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CELLULAR AND MOLECULAR GASTROENTEROLOGY AND HEPATOLOGY

ORIGINAL RESEARCH

HBV X Protein Induces Degradation of UBXN7, a Novel Negative Regulator of NF-κB Signaling, to Promote HBV Replication

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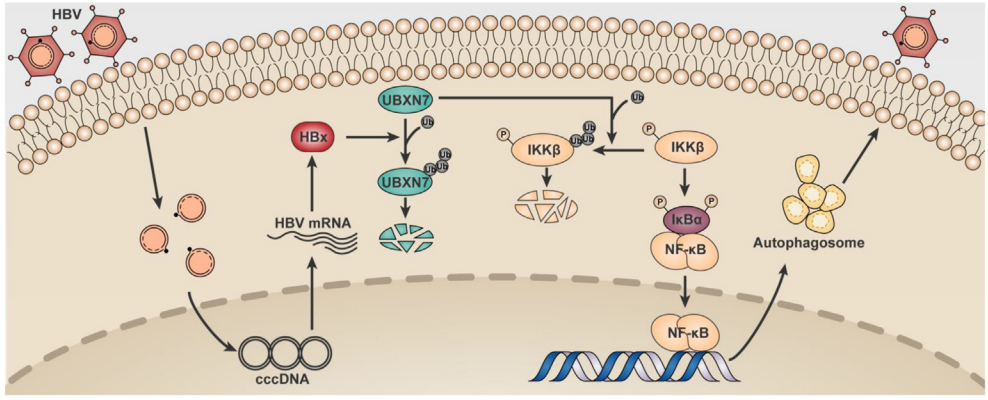

SUMMARY

Our results reveal that HBx can promote HBV replication via degradation of UBXN7, thus maintaining high levels of IKK-β to activate NF-κB signaling and NF-κB–dependent autophagy. Our findings suggest that UBXN7 can be targeted for potential new therapies in HBV-related diseases.

Chronic hepatitis B virus (HBV) infection is a leading cause of hepatocellular carcinoma. However, the function and mechanism of the effect of HBV on host protein ubiquitination remain largely unknown. We aimed at characterizing whether and how HBV promotes self-replication by affecting host protein ubiquitination. In this study, we identified UBXN7, a novel inhibitor for nuclear factor kappa B (NF-κB) signaling, was degraded via interaction with HBV X protein (HBx) to activate NF-κB signaling and autophagy, thereby affecting HBV replication. The expression of UBXN7 was analyzed by Western blot and quantitative reverse transcription polymerase chain reaction in HBV-transfected hepatoma cells and HBV-infected primary human hepatocytes (PHHs). The effects of UBXN7 on HBV replication were analyzed by using in vitro and in vivo assays, including stable isotope labeling by amino acids in cell culture (SILAC) analysis. Changes in HBV replication and the associated molecular mechanisms were analyzed in hepatoma cell lines. SILAC analyses showed that the ubiquitination of UBXN7 was significantly increased in HepG2.2.15 cells compared with control cells. After HBV infection, HBx protein interacted with UBXN7 to promote K48-linked ubiquitination of UBXN7 at K99, leading to UBXN7 degradation. On the other hand, UBXN7 interacted with the ULK domain of IκB kinase β through its ubiquitin-associating domain to facilitate its degradation. This in turn reduced NF-κB signaling, leading to reduced autophagy and consequently decreased HBV replication. *(Cell Mol Gastroenterol Hepatol 2023;15:179–195; https://doi.org/10.1016/j.jcmgh.2022.09.003)*

Keywords: UBXN7; IKK-β; HBx; HBV Replication; Ubiquitination.

Chronic hepatitis B virus (HBV) infection is a major global health problem worldwide. Two hundred fifty-seven million people chronically infected by HBV are at increased risk of death from hepatocellular carcinogenesis.[1]

Antiviral drugs can inhibit HBV replication but cannot completely eliminate HBV. Thus, patients need to take medication for life. Improved understanding of the molecular mechanism of HBV replication may offer novel and better therapies against HBV.

Ubiquitination is a common post-translational modification that plays an essential role in the antiviral mechanism to eliminate viral components.[2,3] Earlier studies have shown that HBV manipulates the ubiquitin system to promote virus replication.[4,5] Recent studies indicate that HBV promotes viral replication through the interaction of hepatitis B X protein (HBx) with various ubiquitinated proteins.[6–8] To clarify the relationship between HBV replication and ubiquitination, we performed stable isotope labeling by amino acids in cell culture (SILAC)-labeling and affinity enrichment of ubiquitinated peptides, followed by high-resolution liquid chromatography with tandem mass spectrometry analysis. Among the proteins found to be differentially ubiquitinated because of HBV was UBXN7.

UBXN7 is a member of the ubiquitin regulatory X (UBX) proteins family, which can interact with a large number of E3 ubiquitin ligases through ubiquitin-associated (UBA) domain at their N termini.[9] Several of them, UBXN1, N9, and N11, have been shown to inhibit the production of retroviruses and lentiviruses and canonical nuclear factor kappa B (NF-κB) signaling.[10] It has also been found that UBXN7 (also called UBXD7) inhibits tumor necrosis factor α–induced NF-κB and human immunodeficiency virus long terminal repeat activities, but the underlying mechanisms are still unclear.[10,11]

The homologous protein of UBXN7 in Arabidopsis and yeast PUX7/Ubx5 has been identified recently as a selective autophagy receptor for CDC48/p97, which is combined with ubiquitin-interaction motif (UIM)-docking site on ATG8 through its UIM motif. It is worth noting that UBXN7 cannot bind with MAP1LC3a and GABARAP isoforms, the 2 main ATG8 sub-clades, through the UIM-UDS surface in humans.[12] Thus, the role of UBXN7 in autophagy remains to be determined.

Here, we showed that HBx encoded by HBV interacts with UBXN7 through aa 55-136 and promotes its ubiquitin-dependent degradation. We further revealed that UBXN7 could interact with IκB kinase β (IKK-β) through its UAS domain to promote IKK-β ubiquitination and subsequently degradation of IKK-β, leading to inactivation of NF-κB signaling and inhibition of autophagy and HBV replication. Our results indicated that HBx promoted HBV replication via degradation of UBXN7 to enhance IKK-β level for activation of NF-κB signaling.

Results

Hepatitis B Virus Reduces UBXN7 Protein Level

To investigate the effect of HBV infection on the ubiquitination of host proteins in hepatocytes, we analyzed the ubiquitylome and proteome in HBV-expressing stable cell line HepG2.2.15 and the corresponding parental cell line HepG2. We performed SILAC labeling and affinity enrichment of ubiquitinated peptides, followed by high-resolution liquid chromatography with tandem mass spectrometry and bioinformatics analyses (Figure 1*A*).[13] Our results showed that HBV infection changes the abundance and ubiquitination of a large number of proteins.[14] Among thus identified proteins was UBXN7, a member of the ubiquitin regulatory X proteins family. In response to HBV infection, UBXN7 protein level was reduced by half in HepG2.2.15 cells (Figure 1*B*, left). In addition, the ubiquitination of the 2 lysine residues in UBXN7 (K84 and K99) were both up-regulated by more than 2-fold in HepG2.2.15 compared with HepG2 (Figure 1*B*, right). To verify the SILAC findings, we analyzed the mRNA and protein levels of UBXN7 in HepG2 and HepG2.2.15 cells by real-time polymerase chain reaction (PCR) and Western blot, respectively. Consistent with the SILAC results, Western blot showed that UBXN7 protein level was significantly lower in HepG2.2.15 cells compared with HepG2 cells (Figure 1*C*, bottom). However, there was no significant difference in UBXN7 mRNA levels between the 2 cell lines (Figure 1*C*, upper), suggesting that HBV regulates UBXN7 protein level post-transcriptionally. To further investigate the regulation of UBXN7 protein level by HBV, we transfected Huh7 or HepG2 cells with pHBV1.3 or a control vector and determined the mRNA and protein levels of UBXN7. Again, the transfection of pHBV1.3 in these 2 cell lines did not affect the mRNA level of UBXN7 but significantly reduced its protein level (Figure 1*D*). Then, we treated HepAD38 cells with tetracycline, which inhibited the production of HBV in HepAD38 cells. After tetracycline treatment, the RNA level of UBXN7 in cells did not change significantly, but the protein level increased significantly (Figure 1*E*). Our results indicated that HBV infection significantly reduced UBXN7 protein level but had no effect on its mRNA level. Thus, we suggested that HBV down-regulates UBXN7 protein expression at the post-transcriptional level.

Hepatitis B X Protein Suppresses UBXN7 Protein Level via the Proteasome Pathway

There are 2 main pathways for protein degradation, proteasome pathways and the lysosome pathways. To determine UBXN7 degradation pathway induced by HBV, we studied the effects of the proteasome inhibitor MG132 and the lysosomal inhibitor NH_4Cl and CQ. We found that HBV reduced UBXN7 protein levels dose-dependently in Huh7 (Figure 2*A*, left) and HepG2 (Figure 2*B*, left). Importantly, treatment with MG132 blocked this reduction, whereas

***Authors share co-first authorship.**

Abbreviations used in this paper: **ELISA, enzyme-linked immunosorbent assay; HA, hemagglutinin; HBV, hepatitis B virus; HBx, HBV X protein; IKK-β, IκB kinase β; NF-κB, nuclear factor kappa B; PCR, polymerase chain reaction; PHHs, primary human hepatocytes; SILAC, stable isotope labeling by amino acids in cell culture; UAS, ubiquitin-associating; Ub, ubiquitin; UBA, ubiquitin-associated; UBX, ubiquitin regulatory X; UIM, ubiquitin-interaction motif; ULD, ubiquitin-like domain; WT, wild-type.**

