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Letter

Non-proteolytic ubiquitination of HBx controls HBV replication

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Dear Editor,

Although effective vaccines and antiviral therapies are available, hepatitis B virus (HBV) infection is still a serious global health threat. Persistent HBV infection remains the principal cause of liver cirrhosis and hepatocellular carcinoma (HCC). HBV is a small DNA virus, owning a ~3.2 kb genome that encodes several proteins: viral DNA polymerase, core antigen (HBcAg), E antigen (HBeAg), three surface antigens (PreS1/PreS2/HBsAg), and a regulatory X protein (HBx) (Lamontagne et al., 2016). X protein, named for its lack of homology with any known proteins, is a 154 aa protein that plays an essential role in HBV biology and regulates the development of HCC (Yang et al., 2022). Although previous studies have strongly expanded our understanding of HBx, the regulation of HBx is not completely elucidated.

The E3 ligase tripartite motif-containing 21 (TRIM21) is the highest affinity cytosolic antibody receptor in human and thus provides intracellular immunity against antibody-attached pathogens (Foss et al., 2015). Recently, TRIM21 was found to ubiquitylate HBV DNA polymerase and suppress HBV replication (Mu et al., 2020). It remains unknown whether TRIM21 regulates other HBV proteins. In the study here, we have proved that human TRIM21 is a critical inhibitory factor for HBV replication by catalyzing non-proteolytic HBx ubiquitination.

First, we performed a whole-genome profiling of the relationships between human E3 ubiquitin (Ub) ligases and HBV integration (Fig. 1A, Supplementary Tables S1–S4), based on a published transcriptome sequencing dataset of 50 HCC samples (88 % HBV-positive) and 5 matched tumor-adjacent normal samples from 50 Chinese patients (GEO#: GSE65486) (Dong et al., 2015). Thirty-six human E3 ligases were found to probably restrict HBV integration to the human genome, of which TRIM21 caught our attention (Fig. 1A). As shown, the expression level of TRIM21 in patients was negatively correlated with the quantity of HBV integration (Fig. 1B), proposing an inhibitory role of TRIM21 toward HBV infection. Moreover, to assess the clinical importance of TRIM21, the expression levels of TRIM21 in the liver biopsies from three HBV-negative patients and five chronic hepatitis B patients (CHB) were detected (Supplementary Fig. S1A and S1B). In particular, a patient with a high HBV DNA level showed an undetected protein level of TRIM21 in the livers. However, the HBV-negative/low patients highly expressed TRIM21 in the livers, showing a potential negative correlation between TRIM21 level and HBV infection. A recently described Cre/loxP-based system generating HBV recombinant covalently closed circular DNA (rcccDNA) allows recapitulation of HBV replication cycle (Qi et al., 2014). So, a hydrodynamic gene injection (HDI)-based mouse model for HBV infection was utilized to test the inhibitory effect of TRIM21 *in vivo* (Supplementary Fig. S1C). The serum levels of HBsAg were readily decreased in mice co-injected with HBV prcccDNA and TRIM21-encoding plasmid, compared with those only injected with prcccDNA (Fig. 1C). Therefore, TRIM21 works as a potential suppressor for HBV infection.

At the cellular level, TRIM21 overexpression inhibited the production of HBcAg, HBeAg, HBsAg, and pgRNA of HBV, which partially recapitulates the impaired phenotype of HBx-deficient HBV (HBx-C22T)

