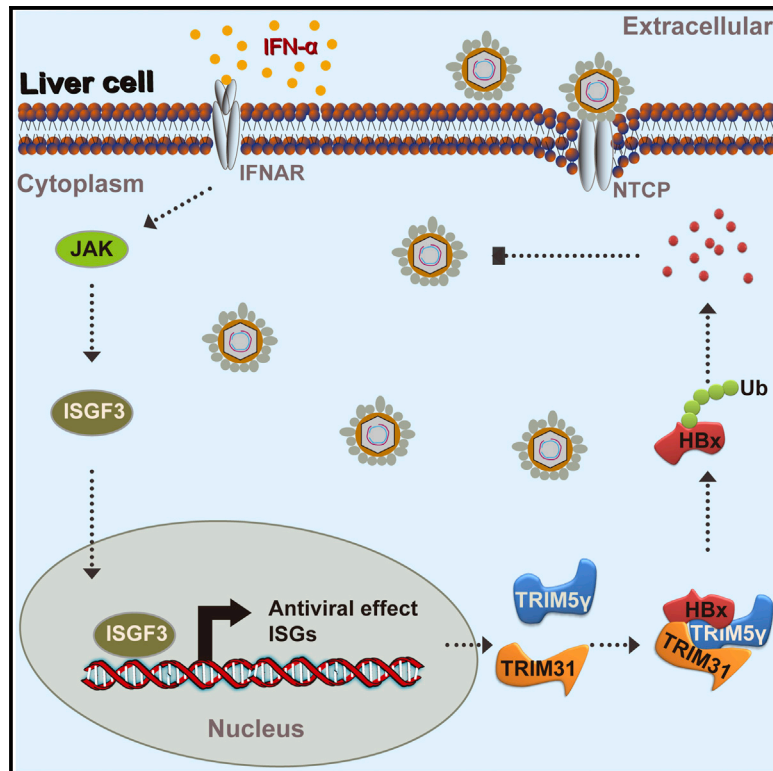


Type-I-IFN-Stimulated Gene TRIM5 γ Inhibits HBV Replication by Promoting HBx Degradation

Graphical Abstract



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In Brief

In brief, Tan et al. find that IFN-induced TRIM5 γ recruits TRIM31 to degrade HBx, resulting in suppression of hepatitis B virus replication.

Highlights

- Twenty-six in 145 interferon-stimulated gene products interact with HBx protein
- Seven HBx-interacting ISGs significantly inhibited HBV replication
- High induction of TRIM5 γ indicated a better IFN- α therapeutic effect
- B-Box domain of TRIM5 γ is sufficient to trigger HBx degradation by recruiting TRIM31



Type-I-IFN-Stimulated Gene TRIM5 γ Inhibits HBV Replication by Promoting HBx Degradation

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SUMMARY

To understand the molecular mechanisms that mediate the anti-hepatitis B virus (HBV) effect of interferon (IFN) therapy, we conduct high-throughput bimolecular fluorescence complementation screening to identify potential physical interactions between the HBx protein and 145 IFN-stimulated genes (ISGs). Seven HBx-interacting ISGs have consistent and significant inhibitory effects on HBV replication, among which TRIM5 γ suppresses HBV replication by promoting K48-linked ubiquitination and degradation of the HBx protein on the K95 ubiquitin site. The B-Box domain of TRIM5 γ under overexpression conditions is sufficient to trigger HBx degradation and is responsible both for interacting with HBx and recruiting TRIM31, which is an ubiquitin ligase that triggers HBx ubiquitination. High expression levels of TRIM5 γ in IFN- α -treated HBV patients might indicate a better therapeutic effect. Thus, our studies identify a crucial role for TRIM5 γ and TRIM31 in promoting HBx degradation, which may facilitate the development of therapeutic agents for the treatment of patients with IFN-resistant HBV infection.

INTRODUCTION

Chronic hepatitis B virus (HBV) infection remains a major public health threat. It is associated with increasing rates of hepatocellular carcinoma (HCC) worldwide. According to the World Health Organization (WHO), 2 billion people are infected with HBV, of which over 240 million are chronically infected, which places these people at a high risk for development of liver cirrhosis and eventu-

ally HCC. Although a vaccine has been available for HBV for almost 30 years, nearly 1 million people die each year due to HBV-related complications, including severe chronic hepatitis, cirrhosis, and primary liver cancer (Mutimer and Lok, 2012).