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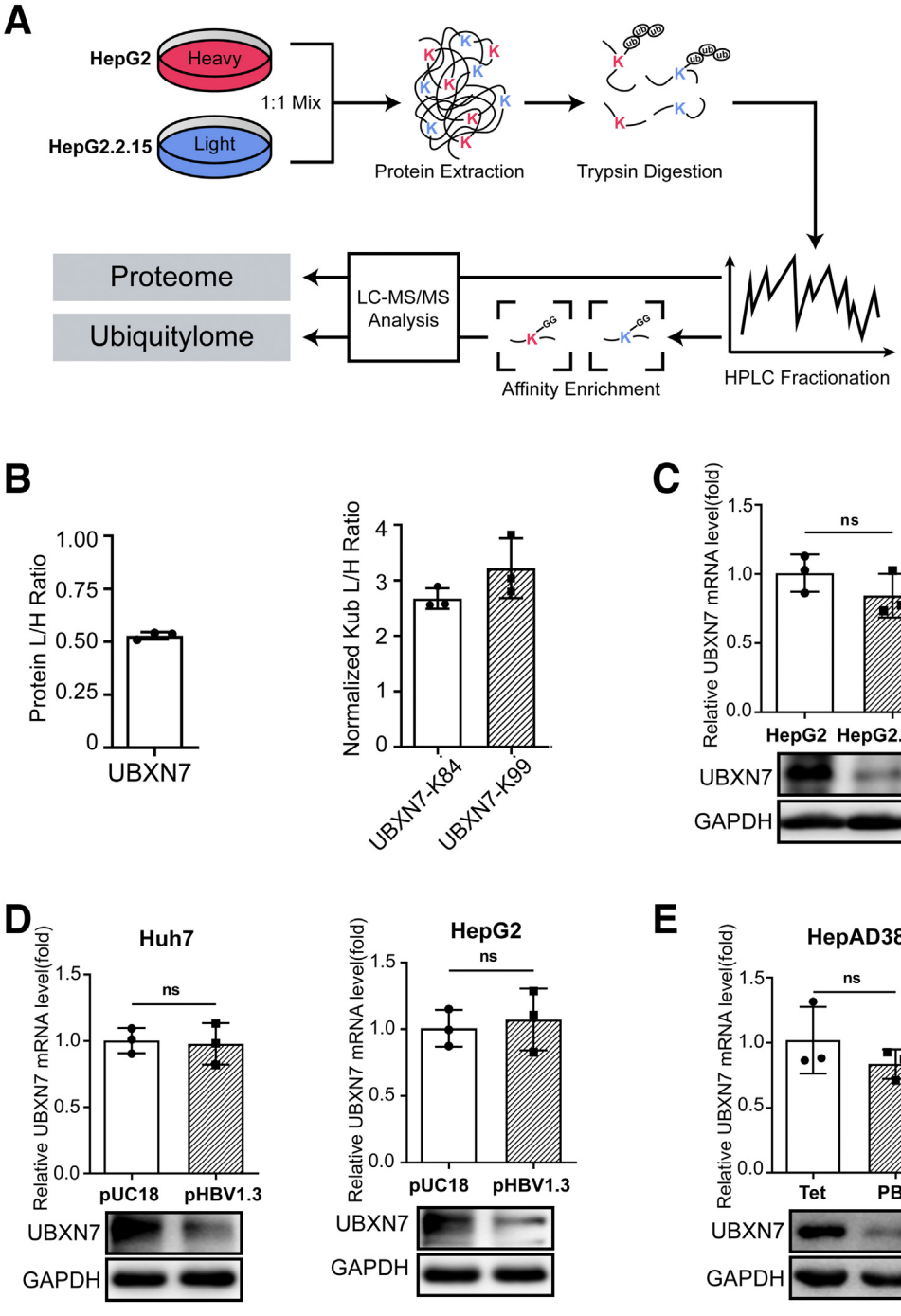
treatment with NH_4Cl and CQ treatment had no effect (Figure 2*A*, middle and right, Figure 2*B*, middle and right). Similar results were obtained in HepAD38 cells with or without tetracycline treatment (Figure 2*C*). These results indicated that HBV down-regulated UBXN7 protein level through the proteasome pathway.

To determine which HBV protein might function to reduce the protein level of UBXN7, we transfected plasmids expressing different viral proteins into hepatic cells. As shown in Figure 2*D*, HBx, but not HBc, HBs, or HBp, significantly reduced UBXN7 protein level. Consistently, when pHBV1.2-Δx plasmid, an HBV plasmid in which the HBx gene was deleted, was transfected into cells, it failed to reduce UBXN7 protein level, unlike that parental wild-type (WT) pHBV1.2 plasmid (Figure 2*E*). Furthermore, like HBV plasmid, transfection of HBx also dose-dependently reduced UBXN7 protein level but not its mRNA level (Figure 2*F*).

To confirm whether the inhibition of UBXN7 by HBx is also through the proteasome pathway, we used the 3 inhibitors as above. Again, we found that HBx reduced UBXN7 protein level in a dose-dependent manner in the presence of NH_4Cl and CQ but not MG132, indicating the involvement of the proteasome pathway (Figure 2*G*). In addition, when we used cycloheximide to block new protein synthesis, we



found that the protein level of UBXN7 decreased gradually with the increasing of treatment time, with a half-life of 9 hours (Figure 2*H*, left). Upon overexpressing HBx, half-life of the UBXN7 protein in cells treated with cycloheximide was only 6 hours (Figure 2*H*, right). Similar results were obtained in HepG2 cells (Figure 2*I*). In addition, cells transfected with pHBV1.2 plasmid also had reduced half-life of UBXN7 protein compared with cells transfected with pHBV1.2-Δx plasmid (Figure 2*J*). These results suggest that HBx shortens the half-life of UBXN7.

Hepatitis B X Protein Promotes K48-Linked Ubiquitination of UBXN7

To determine whether HBV promotes UBXN7 degradation by catalyzing its polyubiquitylation, we first examined the effect of HBV on UBXN7 in an in vivo ubiquitylation assay. Hemagglutinin (HA)-ubiquitin, Flag-UBXN7, and HBV or control vector were co-transfected into Huh7 cells, and UBXN7 proteins were isolated by Flag antibody pull-down, followed by Western blot analysis of the immunoprecipitated proteins. The results showed that HBV significantly up-regulated UBXN7 ubiquitination in the presence of MG132 (Figure 3*A*, left). Similar results were obtained in HepAD38 cells in the absence of tetracycline (Figure 3*A*, right). We further investigated which HBV protein played a role in promoting the ubiquitination of UBXN7. As shown in Figure 3*B*, transfection with HBx expression plasmid increased UBXN7 ubiquitination, whereas transfection with plasmid expressing HBs, HBc, or HBp did not. Next, we showed by coimmunoprecipitation that UBXN7 interacts with HBx, but not with HBs, HBc, or HBp (Figure 3*C* and *D*).

UBXN7 contains 4 domains: UBA domain, ubiquitin-associating (UAS) domain, UIM, and UBX domain. To determine the region of UBXN7 important for its interaction with HBx, we constructed a series of plasmids expressing Flag-tagged mutants UBXN7 (Figure 3*E*, up panel). Coimmunoprecipitation assay showed that the amino acids 55 to 136 in UBXN7 were required to interact with HBx (Figure 3*E*, down panel).

There are 2 types of polyubiquitination, one through K48 and the other through K63 in ubiquitin. To determine whether HBV promotes UBXN7 degradation through K48 or K63 ubiquitination, we transfected plasmid expressing HA-tagged WT ubiquitin (Ub) plasmid (HA-Ub-WT) or mutants in which all lysine residues except K48 or K63 were replaced with arginine (HA-Ub-K48 and HA-Ub-K63). Immunoprecipitation followed by Western blot analyses showed that HBV/HBx promoted the K48-linked ubiquitination of UBXN7 (Figure 3*F* and *G*). In addition, SILAC ubiquitylome results showed that HBV caused an increase of ubiquitination at 2 lysine residues on UBXN7 (K84 and K99) (Figure 1*B*). To validate these results, we constructed two UBXN7 mutants in which lysine 84 or 99 was replaced with arginine (UBXN7-K84R or UBXN7-K99R). We transfected various FLAG-UBXN7 (WT, K84R, K99R), HA-Ub (K48), and MYC-HBx plasmids into Huh7 cells, and the results showed that HBx promoted the K48-linked ubiquitination of the UBXN7-K84R mutant but not UBXN7-K99R mutant (Figure 3*H*). These results indicated that HBV via HBx promoted K48-linked ubiquitination at lysine 99 of UBXN7.

UBXN7 Inhibits the Nuclear Factor Kappa B Signaling via IκB Kinase β

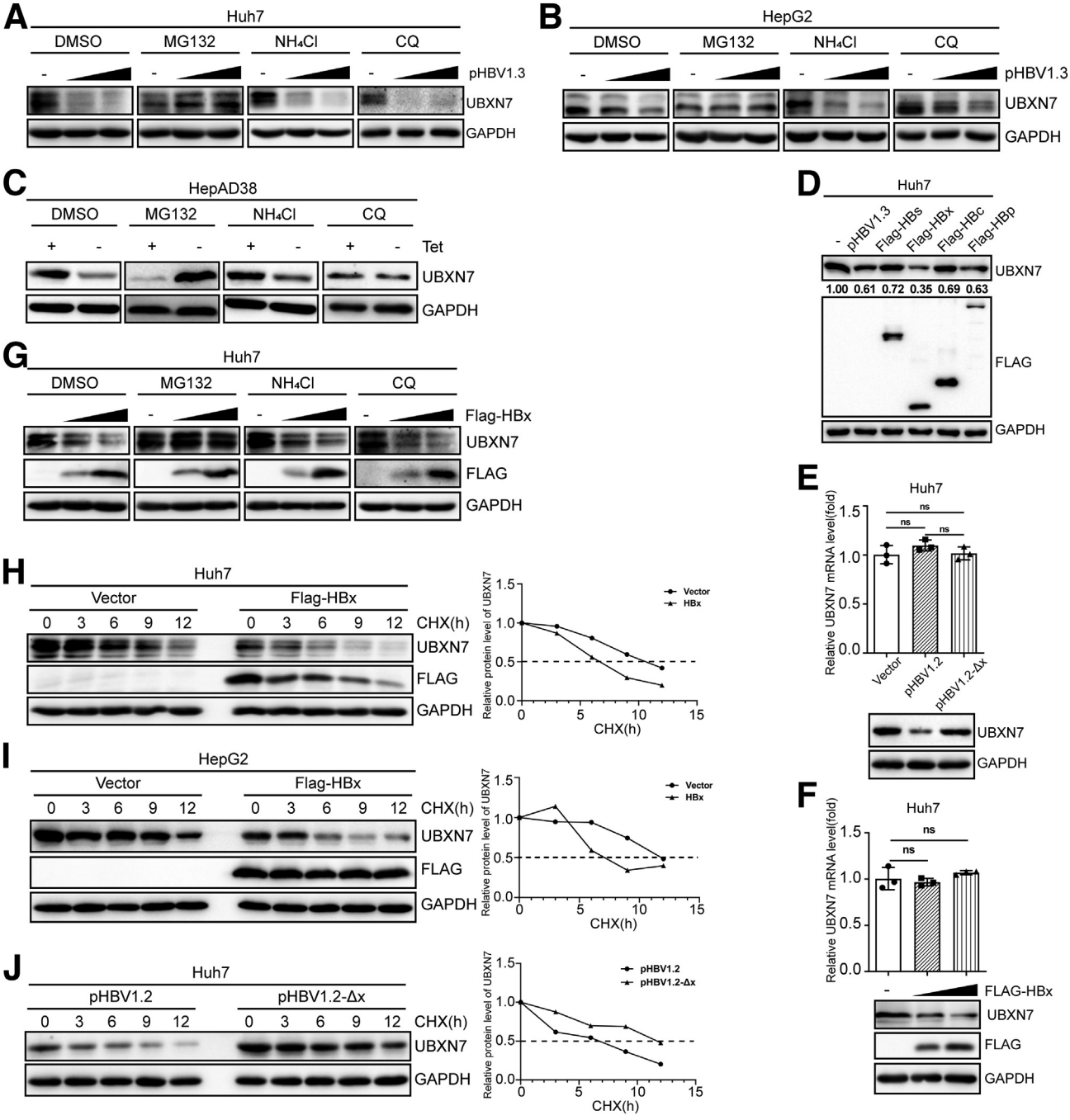
Earlier studies have suggested that UBXN7 might negatively regulate tumor necrosis factor-α–induced NF-κB responsive promoter activity.[11] We hypothesized that in the presence of HBV, UBXN7 also affects the activity of NF-κB pathway. To test this hypothesis, we performed dual-luciferase assays of NF-κB activity in Huh7 cells transfected with pHBV1.3 plasmid plus control vector or UBXN7 expression plasmid; the result showed that the transfection of Huh7 cells with UBXN7 expression plasmid decreased HBV-induced NF-κB responsive promoter activity (Figure 4*A*). To further evaluate the molecular mechanisms that UBXN7 inhibits NF-κB signaling in the presence of HBV, we first examined the binding of UBXN7 to several NF-κB signaling proteins in Huh7 cells. The results showed that UBXN7 interacted with IKK-β, but not with IκBα, p65, TAK1, and IKK-α (Figure 4*B* and *C*). IKK-β is a key upstream kinase in NF-κB signaling. To determine whether the interaction between UBXN7 and IKK-β affects the function of IKK-β in HBV-induced NF-κB signaling, we transiently overexpressed UBXN7 in the presence of HBV. The results showed that UBXN7 reduced the protein level and phosphorylation of IKK-β. Moreover, IκBα was increased, and phosphorylation of IκBα was reduced (Figure 4*D*, left). Similar results were

