in each group were cloned into T vectors, 10 clones were randomly selected and sequenced. For A, C and E, quantitative data are presented as mean±SD from three independent experiments. Three biological replicates were performed for every independent experiment.

Fig.5 UBE2L3 reduces APOBEC3A protein to maintain cccDNA stability. (A) UBE2L3 reduced APOBEC3A protein. The expression of APOBEC3A was analyzed in HBV-infected HepG2-NTCP cotransfected plasmid expressing *UBE2L3* or *UBE2L3* (C86S) with Flag-APOBEC3A. (B) *UBE2L3* decreased the recruitment of APOBEC3A to cccDNA. Cross-linked chromatin from HBV-infected HepG2-NTCP cells cotransfected indicated plasmids was immunoprecipitated with anti-Flag antibody or relevant control IgG followed by PCR with HBV cccDNA selective primers. ChIP results are expressed as % of input. Western blotting analysis were performed to confirm that APOBEC3A were immunoprecipitated in the ChIP experiment. (C) Correlative analysis for the protein levels of UBE2L3 and APOBEC3A in liver biopsies from five CHB patients and five inactive carriers. (D) Reintroduction of APOBEC3A antagonized the effect of UBE2L3 on cccDNA. HBV-infected HepG2-NTCP cells were cotransfected plasmid expressing *UBE2L3* with Flag-APOBEC3A. Hirt-extracted DNA was digested with T5 exonuclease, followed by real-time PCR analysis using cccDNA selective primers and southern blotting analysis. Mitochondrial gene Cox1 was hybridized as the loading control for cccDNA. (E) Reintroduction of APOBEC3A antagonized the effect of UBE2L3 on HBV RNAs. Total HBV RNAs and 3.5-kb RNA were extracted and analyzed by real-time PCR (Left panel) and Northern blotting analysis (Right panel). For B, D and E, quantitative data are presented as

mean±SD from three independent experiments. Three biological replicates were performed for every independent experiment.

Fig.6 UBE2L3 promotes ubiquitination and proteasome-dependent degradation of APOBEC3A. (A) UBE2L3 decreased the half-life of APOBEC3A. HBV-infected HepG2-NTCP cells transfected indicated plasmids were incubated with 10μg/ml cycloheximide, and then harvested at indicated times. APOBEC3A protein level was quantified using Image J, and band intensities was normalized to β-actin (band intensity at t_0 was defined as 100%). (B) Proteasome inhibitor MG132 (10μM; 8 h) prevented the degradation of APOBEC3A induced by UBE2L3 in HBV-infected HepG2-NTCP cells. (C) UBE2L3 interacts with ectopically expressed APOBEC3A protein. The co-immunoprecipitation assay was performed in HBV-infected HepG2-NTCP cells co-transfected with UBE2L3 and APOBEC3A constructs. (D) UBE2L3 increased APOBEC3A ubiquitination. HA–Ub and Flag-APOBEC3A were co-transfected into HBV-infected HepG2-NTCP cells overexpressing UBE2L3; Cells were then treated with MG132 (10μM; 8h) prior to harvest to prevent proteasomal degradation. Cell lysates were subject to immunoprecipitation with anti-Flag antibody and then were immunoblotted with anti-ubiquitin antibody. (E) Mutation of Lys 137 to arginine blocked the UBE2L3-induced degradation of APOBEC3A. Immunoblotting analysis for APOBEC3A in HBV-infected HepG2-NTCP cells transfected with indicated plasmids. (F) APOBEC3A mutation (K137R) blocked UBE2L3-induced ubiquitination in HBV-infected HepG2-NTCP cells.