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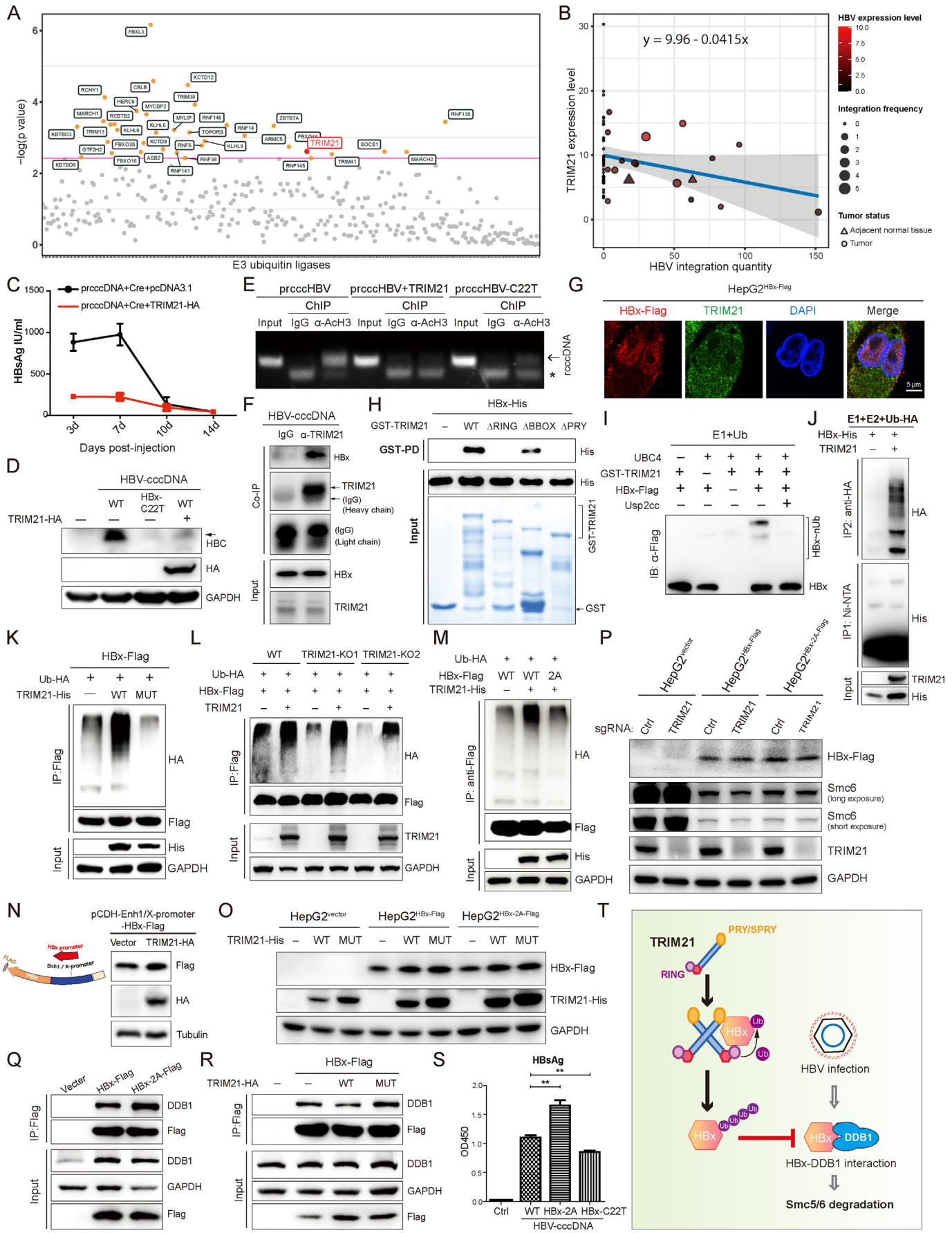