Figure 1. HBV reduces UBXN7 protein level. (*A*) Workflow for quantitative proteome and ubiquitylome analyses in HepG2.2.15 and HepG2 cells. (*B*) UBXN7 protein level was lower in HepG2.2.15 cells compared with parental HepG2 cells. HepG2.2.15 cells were labeled with the "light" form [$^{12}C_6$]-$_L$-lysine/[$^{12}C_6$ $^{14}N_4$]-$_L$-arginine, and the HepG2 cells were labeled with the "heavy" form [$^{13}C_6$]-$_L$-lysine/[$^{13}C_6$ $^{15}N_4$]-$_L$-arginine. UBXN7 protein level (*left*) and normalized ubiquitination levels (*right*) of 2 lysine residues (K84 and K94) in UBXN7 were determined by quantitative proteome and ubiquitylome. Note that in HepG2.2.15 cells, UBXN7 levels were lower and ubiquitination levels at both residues were higher. (*C*) HepG2.2.15 cells have a similar level of UBXN7 mRNA level (up panel) but a lower level of UBXN7 protein compared with HepG2 cells as determined by real-time PCR and Western blot analyses, respectively. GAPDH served as a loading control. (*D*) Transfection with pHBV1.3 (hepatitis B virus 1.3-fold genome plasmid) leads to reduced UBXN7 protein level without affect on UBXN7 mRNA in Huh7 cells (*left*) and HepG2 cells (*right*). Cells were transfected with pHBV1.3 or control pUC18, and UBXN7 mRNA (up panel) protein (bottom panel) levels were determined by real-time PCR and Western blot analyses, respectively. GAPDH served as a loading control. (*E*) HepAD38 cells treated with tetracycline (Tet) (an HBV inhibitor) have similar level of UBXN7 mRNA level (up panel) but higher level of UBXN7 protein compared with control phosphate-buffered saline (PBS) treatment as determined by real-time PCR and Western blot analyses, respectively. GAPDH served as a loading control. (Three independent experiments were performed, and 1 representative dataset is shown. ns $P \geq .05$ in panels *C*–*E*).

found in HepG2.2.15 (Figure 4*D*, right). When UBXN7 was knocked down by using siRNA, the opposite effects were observed (Figure 4*E*). These results collectively suggest that UBXN7 inhibited the HBV-induced NF-κB signaling via interacting with IKK-β.

UBXN7 Inhibits Hepatitis B Virus Transcription and Replication via Nuclear Factor Kappa B Signaling

NF-κB is a central player for HBV replication.[15,16] Earlier studies have found that UBXN7 could significantly inhibit some retroviruses and lentiviruses, but it is unclear whether UBXN7 can inhibit HBV replication. To investigate the effect of UBXN7 on HBV replication, we co-transfected pHBV1.3 and UBXN7 plasmid or UBXN7 siRNA into Huh7 cells. As shown in Figure 5*A*, overexpression of UBXN7 reduced the levels of secreted viral protein HBs and HBe and viral RNA level in a dose-dependent manner. Similar results were found in HepG2 (Figure 5*B*) and HepG2.2.15 cells (Figure 5*C*). Consistently, knocking down UBXN7 had the opposite effects (Figure 5*D*–*F*). Then, we analyzed HBV RNA level in Huh7, HepAD38, and HepG2 cells by Northern blot (Figure 5*G* and *H*), and the results showed that HBV RNA levels in cells with HBx mutant were similar to those in UBXN7 overexpressing cells, which were lower than those



in WT HBV replicon cells, indicating that UBXN7 inhibited HBV transcription. Consistently, knocking down UBXN7 had the opposite effects (Figure 5*I*). Finally, we analyzed HBV DNA level in Huh7 cells by Southern blot (Figure 5*J*). These results collectively showed that UBXN7 reduced NF-κB signaling to inhibit HBV replication.

Ubiquitin-Associating Domain of UBXN7 Is Crucial for Its Effect on Hepatitis B Virus–Induced Nuclear Factor Kappa B Signaling and Autophagy

To investigate whether UBXN7 reduced IKK-β protein level through ubiquitination-dependent proteasome degradation, we co-transfected HA-Ub-WT or HA-Ub-K48 and HA-Ub-K63 mutants with UBXN7 and IKK-β in Huh7 cells. As shown in Figure 6*A*, UBXN7 up-regulated ubiquitination of IKK-β with Ub-WT and Ub-K48 in the presence of MG132 to block proteasome activity (Figure 6*A*). On the other hand, UBXN7 did not affect K63-linked ubiquitination of IKK-β (Figure 6*A*). These results suggested that UBXN7 promoted IKK-β degradation by enhancing its K48-linked ubiquitination.

To determine the regions through which IKK-β interacted with UBXN7, we generated a series of IKK-β deletion constructs (Figure 6*B*, up panel) and co-expressed them with UBXN7. We analyzed the binding to UBXN7 by coimmunoprecipitation and found that the ubiquitin-like domain (ULD) of IKK-β was crucial for its interaction with UBXN7 (Figure 6*B*, down panel). To determine the domain in UBXN7 important for its binding to IKK-β, different UBXN7-deletion constructs were co-expressed with IKK-β. As shown in Figure 6*C*, IKK-β interacted with the UAS domain of UBXN7. It is worth noting that IKK-β represented the first interacting protein for the UAS domain ever discovered.

To determine whether the UAS domain of UBXN7 functions in HBV-induced NF-κB signaling, we transfected UBXN7 or UAS domain deletion-mutant (UBXN7-ΔUAS) plasmid into HepG2.2.15 cells. Western blot results showed that UBXN7-ΔUAS had no inhibitory effect on NF-κB signaling (Figure 7*A*). It has been shown that HBV promotes its own replication by activating NF-κB pathway to induce autophagy. To test whether UBXN7 suppresses HBV replication by inhibiting autophagy, we transfected GFP-LC3, an autophagy marker, and UBXN7 or UBXN7-ΔUAS expressing-plasmids into HepG2.2.15 cells. Confocal microscopy analysis revealed that UBXN7 suppressed HBV-induced autophagy, whereas UBXN7-ΔUAS had no effect (Figure 7*B*). Enzyme-linked immunosorbent assay (ELISA) and real-time quantitative PCR analyses demonstrated that the UAS domain of UBXN7 is essential for UBXN7 to reduce HBsAg and HBeAg secretion and HBV RNA level (Figure 7*C* and *D*).

Then, we transfected pHBV1.2, pHBV1.2-Δx, and FLAG-IKK-β plasmids in Huh7 cells. Northern blot results showed that after transfection of HBx-deleted replicons into cells, UBXN7 was no longer inhibited by HBx, whereas overexpression of IKK-β increased HBV transcription in cells. Combined with our previous results, these results indicated that IKK-β–mediated NF-κB signaling is critical for the function of HBx to regulate viral transcription by inhibiting UBXN7 (Figure 7*E*).

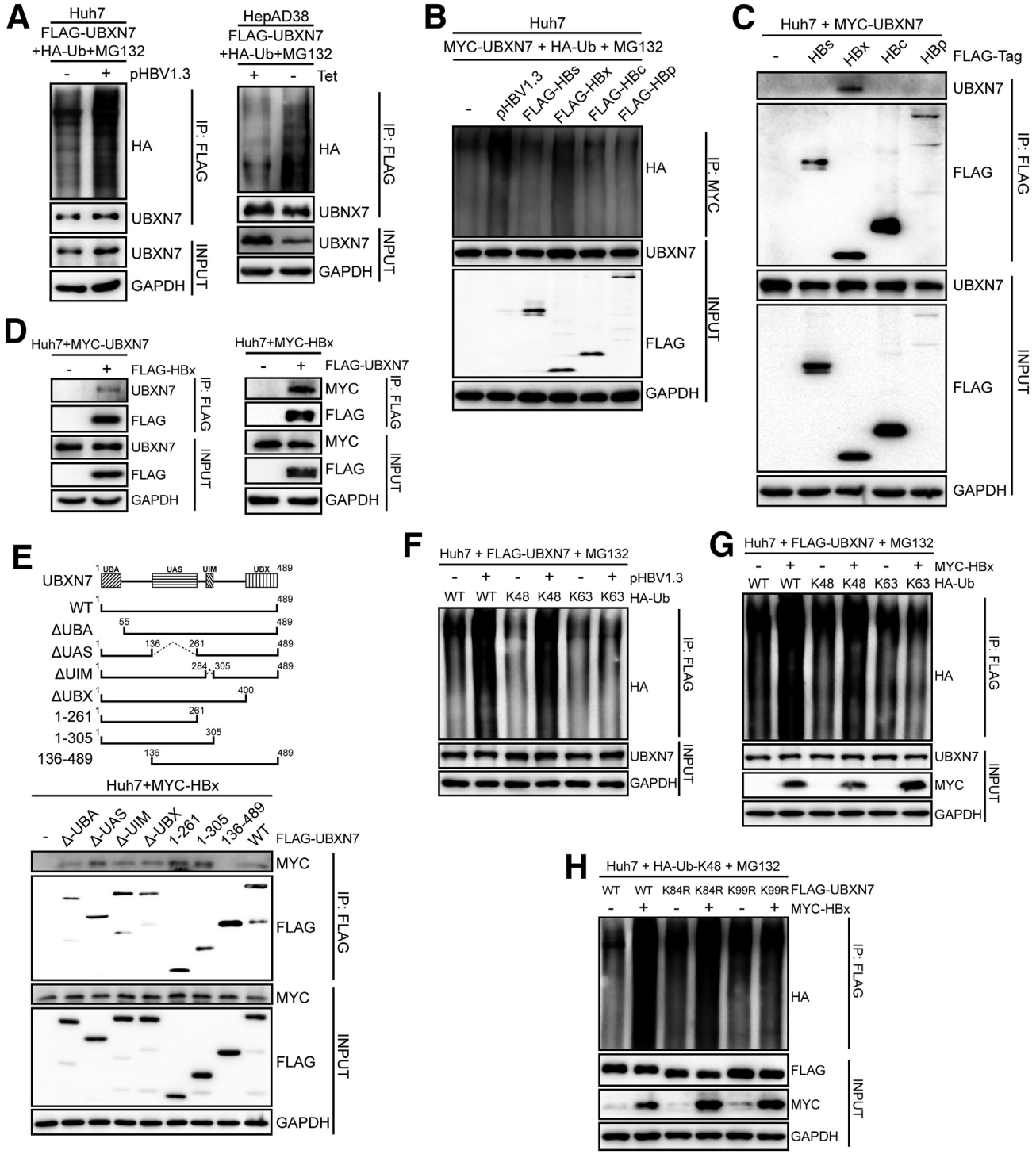
To examine the relevance of these findings to humans, we carried out studies in primary human hepatocytes (PHHs). ELISA and real-time quantitative PCR showed again that HBsAg secretion and HBV pgRNA level in HBV-infected PHH cells were decreased after overexpressing UBXN7. On the other hand, the UAS domain deletion-mutant (UBXN7-ΔUAS) had no such inhibitory effect, indicating that the UAS domain of UBXN7 is required to inhibit HBV replication in human cells as well (Figure 7*F* and *G*). Furthermore, consistent with the result in Figure 7*A*, Western blot results indicated that the UAS region in UBXN7 was crucial for its suppression of LC3expression in PHHs (Figure 7*H*). Similar