Fig.7 Expression of *UBE2L3* correlates with response to interferon treatment. HBV-infected PHHs were transduced with lentivirus expressing shCont or shRNA targeting *UBE2L3* and then were treated with IFN-α (1000 U/ml)) for 9 days. (A) IFN-α treatment decreased UBE2L3 expression. Western blotting was performed using anti-UBE2L3 or anti-APOBEC3A antibody. β-actin was used as a loading control. Intensities of bands were normalized to the amount of β-actin. (B-E) *UBE2L3* silencing reinforces the antiviral activity

of IFN-α. HBV DNA was analyzed by real-time PCR and southern blotting analysis; HBV RNAs were analyzed by real-time PCR; cccDNA was analyzed by real-time PCR. (F) Comparison of UBE2L3 protein in children with CHB before and after IFN-α treatment (Left panel). The *UBE2L3* risk allele (rs59391722 genotype) was associated with HBV DNA suppression and HBeAg loss in response to IFN-α treatment (Right panel). For A-E, quantitative data are presented as mean±SD from three independent experiments. Three biological replicates were performed for every independent experiment.

Figure 1

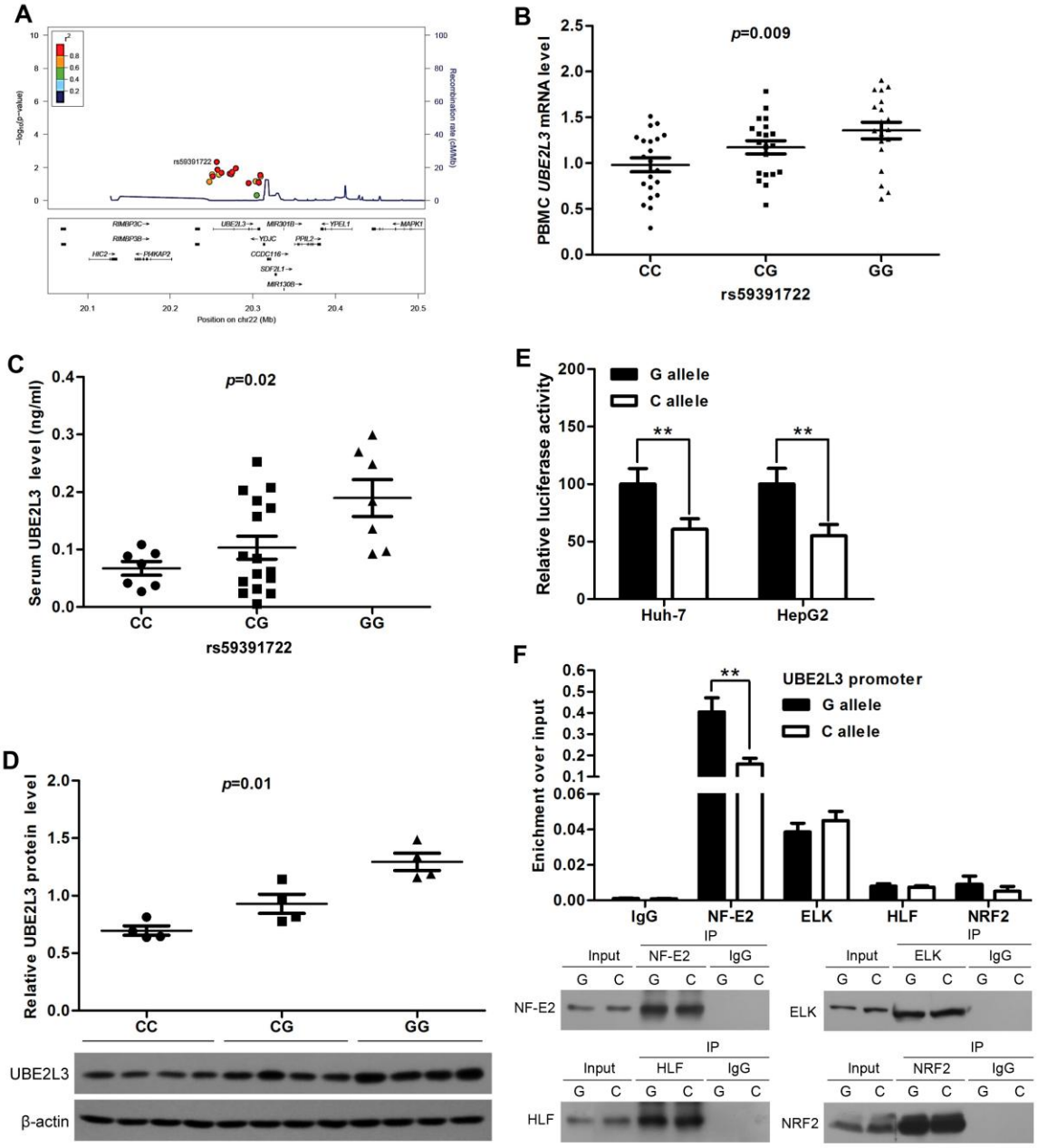

Figure 2

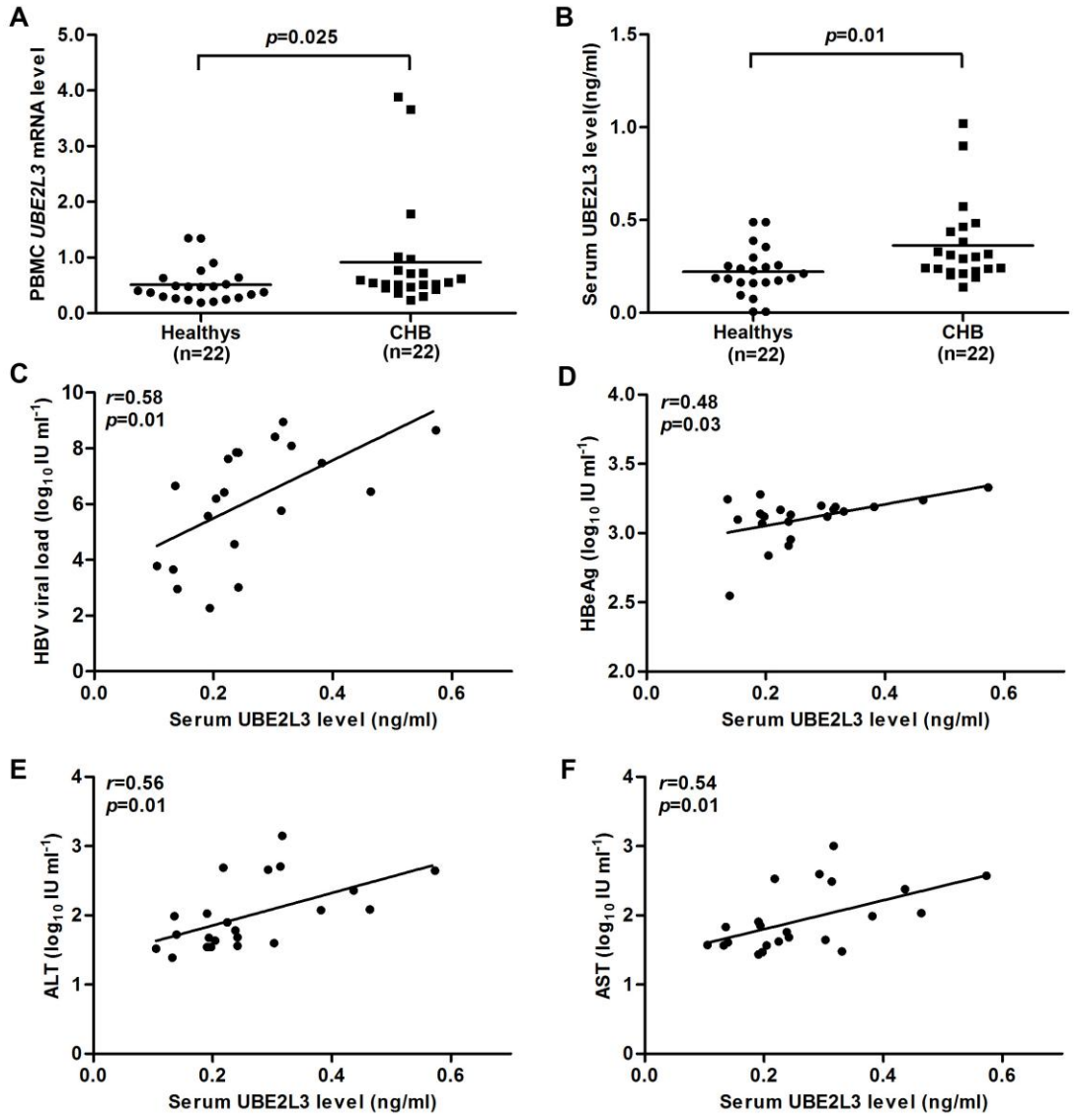