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Fig. 1. TRIM21 inhibits HBV replication by triggering HBx for non-proteolytic ubiquitination. **A** Distribution patterns of human genome-scale E3 ligases owning strong negative correlations with HBV integration. The horizontal red line indicates the correlation threshold of selective sweep selection signals (top 10 %). **B** Correlation between *TRIM21* gene expression and HBV integration degree. The x-axis represents the levels of HBV integrated quantity in each sample, while the y-axis shows the gene expression level of TRIM21 measured in FPKM (Fragments per Kilobase of transcript per Million mapped reads). The size of the dot corresponds to the frequency of HBV integration events on human genome. The color of the dot represents the level of HBV transcriptome expression, and the shape of the dot indicates the status of the sample. **C** TRIM21 suppresses HBV replication in mice. Mice were hydrodynamically co-injected with pCMV-Cre, HBV-prcccDNA, along with or without TRIM21 (n = 10 per group). The levels of serum HBsAg were determined by ELISA. **D** TRIM21 suppresses HBcAg expression in HepG2 cells. Cells were transfected with HBV prcccDNA system with or without TRIM21. Anti-HBcAg blot was conducted in infected cells. **E** Chromatin immunoprecipitation assay revealed that TRIM21 decreased H3 acetylation in HBV mini-chromosome. Chromatin was prepared from HepG2 cells co-transfected with HBV-prcccDNA (WT or HBX-C22T) and TRIM21, then was immunoprecipitated (IP) with control IgG or a specific anti-AcH3 antibody. Immunoprecipitated chromatin was analyzed by RT-PCR with HBV rcccDNA-specific primers. Asterisk indicates non-specific signals which may be primer dimers. **F** TRIM21 interacts with HBx in the recombinant HBV cccDNA system. HepG2 cells transfected with prcccDNA/Cre system were harvested at three days post transfection, and subjected into anti-TRIM21 Co-IP assay. Co-IP samples were analyzed by IB using anti-HBx or anti-TRIM21 antibody. An anti-rabbit light-chain antibody was used in the anti-TRIM21 Western blot. **G** Co-localization of HBx with TRIM21 in HBx-expressing HepG2 stable cells. HepG2 stably expressing HBx-Flag was stained with anti-Flag antibody (Red) and anti-TRIM21 antibody (green). Scale bar, 5 μm. **H** Mapping HBx-interacting domains of TRIM21. GST or GST-fused full-length or truncated TRIM21 were incubated with purified HBx-His protein, and subjected to GST pull-down assay. PD: pull-down. **I** TRIM21 ubiquitylated HBx *in vitro*. HBx-Flag was purified from HEK293T cells using anti-Flag M2 affinity beads and were subjected to ubiquitylation reaction mixture containing E1, UBC4 (E2), Ub, GST-TRIM21. After the reaction, samples were subjected to anti-Flag IB. Usp2cc: the catalytical core of human deubiquitylating enzyme USP2. **J** TRIM21 ubiquitylated HBx in *E. coli*-based system. Reconstituting the *E. coli* ubiquitylation system by transforming pET22b-HBx and YESS^{UB} vector that expresses HA-Ub, E1 (UBA1), E2 (UBCH5a) with or without E3 (TRIM21) into *E. coli* BL21. After a two-step IP, the samples were detected with indicated antibodies. **K** TRIM21 ubiquitylates HBx in mammalian cells. HEK293T cells were co-transfected with plasmids encoding Ub-HA, HBx-Flag, His_6-tagged WT TRIM21 or E3-dead TRIM21-MUT. Flag-tagged proteins were enriched by anti-Flag beads in denaturing buffer, and then subjected to anti-HA IB. **L** TRIM21 deficiency in host cells attenuates HBx ubiquitination. WT or TRIM21-KO HEK293T cells were co-transfected with indicated plasmids for 24 h. Cell lysates were subjected to anti-Flag IP and anti-HA IB. **M** T/S-to-A substitutions at T81/S104 (HBx-2A) attenuated TRIM21-mediated ubiquitylation of HBx. **N** Viral promoter-driven HBx expression cannot be affected by TRIM21 overexpression. HBx-expressing plasmid with HBV Enh1/X-promoter was constructed and co-transfected with TRIM21 plasmid for 48 h. **O** TRIM21 overexpression in HBx-expressing HepG2 stable cells does not promote HBx degradation. Empty vector, TRIM21, or TRIM21-MUT plasmids were transfected into the HepG2 cell lines stably expressing Flag-tagged HBx or HBx-2A, for 24 h. HBx protein levels were then determined by anti-Flag immunoblotting analysis. **P** Knockdown of TRIM21 cannot lead to HBx accumulation in HBx-expressing HepG2 stable cells. The pLenti-CRISPRv2 plasmids expressing non-target (Ctrl) sgRNA or TRIM21-sgRNA, psPAX2, and pMD2.G, were co-transfected into HEK293T to generate lentivirus. HepG2 cells were then subjected to lentivirus transduction, blasticidin selection, and IB analysis with indicated antibodies. **Q** HBx-2A mutant shows a stronger interaction with DDB1 than HBx. HEK293T expressing Flag-tagged WT HBx or HBx-2A were subjected to anti-Flag IP, and subsequently anti-DDB1 IB. **R** TRIM21 disrupts the recruitment of endogenous DDB1 to HBx. Lysates of HEK293T expressing HBx-Flag and HA-TRIM21 or its E3-dead mutant were lysed and subjected to anti-Flag IP, followed by anti-DDB1 blot. **S** Introduction of HBx-2A mutation into HBV genome increased the expression of HBs and HBe antigens in the culture medium of HepG2 cells. Data were represented as mean ± s.d. from three biological replicates, $^{**}P < 0.01$ (Student's *t*-test). **T** Schematic illustration of the mechanism by which TRIM21 inhibits HBV replication. Host TRIM21 homodimer recruits HBx with its PRYSPRY and RING domains, and catalyzes Ub conjugates on HBx, which disrupts its interaction with host DDB1 and finally suppresses HBV replication in human cells.