Figure 2. (***See previous page***). **HBx reduces UBXN7 protein level.** (*A–C*) HBV reduces UBXN7 protein level in a proteasome-dependent manner. Huh7 (*A*) and HepG2 (*B*) cells were transfected with control vector or pHBV1.3 (hepatitis B virus 1.3-fold genome) plasmid and then cultured in presence of MG132 (a proteasome inhibitor), ammonium chloride (NH_4Cl), chloroquine (CQ), or control dimethyl sulfoxide (DMSO) as indicated. Western blot of protein extracts of cultured cells showed that HBV decreased UBXN7 protein level after DMSO, NH_4Cl, and CQ treatments, whereas MG132 treatment prevented reduction in UBXN7 protein level due to HBV. (*C*) HepAD38 cells treated with tetracycline (Tet) or phosphate-buffered saline (PBS) (control) and then treated with DMSO (control) or MG132, NH4Cl, or CQ as indicated. UBXN7 protein level was determined by Western blot. (*D* and *E*) HBx encoded by HBV reduces UBXN7 protein level without affecting the mRNA level. (*D*) Huh7 cells were transfected with control vector or pHBV1.3, HBs-, HBx-, HBc-, HBp-expression plasmids, and UBXN7 protein level was determined by Western blot analysis. Note that of the HBV encoded proteins, only HBx markedly reduced UBXN7 protein level. (*E*) HBx is required for reduction of UBXN7 protein level after pHBV transfection. Huh7 cells were transfected with pHBV1.2 (hepatitis B virus 1.2-fold genome) plasmid or pHBV1.2-Δx (HBx deletion) plasmids; the UBXN7 mRNA (*upper*) and protein (*bottom*) levels were measured by real-time PCR and Western blot, respectively. (*F*) Huh7 cells were transfected with control vector or HBx-expression plasmid, and UBXN7 mRNA level was measured by real-time PCR. Note that UBXN7 mRNA level was not affected by HBx overexpression. (*G*) HBx reduces UBXN7 protein level in a proteasome-dependent manner. The experiment was performed as (*A*) except for the use of HBx-expression plasmid for transfection. Note that HBx caused a reduction in UBXN7 protein level except when MG132 was present. (*H* and *I*) HBx shortens half-life of UBXN7 protein. Huh7 (*H*) and HepG2 (*I*) cells were transfected with control vector or HBx-expression plasmid and then treated with cycloheximide (10 μg/mL). Cells were harvested at indicated times, and UBXN7 protein level was measured by Western blot (*left*). The optical density (O.D.) ratios of UBXN7/GAPDH were determined and shown on right, revealing a reduced half-life in presence of HBx. (*J*) HBV reduces UBXN7 half-life in a HBx-dependent manner. Huh7 cells were transfected with pHBV1.2 or pHBV1.2-Δx (HBx deletion) plasmid, and the cells were analyzed as in (*F*). Note that pHBV1.2 but not pHBV1.2-Δx caused a reduction in half-life when compared with that with vector transfection in (*H*). (Three independent experiments were performed, and representative data are shown. ns $P \geq .05$ in *E* and *F*).

results were obtained in HepG2-NTCP cells (Figure 7*I* and *J*). These results indicated that UBXN7 suppressed HBV-induced NF-κB signaling to inhibit autophagy via its UAS domain, thereby reducing HBV replication.

UBXN7 Suppresses Hepatitis B Virus Replication in Vivo

To examine whether UBXN7 can inhibit HBV replication in vivo, we introduced pHBV1.3 and UBXN7 or control plasmids into mice through hydrodynamic injection and killed the mice 4 days later. ELISA results showed that UBXN7 significantly reduced the secretion of HBsAg and HBeAg in plasma (Figure 8*A*). Real-time quantitative PCR, immunohistochemical, and immunofluorescence analyses of the liver samples showed that UBXN7 suppressed HBV RNA level and core protein level (Figure 8*B–D*). Western blot showed that UBXN7 inhibits the IKK-β protein level in vivo (Figure 8*E*). Taken together, these results indicate that



UBXN7 suppressed HBV replication through the NF-κB pathway in vivo (Figure 8*F*).

Discussion

Herein, we discovered a novel HBV replication inhibitor, UBXN7, a E3 ubiquitin ligase adaptor. We showed that HBx interacted with UBXN7 and increased its K48 ubiquitination to promote its proteasome-dependent degradation. Furthermore, our results demonstrated that UBXN7 interacted with the ULD domain of IKK-β to increase IKK-β K48 ubiquitination for its degradation, leading to the inhibition of the NF-κB signaling and autophagy. Together, our results indicated that HBx binds UBXN7 to promote its degradation, thus blocking UBXN7-induced IKK-β degradation to maintain NF-κB action, which in turn induces autophagy to help HBV replication.

HBx, a key viral oncoprotein, plays crucial roles in HBV replication.[6] A number of studies have shown that HBx acts as a transcriptional transactivator, suggesting that HBx directly or indirectly modulates a large number of cellular genes for HBV replication by interacting with transcription factors.[17] On the other hand, studies have also shown that HBx shares many features with cellular DDB1 cullin accessory factor that can recruit substrates to CRL4 for ubiquitination and proteasomal degradation.[18–20] Lee et al[21] demonstrated an interaction between HBx and DDB1 (a CUL4 adaptor). Furthermore, Adrien et al and Christopher et al independently identified HBx-induced HBV replication via DDB1-CUL4-ROC1 (CRL4) E3 ligase to target the structural maintenance of chromosomes (Smc) complex Smc5/6 for degradation.[7,8] In addition, Neetu et al showed that HBx directly interacted with the F box region of Skp2 and destabilized the CUL1-SKP2 E3 ligase complex, resulting in blockage of the ubiquitination degradation of MYC, PAX8.[15,22,23] Finally, our previous studies identified HBx-binding and stabilizing MYC to facilitate the replication of HBV.[15] In this study, our unbiased global SILAC analysis revealed that HBV enhanced UBXN7 ubiquitination. Further studies showed that HBx interacted with UBXN7 to induce K48 ubiquitination of UBXN7 for its degradation via the proteasome pathway, suggesting that HBx modulated UBXN7 at the post-translational level in a role distinct from a viral transactivator.

UBXN7, a member of the UBA-UBX family, can interact with a large number of E3 ubiquitin ligases and serves as the substrate-binding adaptor[12,24] and contains 4 domains: UBA domain, UAS domain, UIM motif, and UBX domain. Earlier studies have identified binding partners for UBA, UIM, and UBX domains. Here, we identified that the UAS domain can bind to IKK-β and enhance its K48 ubiquitination.

NF-κB plays a central role in HBV-relative hepatocarcinogenesis. In unstimulated cells, NF-κB is bound to IκBα (the inhibitory protein of NF-κB) and remains in the cytoplasm. Extracellular stimuli lead to activation of the IκB kinase (IKK) complex and phosphorylates IκBα for its degradation, which results in p65/p50 release from IκBα and subsequent translocation from the cytoplasm to the nucleus. Although previously a number of reports suggested that HBx and large HBs activate NF-κB to induce HBV replication,[15,17,25] the mechanisms for NF-κB activation appear to vary. Markus et al suggested that LHBs or MHBs induce the activation of NF-κB via its transactivating activity under pro-oxidant state or ER stress.[26,27] Our previous results reported that HBx functions as a transactivator to down-regulate miR-192-3p to enhance XIAP expression, which phosphorylates IκBa to activate NF-κB signal pathway.[17] On the other hand, Jing et al found that HBx could not co-precipitate with the subunits p65 or p50 and

Figure 3. (***See previous page***). **HBx interacts with UBXN7 and up-regulates K48-linked ubiquitination at lysine 99 of UBXN7.** (*A*) HBV up-regulates UBXN7 ubiquitination. Huh7 cells were co-transfected with FLAG-UBXN7, HA-Ub, and pHBV1.3 plasmids as indicated and treated with MG132 (*left*). HepAD38 cells were co-transfected with FLAG-UBXN7 and HA-Ub plasmids as indicated and treated with MG132 and Tet or control PBS (*right*). Cell extracts were used for immunoprecipitation with anti-FLAG antibody, followed by Western blot analysis with indicated antibodies. (*B*) HBx of HBV up-regulates UBXN7 ubiquitination. Huh7 cells were co-transfected with MYC-UBXN7, HA-Ub, and pHBV1.3 or plasmids expressing various FLAG-HBV-encoded proteins and treated with MG132. Cell extracts were used for immunoprecipitation with anti-MYC antibody, followed by Western blot analyses for indicated proteins. Note that transfect with HBV or plasmid expressing HBx but not other HBV-encoded proteins increased UBXN7 ubiquitination. (*C* and *D*) HBx interacts with UBXN7. (*C*) Huh7 cells were co-transfected with MYC-UBXN7 and FLAG-HBs/HBx/HBc/HBp plasmids as indicated. (*D*) Huh7 cells were co-transfected with FLAG-HBx and MYC-UBXN7 plasmids (*left panel*) or FLAG-UBXN7 and MYC-HBx plasmids (*right panel*). Cell lysates were prepared and used for co-immunoprecipitation with anti-FLAG antibody followed by Western blot analysis. (*E*) The region from aa 55-136 of UBXN7 is required for its interaction with HBx. Huh7 cells were transfected with various FLAG-UBXN7 mutants and MYC-HBx plasmids as indicated, and co-immunoprecipitation and Western blot analyses were done. Note that all mutants, except the one with deletion of the first 135 aa (aa 136-489), could interact with HBx, suggesting that aa 55-136, but not any other known domains, are essential for the binding. (*F*) HBV up-regulates K48-linked ubiquitination of UBXN7. Huh7 cells were co-transfected with FLAG-UBXN7, various HA-Ub (WT, K48, K63) and pHBV1.3 plasmids and treated with MG132, followed by immunoprecipitation and Western blot analyses. Note that HBV enhanced the incorporation of HA-Ub WT or K48, in which all lysine residues except K48 were replaced with arginine, but not HA-Ub K63, into UBXN7. (*G*) HBx up-regulates K48-linked ubiquitination of UBXN7. Huh7 cells were transfected with FLAG-UBXN7, various HA-Ub (WT, K48, K63) and MYC-HBx plasmids and treated with MG132, followed by immunoprecipitation and Western blot analyses. Note that HBx enhanced the incorporation of HA-Ub WT or K48, but not K63, into UBXN7. (*H*) HBx up-regulates K48-linked ubiquitination at lysine 99 on UBXN7. Huh7 cells were transfected with various FLAG-UBXN7 (WT, K84R, K99R), HA-Ub (K48), and MYC-HBx plasmids as indicated and treated with MG132. Cell lysates were prepared and used for immunoprecipitation and Western blot analyses. Note that HBx failed to enhance K48-linked ubiquitination of UBXN7 K99R.