Figure 3

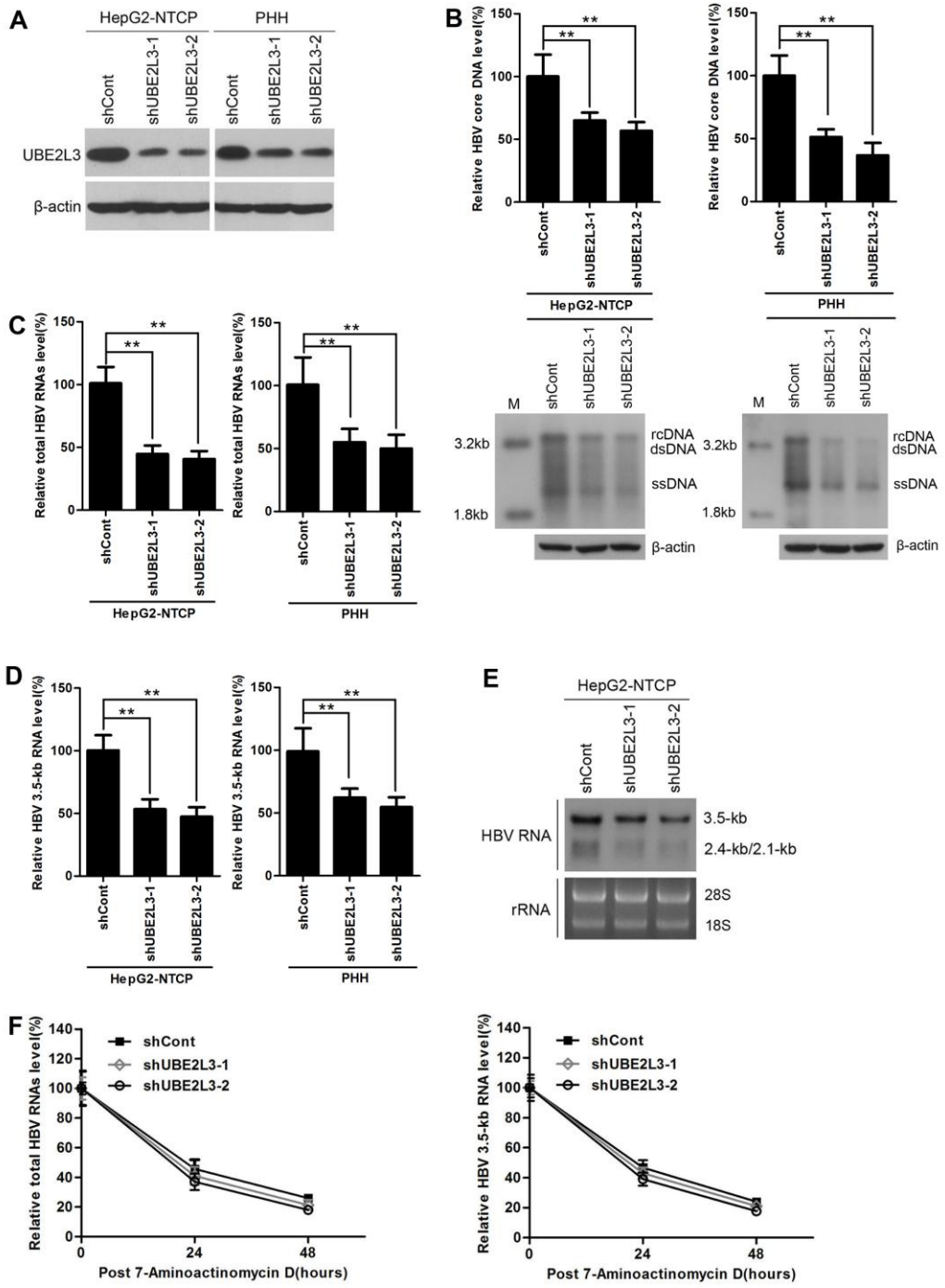

Figure 4

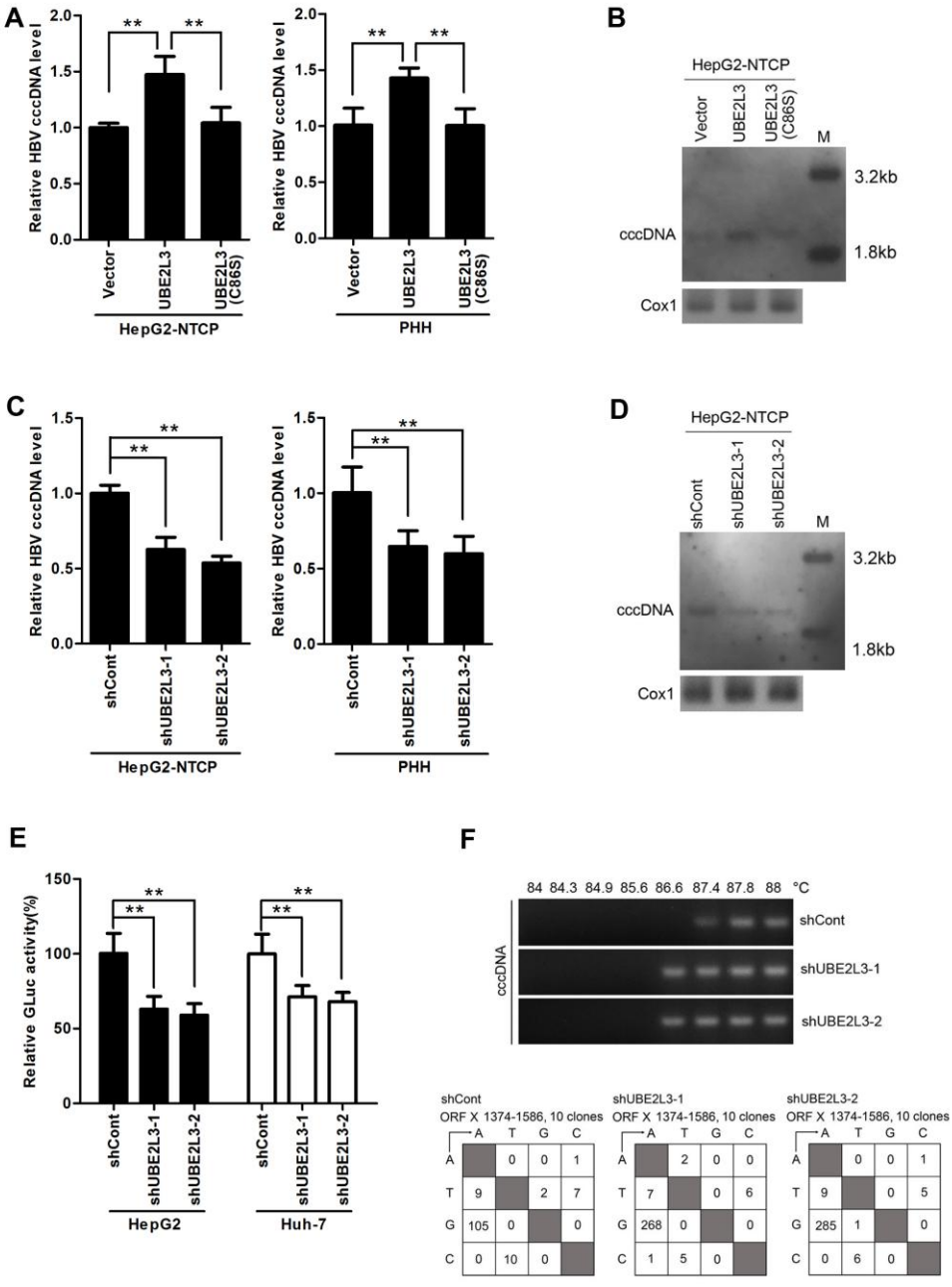

Figure 5

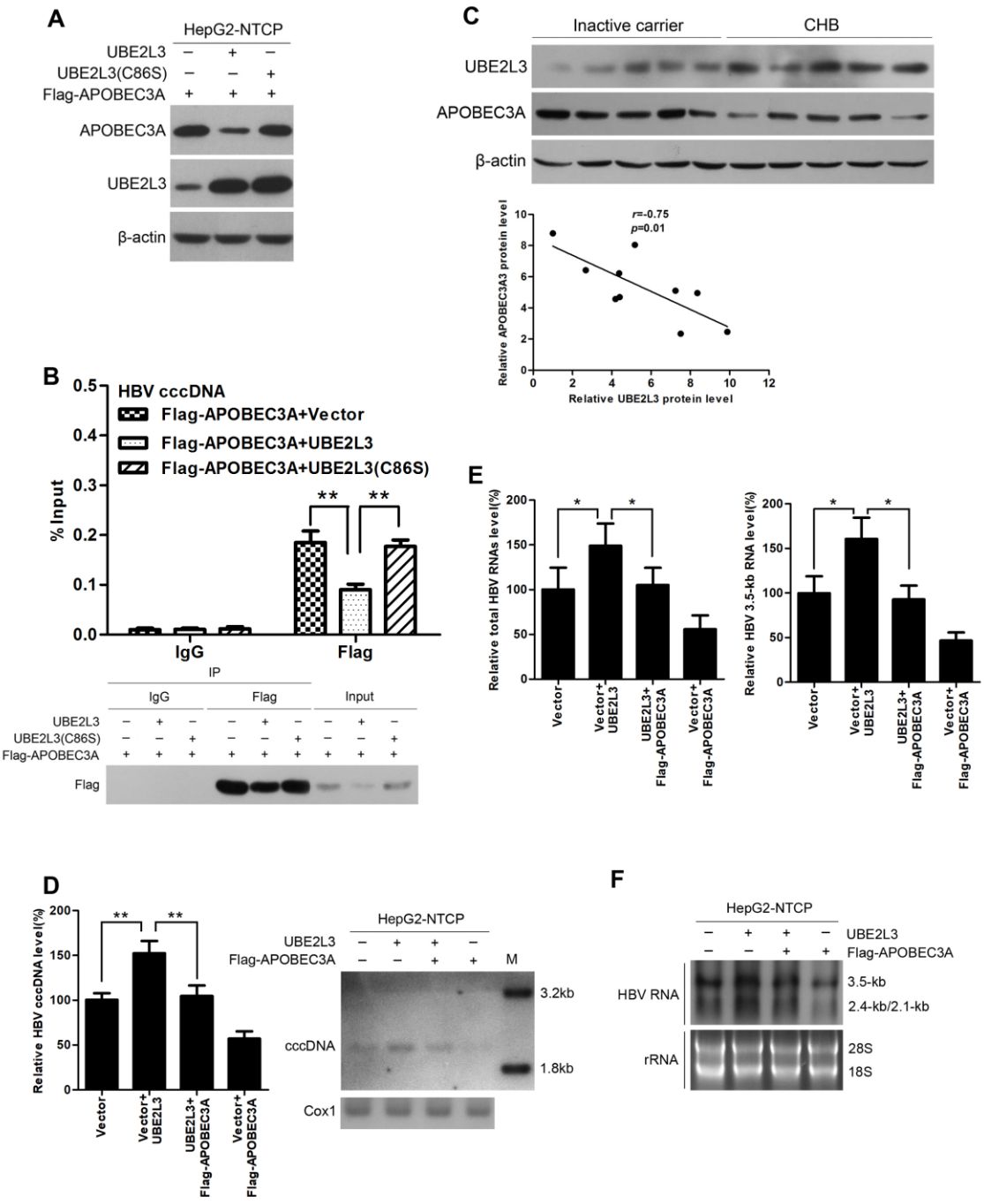

Figure 6

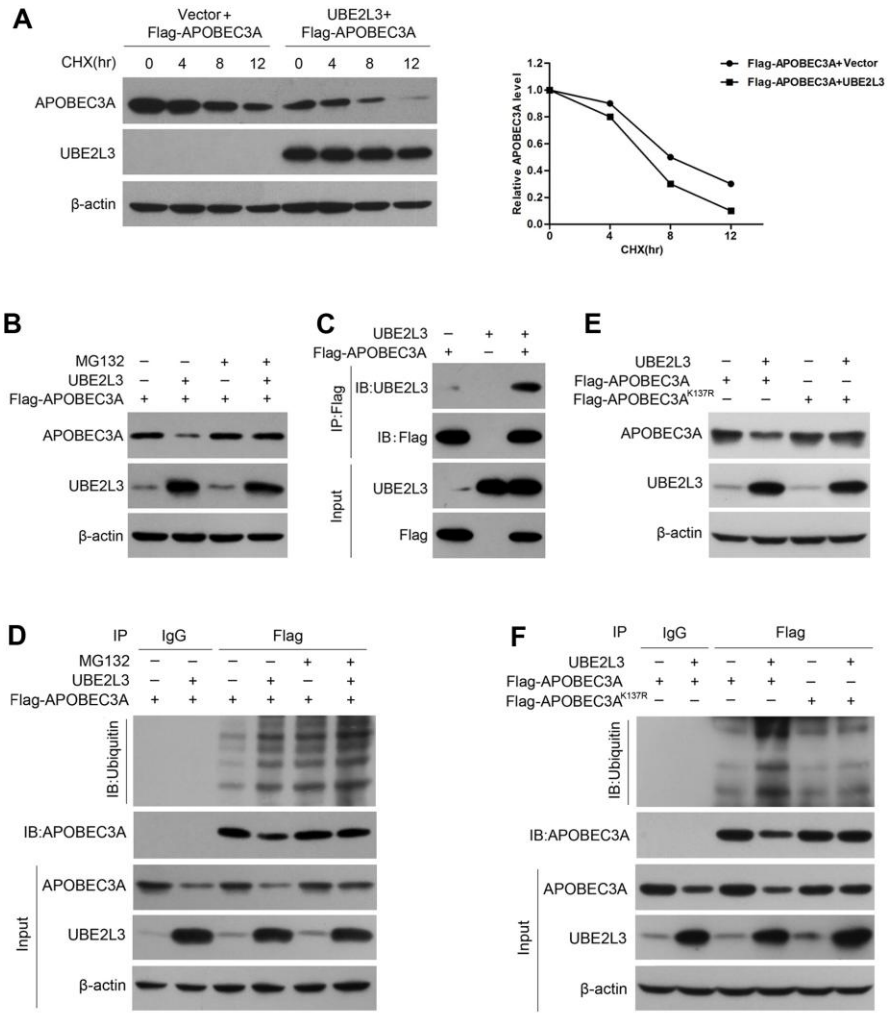

Figure 7