(Fig. 1D, Supplementary Fig. S1E and S1F). However, an E3 activity-dead TRIM21 (TRIM21-MUT) lost the ability partly to restrict HBV replication (Supplementary Fig. S1D and S1E). Because HBx can promote acetylation of cccDNA-bound histones, HBx deficiency impaired histone H3 acetylation in the cccDNA-containing chromosome, similar to TRIM21 overexpression (Fig. 1E). Thus, these results propose a possibility that human TRIM21 may have potential HBx-associated functions.

To test the hypothesis, a series of assays were then carried out to verify HBx interaction with TRIM21. Firstly, HBx readily captured TRIM21 in a co-immunoprecipitation (Co-IP) assay, suggesting that HBx can form a complex with TRIM21 in human cells (Supplementary Fig. S2A). Moreover, in the recombinant cccDNA system, enrichment of endogenous TRIM21 was found to recruit HBx expressed by the HBV genome (Fig. 1F). A GST pull-down assay also demonstrated that TRIM21 was captured by GST-HBx fusion protein, but not alone GST (Supplementary Fig. S2B). Additionally, the co-localization of HBx and endogenous TRIM21 was observed, in both the nucleus and the cytosol of HBx-expressing HepG2 stable cells (HepG2$^{HBx\text{-}Flag}$), in an immunofluorescence assay (Fig. 1G). Thus, HBx indeed binds to TRIM21.

TRIM21 has a RING finger domain, a B-Box domain, a coiled-coil domain, and a C-terminal PRYSPRY domain (Supplementary Fig. S2C) (Keeble et al., 2008). To investigate which domain is required for HBx binding, several truncated mutants of TRIM21 were constructed and purified, and sequentially subjected to a GST pull-down assay. Deletion of RING or PRYSPRY domain of TRIM21 completely abolished its binding to HBx, while the B-Box-deleted mutant still showed a robust interaction (Fig. 1H). As TRIM21 usually exists as a cross-linked homodimer, in which the PRYSPRY domain of one TRIM21 protomer is spatially close to the RING domain of another promotor (Foss et al., 2015), we speculate that the PRYSPRY and RING domains of TRIM21 dimer may form a docking conformation for HBx.

Since TRIM21 is a RING-type E3 ligase, we next explored whether TRIM21-HBx interaction could induce HBx ubiquitination. To this end, we resembled an *in vitro* ubiquitylation system containing ubiquitin, ATP, E1, E2, TRIM21, and HBx. As shown, HBx was ubiquitylated by TRIM21 *in vitro* (Fig. 1I). Treatment with the catalytic core of ubiquitin-specific protease 2 (Usp2cc) completely eliminated TRIM21-catalyzed signals, indicating it is real ubiquitinated HBx. An *E. coli*-based ubiquitylation system was reconstituted as described before (Li et al., 2020), through co-expression of HBx and other ubiquitylation components in *E. coli* cells (Supplementary Fig. S2D). HBx was found to be markedly ubiquitylated once TRIM21 was presented (Fig. 1J). Moreover, expression of WT TRIM21, but not E3-dead TRIM21 mutant, increased HBx ubiquitylation in 293T (Fig. 1K). Additionally, we constructed *TRIM21* knockout (KO) HEK293T via CRISPR/Cas9 technology (Supplementary Fig. S2E), and found that TRIM21 deficiency significantly attenuated the ubiquitination level of HBx (Fig. 1L). Meanwhile, overexpression of TRIM21 readily rescued HBx ubiquitination. Notably, depletion of TRIM21 did not completely block HBx ubiquitination, suggesting that other E3 ligases may also target HBx in human cells (Fig. 1L). Similar results were also observed in TRIM21-knockdown HepG2$^{HBx\text{-}Flag}$ cells (Supplementary Fig. S2F). These data demonstrate that TRIM21 directly catalyzes the ubiquitination of HBx.