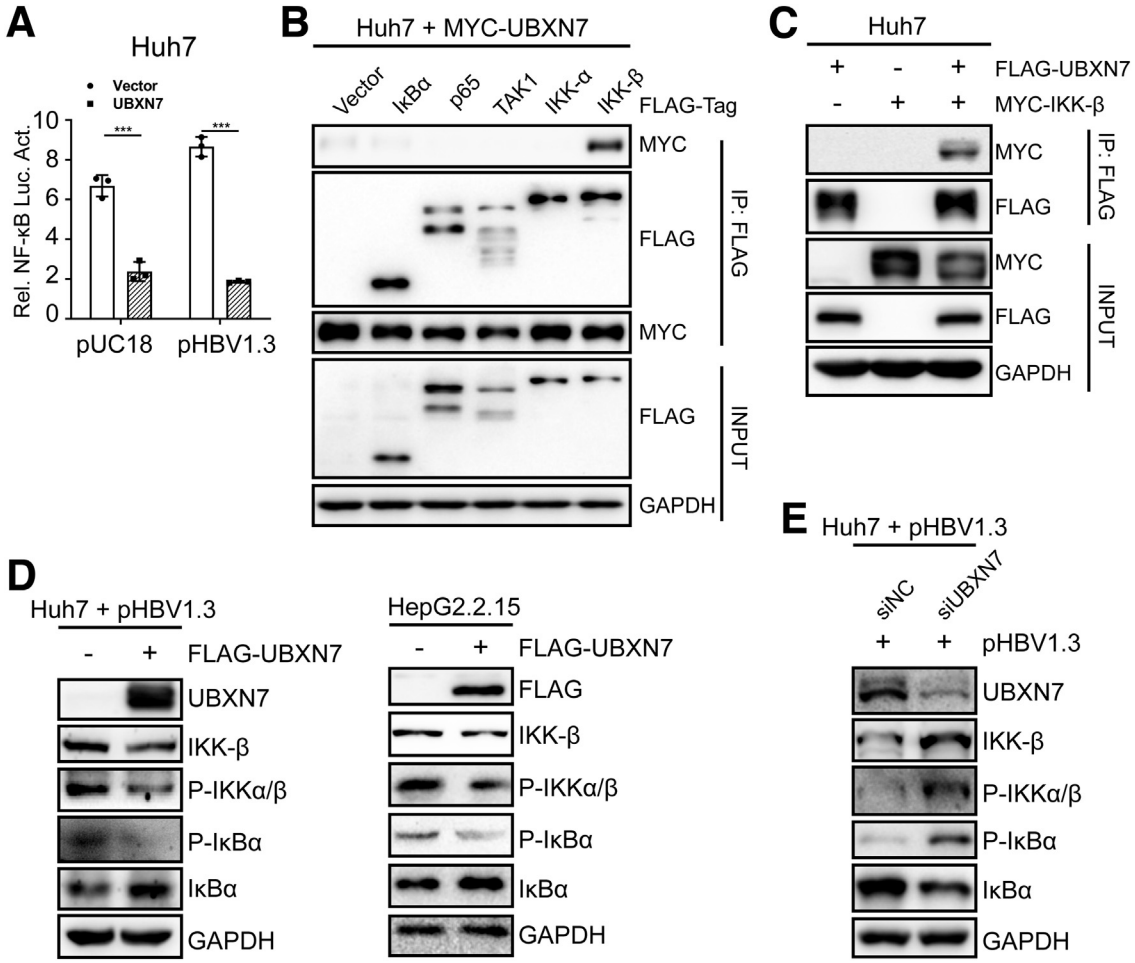
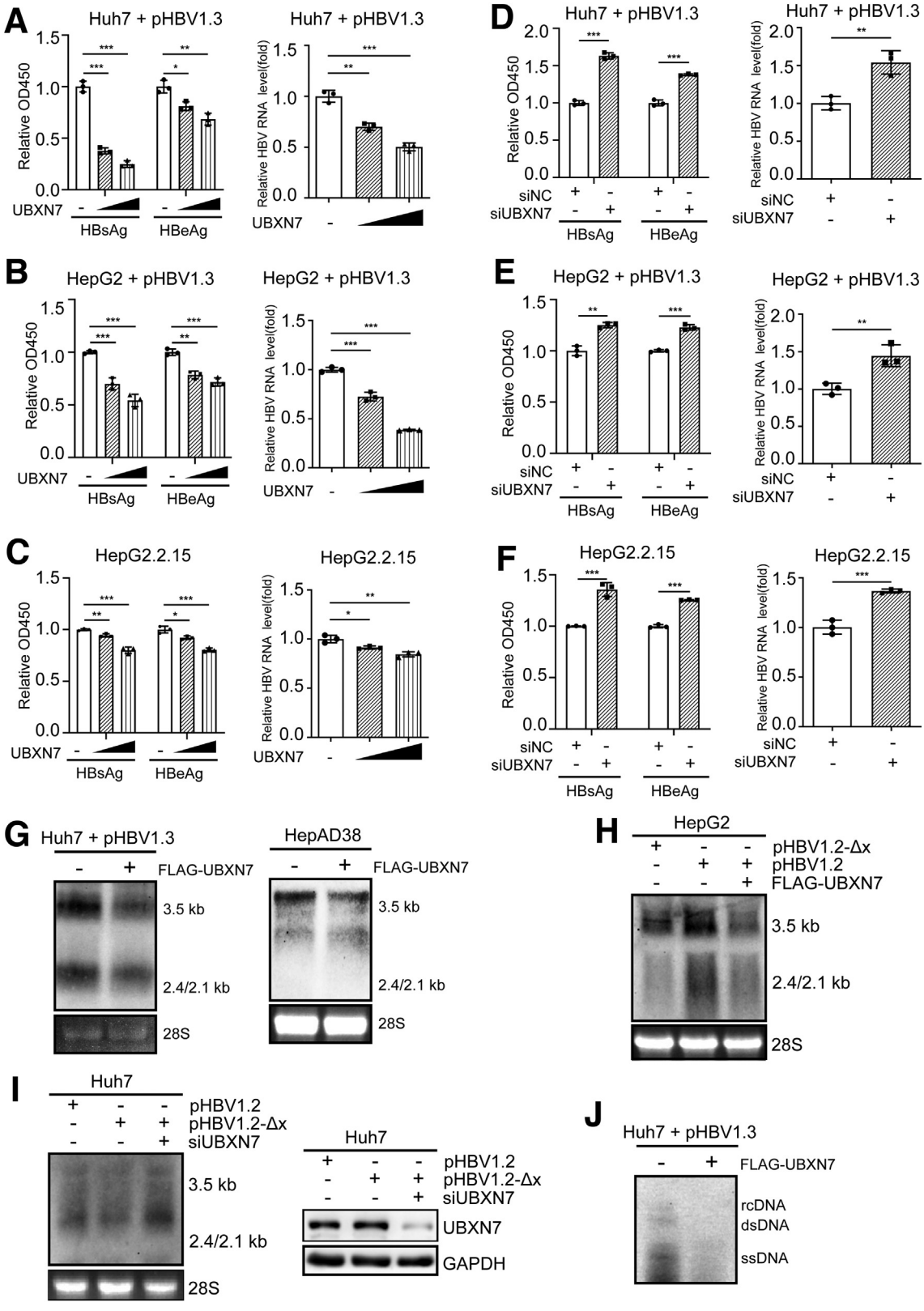


Figure 4. UBXN7 inhibits NF-κB signaling pathway by promoting the degradation of IκB kinase β (IKK-β). (*A*) UBXN7 inhibits NF-κB–dependent luciferase reporter activity. Huh7 cells were co-transfected with NF-κB–responsive promoter NF-κB-Luc, FLAG-UBXN7, and control vector or pHBV1.3 plasmids, and the cells were subjected to luciferase assay as a measure of relative NF-κB activity. (*B* and *C*) IKK-β interacts with UBXN7. (*B*) Huh7 cells were co-transfected with MYC-UBXN7 and FLAG-IκBα/P65/TAK1/IKK-α/IKK-β plasmids as indicated. Cell lysates were prepared for co-immunoprecipitation with anti-FLAG antibody and Western blot with indicated antibodies. Note that only IKK-β binded with UBXN7. (*C*) Immunoprecipitation of UBXN7 confirms its binding to IKK-β. Huh7 cells were co-transfected with MYC-IKK-β and Flag-UBXN7. Cell lysates were prepared for co-immunoprecipitation and Western blot analyses. (*D*) Overexpression of UBXN7 inhibits HBV-induced NF-κB signaling. Huh7 cells were co-transfected with pHBV1.3 plasmids and control vector or FLAG-UBXN7 plasmids as indicated (*left*). HepG2.2.15 cells were transfected with control vector or FLAG-UBXN7 plasmids as indicated (*right*). Levels of proteins were examined by Western blot analysis. Note reduction of P-IκBα by UBXN7, suggesting an inhibition of NF-κB signaling. (*E*) Knockdown of UBXN7 enhances HBV-induced NF-κB signaling. Huh7 cells were co-transfected with pHBV1.3 plasmids and control oligo or siUBXN7 oligo, and levels of proteins were examined by Western blot analysis. (Three independent experiments were performed, and representative data are shown. $^{*}P < .05$, $^{***}P < .001$ in *A*).

the suppressor IκBα and suggested that HBx affected NF-κB signaling pathways through regulating the Notch signaling pathway in L02 cells.[28] Finally, Lim et al[29] hinted that HBx physically interacts with p22-FLIP and NEMO and potentially forms a ternary complex to induce NF-κB activation. Our findings here reveal a novel mechanism by which HBx regulates NF-κB activation via regulating the stability of IKKs kinase. Our results showed that HBx interacted with UBXN7 to promote ubiquitination and degradation of UBXN7. This in turn prevents UBXN7-induced destabilization of IKK-β, leading to NF-κB activation under HBV infection. Our results appear to differ from those of Hu et al,[10] who reported that UBXN1, N9, and N11, which are the same family of UBXN7, blocked the canonical NF-κB pathway by binding to Cullin1 to inhibit IκBα degradation for re-activating quiescent human immunodeficiency virus from latent viral reservoirs in chronically infected individuals. In addition, our results showed that UBXN7 selectively binded to IKK-β via ULD domain but not to IκBα, p65, TAK1, and IKK-α, suggesting that IKK-β may be a

natural target of UBXN7-mediated ubiquitination in the absence of HBV infection. Furthermore, our mutational studies suggested that the UAS domain of UBXN7 was essential for binding to IKK-β and its effect on HBV replication. Therefore, UBXN7 is a crucial regulator in HBV viral transcription and subsequent viral protein production. In



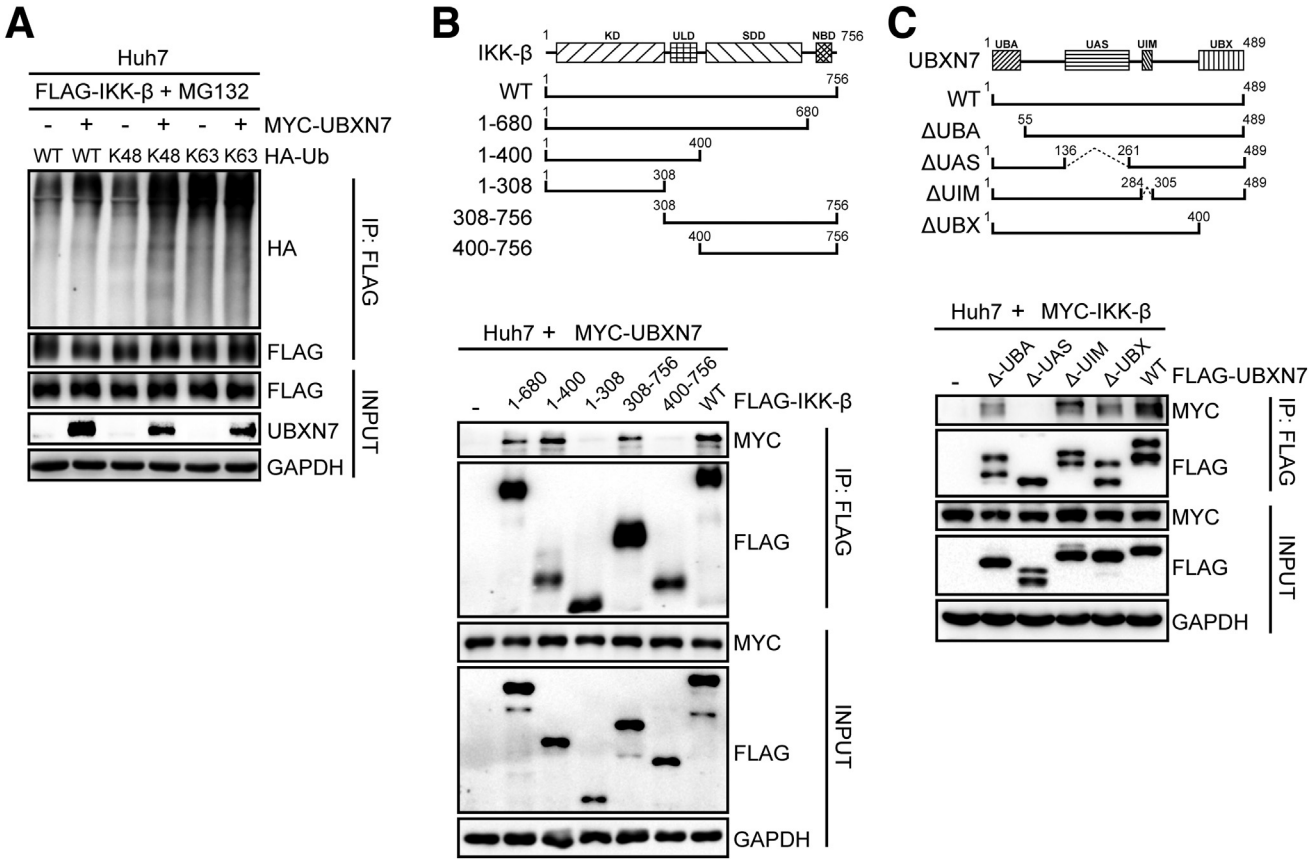
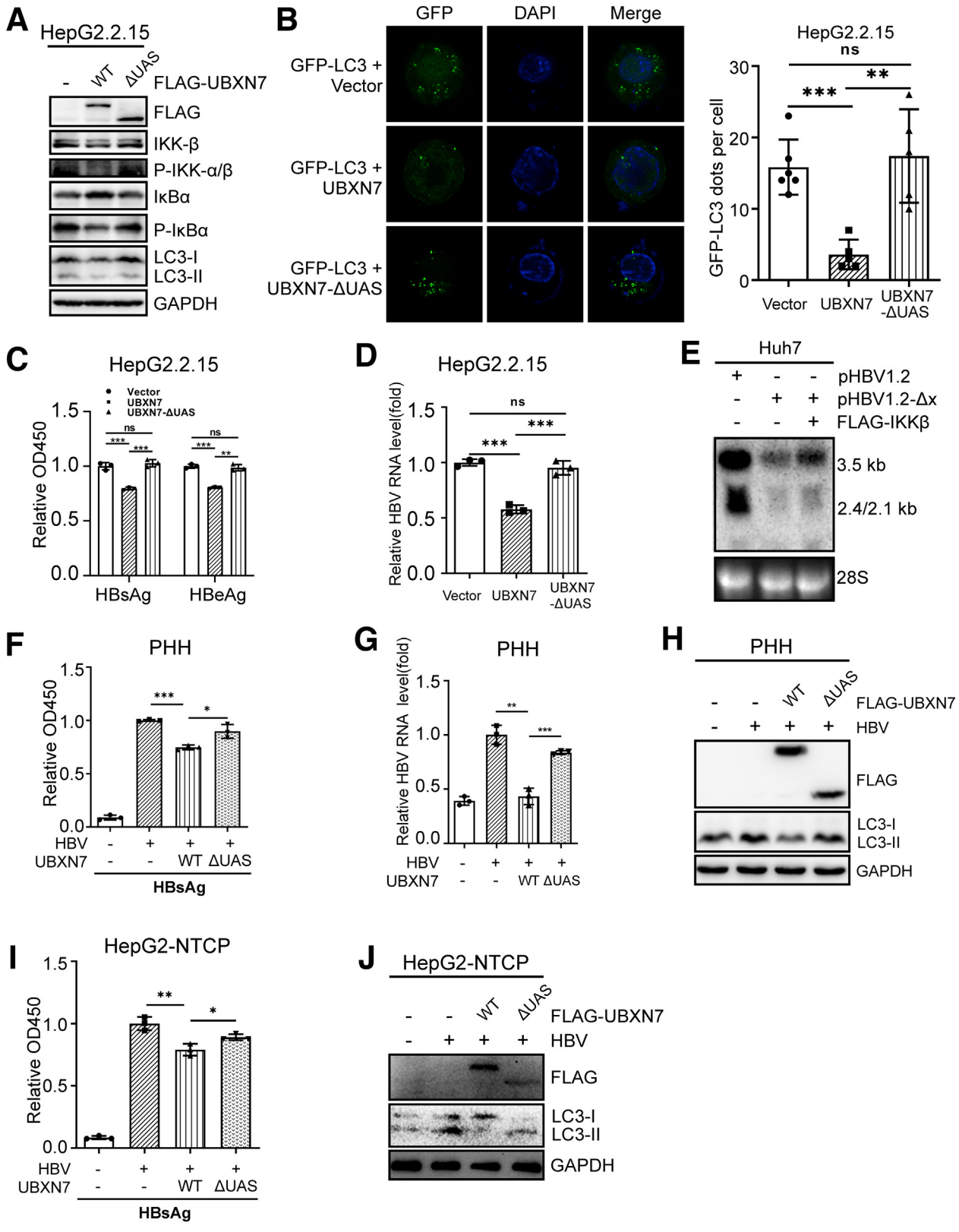


Figure 6. UBXN7 interacts with IKK-β via the UAS domain and up-regulates K48-linked ubiquitination of IKK-β. (*A*) UBXN7 up-regulates K48-linked ubiquitination of IKK-β in the presence of MG132. Huh7 cells were transfected with FLAG-IKK-β, various HA-Ub (WT, K48, K63) and MYC-UBXN7 plasmids. Cells were cultured in presence of MG132 and used for immunoprecipitation and Western blot analysis. Note that K48-linked ubiquitination of UBXN7 was increased after MG132 treatment. (*B*) The ULD domain of IKK-β is necessary and sufficient for its interaction with UBXN7. Huh7 cells were transfected with various FLAG-IKK-β mutants and MYC-UBXN7 plasmids as indicated, and cell extracts were used for co-immunoprecipitation analysis. Note that any mutant contains the ULD domain could bind UBXN7, whereas those lacking it could not. (*C*) UAS domain of UBXN7 is required for its interaction with IKK-β. Huh7 cells were transfected with various FLAG-UBXN7 mutants and MYC-IKK-β plasmids as indicated, and cell extracts were used for co-immunoprecipitation analysis.

fact, reduction of IKK-β protein level by UBXN7 only occurred in the absence of HBx expression, suggesting that the effect of UBXN7 on IKK-β protein levels is also dependent on HBx, most likely via the interaction between HBx and DDB1-containing E3 ubiquitin ligase. Thus, UBXN7 treatment may lead to destabilization of IKK-β, which may

Figure 5. (***See previous page***). **UBXN7 inhibits HBV replication.** (*A–C*) Overexpression of UBXN7 inhibits HBV protein and RNA levels in transfected cells in a dose-dependent manner. Huh7 (*A*) and HepG2 (*B*) cells were transfected with pHBV1.3 plasmids and control vector (-) or FLAG-UBXN7 plasmids as indicated. HepG2.2.15 (*C*) cells were transfected with control vector (-) or FLAG-UBXN7 plasmid as indicated. Levels of secreted hepatitis B surface antigen (HBsAg) and hepatitis B e antigen (HBeAg) were determined by ELISA from the supernatants (*left*), and levels of HBV RNA were determined by real-time PCR (*right*). (*D–F*) Knockdown of UBXN7 enhances HBV protein and RNA levels in transfected cells. Huh7 (*D*) and HepG2 (*E*) cells were transfected with pHBV1.3 plasmids and control oligo or siUBXN7 oligo as indicated. HepG2.2.15 (*F*) cells were transfected with control oligo or siUBXN7 oligo as indicated. Levels of the secreted HBsAg and HBeAg were determined by ELISA from the supernatants (*left*), and levels of HBV RNA were determined by real-time PCR (*right*). (*G*) Northern blot analyses of HBV RNA level show that UBXN7 inhibits HBV replication. Huh7 cells were transfected as in (*A*) (*left*), and HepAD38 cells were transfected with control vector or FLAG-UBXN7 plasmids as indicated (*right*). HBV RNA levels were analyzed. (*H*) Northern blot analyses of HBV RNA level show that overexpression of UBXN7 inhibits HBV transcription. HepG2 cells were co-transfected with pHBV1.2-Δx, pHBV1.2, and FLAG-UBXN7 plasmids as indicated. (*I*) Northern blot (*left panel*) and Western blot (*right panel*) analyses of HBV RNA level and UBXN7 protein level show that knockdown of UBXN7 enhances HBV transcription. Huh7 cells were co-transfected with pHBV1.2-Δx, pHBV1.2, and FLAG-UBXN7 plasmids as indicated. (*J*) Southern blot analyses of HBV DNA level show that UBXN7 inhibits HBV replication. Huh7 cells were transfected as in (*A*), and HBV DNA levels were analyzed. (Three independent experiments were performed, and 1 representative dataset is shown. $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$ in *A–F*).