Several potential ubiquitination sites were next identified by Mass Spec analysis, and two of them (T81 and S104) were further verified by ubiquitylation assays (Supplementary Fig. S2G and S2H). Simultaneous mutation of T81/S104 to Ala (HBx-2A) robustly decreased HBx ubiquitination in HBx-2A-expressing HepG2 stable cells (HepG2$^{HBx\text{-}2A\text{-}Flag}$), compared with that in HepG2$^{HBx\text{-}Flag}$ cells (Supplementary Fig. S2F). Consistently, HBx-2A underwent less ubiquitination catalyzed by TRIM21 in 293T (Fig. 1M). Moreover, TRIM21 catalyzed the formation of polyUb chains on HBx with complicated linkages, because several Ub mutants carrying only one lysine shown similar abilities to form polyUb

chains on HBx (Supplementary Fig. S2I). Thus, TRIM21 catalyzes non-lysine ubiquitylation of HBx with multiple Ub linkages.

Importantly, as shown in Fig. 1N and Supplementary Fig. S3A, HBx expression driven by HBV enhancer I/X (Ehn1/X) promoter was not down-regulated by TRIM21 overexpression, which suggested that TRIM21 did not affect HBx synthesis. Also, CMV promoter-driven co-expression of HBx with TRIM21 did not decrease HBx protein level (Supplementary Fig. S2A), probably indicating a non-proteolytic function of the ubiquitylation. To validate this, HepG2^{vector}, HepG2$^{HBx\text{-}Flag}$, and HepG2$^{HBx\text{-}2A\text{-}Flag}$ stable cell lines were first constructed by lentivirus transduction (Supplementary Fig. S3B). HepG2$^{HBx\text{-}Flag}$ or HepG2$^{HBx\text{-}2A\text{-}Flag}$ cells stably expressed HBx or its mutant HBx-2A, avoiding the issue of inconsistent transfection efficiency in the co-transfection of two plasmids. Overexpression of WT TRIM21 or TRIM21-MUT in HepG2$^{HBx\text{-}Flag}$ or HepG2$^{HBx\text{-}2A\text{-}Flag}$ cells did not decrease the protein level of HBx or HBx-2A (Fig. 1O, Supplementary Fig. S3C). Instead, a weak increase of HBx protein level was continually observed in HepG2 cells overexpressing TRIM21, which could be explained by a possibility that TRIM21-mediated non-proteolytic ubiquitination could disrupt HBx degradation induced by other human E3 ligases, because one special Ub linkage conjugated on substate usually could interfere with the formation of other different Ub linkages (Sheng et al., 2020, 2023). Moreover, knockdown of *TRIM21* gene in HepG2$^{HBx\text{-}Flag}$ or HepG2$^{HBx\text{-}2A\text{-}Flag}$ cells did not induce HBx accumulation (Fig. 1P). Together, TRIM21-catalyzed non-lysine ubiquitination of HBx is a non-proteolytic modification.

Because previous studies had shown that HBx recruits a DDB1-contained E3 ligase to degrade host Smc5/6 complex and facilitate HBV transcription and replication (Decorsière et al., 2016), we further explored whether TRIM21-mediated HBx ubiquitylation might modulate DDB1 recruitment to HBx. In a Co-IP assay, the ubiquitylation-deficient HBx-2A showed a stronger capacity to recruit DDB1 than WT HBx (Fig. 1Q), indicating that the ubiquitinated HBx has weaker interaction with DDB1. Furthermore, it was WT TRIM21, but not the E3-dead TRIM21, that reduced endogenous DDB1 association with HBx (Fig. 1R), which suggests that TRIM21 may facilitate the stability of Smc5/6. Indeed, knockdown of *TRIM21* gene in HepG2$^{HBx\text{-}Flag}$ cells, but not in HepG2$^{HBx\text{-}2A\text{-}Flag}$ cells, led to a decrease in Smc6 protein level (Fig. 1P). Thus, TRIM21-catalyzed ubiquitylation of HBx disrupts the HBx-DDB1 interaction.