A
HepG2.2.15
- WT ΔUAS
FLAG-UBXN7
FLAG
IKK-β
P-IKK-α/β
IκBα
P-IκBα
LC3-I
LC3-II
GAPDH
B
GFP
DAPI
Merge
GFP-LC3 + Vector
GFP-LC3 + UBXN7
GFP-LC3 + UBXN7-ΔUAS
HepG2.2.15
ns
**
***
GFP-LC3 dots per cell
Vector
UBXN7
UBXN7 -ΔUAS
C
HepG2.2.15
Vector
UBXN7
UBXN7-ΔUAS
Relative OD450
HBsAg
HBeAg
D
HepG2.2.15
Relative HBV RNA level(fold)
Vector
UBXN7
UBXN7 -ΔUAS
E
Huh7
pHBV1.2
pHBV1.2-Δx
FLAG-IKKβ
3.5 kb
2.4/2.1 kb
28S
F
PHH
Relative OD450
HBV
UBXN7
WT ΔUAS
HBsAg
G
PHH
Relative HBV RNA level(fold)
HBV
UBXN7
WT ΔUAS
H
PHH
FLAG-UBXN7
HBV
FLAG
LC3-I
LC3-II
GAPDH
I
HepG2-NTCP
Relative OD450
HBV
UBXN7
WT ΔUAS
HBsAg
J
HepG2-NTCP
FLAG-UBXN7
HBV
FLAG
LC3-I
LC3-II
GAPDH

contribute to reduced activates NF-κB signaling. In addition, because NF-κB signaling is involved in hepatocellular carcinogenesis, reducing NF-κB signaling may also reduce hepatocellular carcinogenesis in patients with chronic hepatitis B. Further investigations into this are clearly warranted.

In summary, we have shown that HBx interacts with UBXN7 to promote K48-linked ubiquitination of UBXN7 for its degradation. Our results reveal that IKK-β is the first reported binding partner of the UAS domain of UBXN7 and that its interaction with UBXN7 leads to increased IKK-β ubiquitination and proteasomal degradation. We further show that this UBXN7-induced degradation of IKK-β decreases the activation of NF-κB signaling and autophagy, thereby inhibiting HBV replication. Moreover, we have shown that HBx can promote HBV replication via degradation of UBXN7, thus maintaining high levels of IKK-β to activate NF-κB signaling and NF-κB–dependent autophagy (Figure 8*F*). Our findings suggest that UBXN7 can be targeted for potential new therapies in HBV-related diseases.

Materials and Methods

Cell Culture

HEK293, Huh7, and HepG2 cells were cultured in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum. HepAD38 and HepG2-NTCP were maintained in the same medium but with 50 μg/mL Collagen Type 1 Rat tail spreading on the bottom in advance. Tetracycline can suppress HepAD38 to replicate HBV. HepG2.2.15 was cultured in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum and 400 μg/mL G418. PHHs were purchased, cultured, and infected as described.[17] All cells were maintained at 37°C in humidified CO_2 (5%) incubators.

Plasmids and Transfection

The pHBV1.3 plasmid was provided by Guangxia Gao (Chinese Academy of Sciences, China). pEF-UBXN7 and pEF-IKK-β were generated by PCR cloning into pEF vector. UBXN7 mutants with an arginine substitution at residue lysine 84 (K84R) and lysine 99 (K99R) residues were generated by PCR-based mutations on pEF-UBXN7. Multiple truncated mutants of UBXN7 (Δ-UBA, Δ-UAS, Δ-UIM, Δ-UBX, 1-261, 1-305, and 136-489) and IKK-β (1-680, 1-400, 1-308, 308-756, and 400-756) were generated by PCR-based mutations on pEF-UBXN7 or pEF-IKK-β. NF-κB luciferase reporter plasmid, pEF-IκBα, pEF-P65, pEF-TAK1, pEF-IKK-α, pRL-TK, pRK-Ub, and pRK-Ub mutant plasmids were generated by PCR cloning into pEF or pRK vector as described.[30] All primers are listed in Table 1. All plasmids were confirmed by DNA sequencing.

Chemicals, Antibodies, and Other Reagents

Cyclohexamide (C7698), chloroquine (C6628), ammonium chloride (A9434), MG-132 (M7449), and protease inhibitor cocktail (P1860) were purchased from Sigma-Aldrich (St Louis, MO). Tetracycline (MB5564) was purchased from Meilunbio (Dalian, China). Anti-phospho-IKKα/β (#2697), anti-total-IKKβ (#2684), anti-phospho-IκBα (#9246S), anti-total-IκBα (#4812S), and anti-LC3I/II (#4108) antibodies were purchased from Cell Signaling Technology (Danvers, MA). Anti-β-tubulin (10094-1-AP), anti-GAPDH (10494-1-AP), anti-Myc-Tag (16286-1-AP), anti-Flag-Tag (20543-1-AP), and anti-HA-Tag (51064-2-AP) antibodies were purchased from Proteintech (Wuhan, China). Anti-HBcAg (MAB16990) antibody was purchased from Millipore (Temecula, CA). Anti-UBXN7 (NBP 2-2 2223) antibody was purchased from Novus Biologicals (Littleton, CO). Horseradish peroxidase–conjugated secondary antibody (71045-M and 12-348) and Flag Beads (M8823) were purchased from Sigma-Aldrich. Collagen Type 1 Rat tail (354236) was purchased from Corning (New York, NY).

Small interfering RNAs (siRNAs) (stB0010599) were purchased from RiboBio (Guangzhou, China). Cells were co-transfected with 3 siRNAs targeting UBXN7 at a concentration of 50 nmol/L according to the manufacturer's

Figure 7. (***See previous page***). **UBXN7 inhibits HBV replication in an autophagy-dependent manner.** (*A*) UAS domain is required for UBXN7 to inhibit NF-κB signal pathway. HepG2.2.15 cells were transfected with FLAG-UBXN7 or FLAG-UBXN7-ΔUAS mutant plasmid. Levels of indicated proteins were detected. Note that deletion of UAS domain abolished the ability to UBXN7 to reduce NF-κB signaling as reflected by reduction in p-IκBα. (*B*) UAS domain is required for UBXN7 to reduce autophagy. Cells were transfected with GFP-LC3, a marker for autophagy, and control or UBXN7 expression plasmid and analyzed by confocal microscopy (*left*). The GFP-LC3 puncta were quantified (*right*). Note that overexpressing UBXN7 decreased autophagy, and deletion of UAS domain abolished this ability. (*C* and *D*) UAS domain of UBXN7 is required for its ability to inhibit HBV RNA and protein levels in HepG2.2.15. Cells were transfected as in (*A*). The secreted HBsAg and HBeAg (*C*) and HBV RNA (*D*) were determined. (*E*) IKK-β–mediated NF-κB signaling is critical for the function of HBx to regulate viral transcription by inhibiting UBXN7. Huh7 cells were transfected with pHBV1.2, pHBV1.2-Δx, and FLAG-IKK-β plasmids. Levels of HBV RNA were detected by Northern blot. (*F–H*) UAS domain of UBXN7 is required for its ability to inhibit HBV RNA and protein levels and to induce autophagy in primary human hepatocytes (PHHs). PHH cells were infected with HBV viruses, followed by transfection with FLAG-UBXN7 or FLAG-UBXN7-ΔUAS mutant plasmid. Levels of the secreted HBsAg (*F*), HBV pgRNA (*G*), and autophagy marker LC3 (*H*) were then determined. Note increased level of LC3-II in presence of UBXN7 but not the UAS deletion mutant. (*I* and *J*) UAS domain of UBXN7 is required for its ability to inhibit expression of HBsAg and to induce autophagy in HepG2-NTCP cells. HepG2-NTCP cells were infected with HBV viruses, followed by transfection with FLAG-UBXN7 or FLAG-UBXN7-ΔUAS mutant plasmid. Levels of secreted HBsAg (*I*) and autophagy marker LC3 (*J*) were then determined. Note increased level of LC3-II in presence of UBXN7 but not the UAS deletion mutant. (Three independent experiments were performed, and representative data are shown. ns $P \geq .05$, $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$ in *B–D*, *F*, *G*, and *I*).

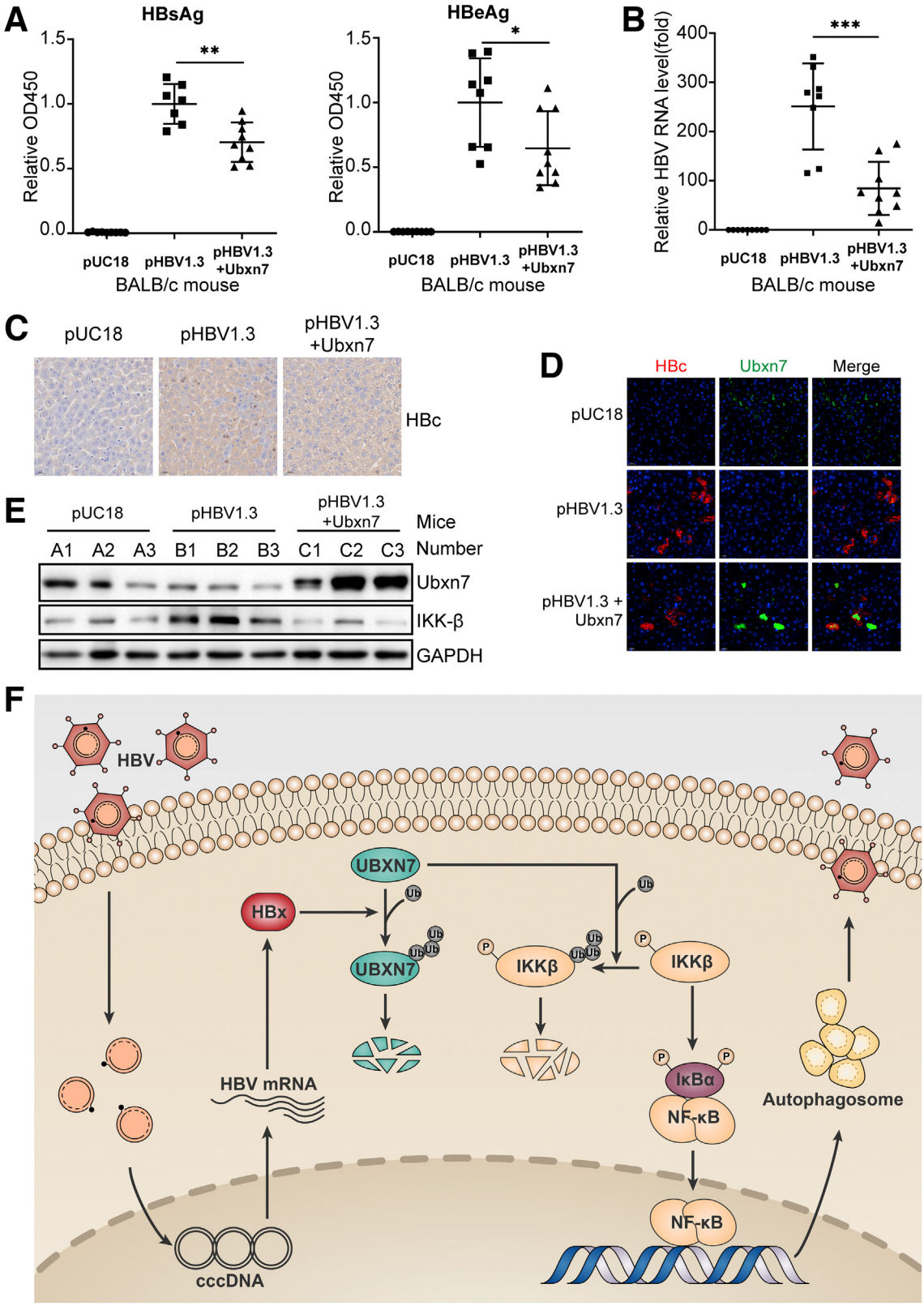

A
HBsAg
Relative OD450
1.5
1.0
0.5
0.0
**
pUC18
pHBV1.3
pHBV1.3 +Ubxn7
BALB/c mouse
HBeAg
*
B
Relative HBV RNA level(fold)
400
300
200
100
0
***
C
pUC18
pHBV1.3
pHBV1.3 +Ubxn7
HBc
D
HBc
Ubxn7
Merge
E
Mice
A1 A2 A3 B1 B2 B3 C1 C2 C3
Number
Ubxn7
IKK-β
GAPDH
F
HBV
UBXN7
HBx
Ub
P
IKKβ
HBV mRNA
IκBα
NF-κB
Autophagosome
cccDNA

Table 1. List of the Primer and siRNA Sequences

Name	Sequences
UBXN7-FP	5-CCGGAATTCATGGCTGCCCACGGGGGCTCC-3
UBXN7-RP	5-CTAGTCTAGATTAATTTCTTTCCTGTACAAAGACAGTCT-3
HBV-HBx-FP	5-TCTGTGCCTTCTCATCTGC-3
HBV-HBx-RP	5-TCGGTCGTTGACATTGCTG-3
IKK-β-FP	5-CCGTAGCTAGCATGAGCTGGTCACCTTCCCTGACA-3
IKK-β-RP	5-GGCGACGCGTTCATGAGGCCTGCTCCAGGCAGCT-3
HBV-RNAs-FP	5-GCACTTCGCTTCACCTCTGC-3
HBV-RNAs-RP	5-CTCAAGGTCGGTCGTTGACA-3
HBV-pgRNA-FP	5-TGTTCAAGCCTCCAAGCT-3
HBV-pgRNA-RP	5-GGAAAGAAGTCAGAAGGCAA-3
LC3B-FP	5-GCCGCACCTTCGAACAAAGA-3
LC3B-RP	5-GATTGGTGTGGAGACGCTGA-3
si-h-UBXN7-1	5-GCAAGTGTCTCTACTGTCA-3
si-h-UBXN7-2	5-GTTGCGGTATCCAGATGGA-3
si-h-UBXN7-3	5-CCATTGATTTGATGCATAA-3

instructions, and protein or RNA was extracted 48 hours after transfection for experiments.

RNA Extraction and Real-Time Quantitative Polymerase Chain Reaction

Total RNA was extracted from cells by using TRIzol reagent (Invitrogen, Thermo Fisher Scientific, Waltham, MA) and subjected to reverse transcription by using M-MLV Reverse Transcriptase Kit (Invitrogen, Thermo Fisher Scientific). Real-time quantitative PCR was performed with the SYBR Select Master Mix (Life Technologies, Carlsbad, CA) with indicated primers.

Quantitative Ubiquitylome and Proteome Analyses

SILAC was performed as described.[13] The HepG2.2.15 and HepG2 cells were cultured in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum and either the "light" form [$^{12}C_6$]-$_L$-lysine/[$^{12}C_6{}^{14}N_4$]-$_L$-arginine or the "heavy" form [$^{13}C_6$]-$_L$-lysine/[$^{13}C_6{}^{15}N_4$]-$_L$-arginine for more than 7 generations by using SILAC Protein Quantitation Kit according to manufacturer's instructions. After SILAC labeling, the cell samples were collected for protein extraction, trypsin digestion, high-performance liquid chromatography fractionation, affinity enrichment, and liquid chromatography with tandem mass spectrometry analysis to identify the host protein and ubiquitination changes.

Co-Immunoprecipitation and Western Blot Analyses

Cells were co-transfected with 5 μg each of 2 differently tagged plasmids for 48 hours. Cells were lysed with IP buffer: 20 mmol/L Tris-Cl (pH 8.0), 150 mmol/L NaCl, 2 mmol/L EGTA, 1% NP-40, and protease inhibitor cocktails. The lysates were centrifuged at 12,000 rpm for 5 minutes. Equal number of supernatants were incubated with anti-Flag- or anti-Myc-beads overnight at 4°C. Beads were washed 4 times with beads washing buffer (20 mmol/L Tris-Cl [pH 7.5], 300 mmol/L NaCl) before Western blot analysis. For Western blot, protein samples were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis on a 4%-20% gradient Tris-glycine gel and transferred to a nitrocellulose membrane. The membranes were blocked for 1 hour at room temperature with 5% skim milk in TBS-T (0.1% Tween 20), followed by incubation overnight at 4°C with primary antibodies. After washing in TBS-T 3 times, the membranes were incubated with horseradish peroxidase–conjugated secondary antibodies for 1 hour at room temperature. The immunoreactive bands were visualized with enhanced chemiluminescence system (Yeasen, China).

Figure 8. (***See previous page***). **Overexpressed UBXN7 inhibits HBV replication in vivo.** (*A* and *B*) UBXN7 inhibits expression of HBsAg and HBeAg and HBV RNA in vivo. The pHBV1.3 and UBXN7 expression plasmid or control plasmid were hydrodynamically injected into BALB/c mice, and the mice were killed 4 days later. HBsAg and HBeAg levels (*A*) in the blood were determined by ELISA, and HBV RNA level (*B*) in the liver was determined by real-time PCR. (*C* and *D*) UBXN7 reduces levels of HBc in vivo. HBc protein level in the liver of the BALB/c mice in (*A*) was determined by immunohistochemistry (*brown signal*) (*C*) and immunofluorescence (*D*). (*E*) UBXN7 inhibits NF-κB signaling in vivo. UBXN7 and IKK-β protein levels in the liver of the BALB/c mice in (*A*) were determined by Western blot analysis. A1-3, B1-3, and C1-3 indicate 3 different animals for each group. (*F*) A proposed model. HBV down-regulates UBXN7 and thus prevents UBXN7-targeted IKK-β degradation, leading to increased HBV transcription and replication via enhanced NF-κB signaling. (Three independent experiments were performed, and representative data are shown for *A–E*. $^*P < .05$, $^{**}P < .01$, $^{***}P < .001$ in *A* and *B*).

Northern and Southern Blot Analysis

Cells were co-transfected with 10 μg plasmids for 48 hours. Total RNA was electrophoresed in a 1% agarose formaldehyde gel and was transferred onto a nylon membrane. Intracellular HBV core particle-associated DNA was extracted, and Southern blot analysis was performed as described previously.[31] The blot was detected by using the DIG Northern starter kit (Roche Diagnostics, Indianapolis, IN) for Northern blot corresponding to nucleotides 1072 to 2171 of the HBV genome and for Southern blot corresponding to nucleotides 157 to 1068 of the HBV genome.

Luciferase Activity Assay

Cells were co-transfected with 200 ng firefly luciferase reporter vector, 20 ng internal control renilla luciferase control vector (pRL-TK), and either 400 ng UBXN7 expression construct or control vector for 36 hours. Luciferase activities in cell lysates were detected by using the Dual-Glo system (Promega, Madison, WI). The firefly luciferase activity was normalized by renilla luciferase activity.

Enzyme-Linked Immunosorbent Assay

Cells were co-transfected with 1 μg pHBV1.3 and 1–2 μg of either UBXN7 expression construct or the control vector for 48 hours. The levels of HBV surface antigen and HBV e antigen in the supernatant were determined by using ELISA (Kehua Bio-engineering, Shanghai, China).

Hydrodynamics-Based Transfection in Mice

BALB/c mice at 10 weeks old were purchased from the Hubei Provincial Center for Disease Control and Prevention (Wuhan, China). Briefly, 10 μg pHBV1.3 and 20 μg of either UBXN7 expression construct or the control vector were premixed with normal saline in a volume equivalent to 10% body weight and injected via tail vein within 6–8 seconds. Ninety-six hours later, the liver tissue was isolated for immunohistochemical staining and Western blot analysis. Sera were collected for ELISA analysis of HBV surface antigen and HBV e antigen. All mice were housed in a pathogen-free mouse colony, and the animal experiments were performed according to the Guide for the Care and Use of Medical Laboratory Animals (Ministry of Health, People's Republic of China, 1998).

Autophagosome Formation Assay

Cells were co-transfected with 500 ng GFP-LC3 and 1 μg of either UBXN7 (WT or ΔUAS) expression construct or the control vector for 48 hours. The cells were fixed, and the nuclei were stained with 4-6-diamidino-2-phenylindole (Promoter Biotechnology). The green fluorescent protein-LC3 fluorescence was observed under a confocal fluorescence microscope (Leica SP8, Wetzlar, Germany).

Statistical Analysis

All experiments were performed at least 3 times. Statistical significance was determined by using unpaired Student t test between 2 groups. $P < .05$ was considered significant ($^*P < .05$, $^{**}P < .01$, $^{***}P < .001$). All data analyses were carried out by using the statistical analysis software GraphPad Prism, version 6.0 (GraphPad Software, San Diego, CA).

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