To explore whether TRIM21-catalyzed ubiquitylation could play roles in HBV replication, an HBV mutant carrying HBx-2A (HBV-2A) was constructed. HBV-2A was found to have an increased expression in HBsAg and HBeAg (Fig. 1S, Supplementary Fig. S3D). Because the interaction of HBx with DDB1 promotes the stimulatory activity of HBx (van Breugel et al., 2012), a HBV enhancer I-associated reporter assay was conducted. As shown, the overexpression of HBx readily increased the activity of HBV enhancer I (Supplementary Fig. S3E). More importantly, the enhancer I activity was much higher in cells expressing HBx-2A than HBx, proposing that the ubiquitin conjugates on HBx might decrease its ability to promote enhancer I activity, which finally prevents HBV replication. Together, non-proteolytic ubiquitylation of HBx suppresses HBV replication in human cells by disrupting the HBx-DDB1 interaction (Fig. 1T).

In this work, we have shown that human TRIM21 inhibits HBV replication via targeting HBx. During the preparation of our manuscript, a very relevant article has recently been published (Song et al., 2021), basically consistent to our study here. Firstly, TRIM21 was found to bind to HBx in both studies. Song et al. examined the association between TRIM21 and HBx through Co-IP, while we proved TRIM21-HBx interaction via not only *in vivo* assays but also *in vitro* GST pull-down assay. Moreover, we found that the RING and PRYSPRY domains of TRIM21 are responsible for HBx binding, which is consistent with their conclusion that RING and PRYSPRY domains are necessary for the antiviral role of TRIM21. Secondly, both studies investigated HBx ubiquitylation. It is worth noted that we performed several ubiquitination methods to fully elucidate HBx ubiquitylation catalyzed by TRIM21, including *in vivo* and *in vitro* ubiquitination assays, and an *E. coli*-based reconstituted ubiquitination system. Thirdly, both studies demonstrated that TRIM21 overexpression could suppress HBV replication in mice. Lastly, both studies revealed a similar mechanism. While Song et al. found that TRIM21 rescued Smc6 expression, our study showed that TRIM21 can disrupt HBx-DDB1 interaction and TRIM21 deficiency further decreases Smc6 in HBx-expressing HepG2 cells. Thus, these consistent results from two independent groups strongly support that TRIM21 targets HBx.

However, the biggest difference between these two independent studies occurred in exploring HBx stability. Song et al. concluded that TRIM21 targets HBx for degradation, but we did not observe HBx degradation in HBx-expressing HepG2 stable cells with TRIM21 overexpression. Our data found that the complicated Ub conjugates on HBx is non-proteolytic. We cannot exactly figure out how the difference arises, but we have noticed that Song et al. explored HBx degradation only using a co-transfection system. Some literature had demonstrated that, in a transient co-transfection system, inconsistent transfection efficiency or mutual translational inhibition of co-transfected plasmids may occur, depending on plasmid concentrations in the co-transfection system (Nejepinska et al., 2012, 2014; Stepanenko and Heng, 2017). Therefore, HBx degradation observed by Song et al. may be an artifact phenomenon of the co-transfection of HBx with TRIM21 plasmid. Although Song et al. also claimed that knockdown of TRIM21 by siRNA increased the HBx level in cells, we did not observe HBx accumulation in TRIM21-knockdown HepG2$^{HBx\text{-}Flag}$ cells. Notably, antibody-dependent HBx degradation by TRIM21 is an antibody-specific process (Zhang et al., 2018), which could not demonstrate the direct role of TRIM21 on HBx stability. Further investigations are still needed to clarify the real effect of TRIM21 on HBx stability. Together, the study here demonstrates that HBx is a non-proteolytic target of host E3 ligase TRIM21. Our results offer a novel insight into HBx regulation and provide a potential therapeutic target for HBV infection.

Footnotes

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