HBV is a partially double-stranded DNA virus belonging to the *Hepadnaviridae* family (Evans and Seeger, 2007). After entry into host cells, the viral genome translocates to the nucleus and is converted to covalently closed circular DNA (cccDNA), which is the transcription template for all HBV viral RNAs. Currently available HBV therapeutics (including INF- α and antiviral drugs) fail to eradicate the cccDNA reservoir from infected hepatocytes, despite suppressing replication of new viral DNA. Consequently, the persistent cccDNA results in post-treatment viral rebound (Nassal, 2015; Reville and Locarnini, 2016).

The HBV-encoded regulatory protein hepatitis B virus X protein (HBx) stimulates HBV gene expression from the cccDNA template; however, the mechanism by which HBx facilitates HBV replication is not clear (Keasler et al., 2007). HBx is a 154-amino-acid protein with an N-terminal negative regulatory domain and C-terminal transactivation or coactivation domain. HBx interacts with several cellular proteins, and its role in virus replication may be mediated through these interactions. The best-characterized HBx binding partner is the damage-specific DNA binding protein 1 (DDB1) (Sitterlin et al., 1997). The interaction between HBx and DDB1 is conserved among the HBx proteins from all mammalian hepadnaviruses and woodchuck hepatitis virus (WHV) X protein (Sitterlin et al., 1997). This binding is essential for HBV replication (Hodgson et al., 2012). It was recently reported that an HBx-DDB1-CUL4-ROC1 E3 ligase complex targets the SMC5/6 complex to enhance HBV gene expression from episomal cccDNA (Mitra and Guo, 2016; Murphy et al., 2016).

IFN therapy is an approved treatment modality for chronic HBV infection. However, the poor response and substantial side effects associated with this treatment impact its clinical utility (Tan et al., 2018b). HBV has been shown to inhibit IFN-stimulated genes (ISGs) and even the production of IFN in infected



patients (Khan et al., 2016; Lim et al., 2017; Liu et al., 2015; Xu et al., 2016). However, the mechanism of HBV-mediated inhibition of ISGs is poorly understood. Therefore, unraveling the molecular mechanisms of the interaction between IFN and HBV during the course of viral infection is a key imperative, which may help improve the efficacy of IFN therapy.

The induction of the type I IFN family (IFN- α , - β , - ϵ , - κ , and - ω) is perhaps the most important innate immune response to viral infection. Since its discovery in 1957, type I IFN has been recognized as the major antiviral cytokine in vertebrates (Hayes and Chayama, 2017; Isaacs and Lindenmann, 1957). Most nucleated vertebrate cells are able to both produce and respond to type I IFN. The antiviral function of type I IFN is carried out through its binding to the type I IFN receptor (IFNAR1), activation of the JAK/STAT pathway, and subsequent induction of about 300 ISGs (Ivashkiv and Donlin, 2014), which can inhibit different stages of the viral life cycle (Chen et al., 2014; Liu et al., 2013). Some ISGs have already been shown to inhibit HBV replication. For example, TRIM22 was shown to confer antiviral immunity against HBV by its inhibitory effect on viral core promoter activity (Gao et al., 2009). We reported that TRIM14 inhibited HBV replication by blocking the formation of the DDB1-HBx-Smc complex (Xu et al., 2019), and APOBEC3A/B was shown to play a critical role in the degradation of nuclear HBV cccDNA (Lucifora et al., 2014). Unfortunately, there is currently no effective treatment for patients who respond poorly to IFN therapy. Therefore, characterization of the ISGs that specifically modulate HBV infection and replication is a key imperative. In the present study, we conducted a high-throughput bimolecular fluorescence complementation (BiFC) assay screen and identified 41 out of 145 ISG products that interact with the HBx protein. The interaction of 26 ISGs was further confirmed by co-immunoprecipitation (coIP). Seven HBx-interacting ISGs (GBP2, PVRL4, CBF β , TRIM38, TRIM5 γ , TRIM25, and Gadd45 γ) showed strong inhibitory effects on HBV replication. The TRIM5 family was famous since the discovery of TRIM5 α as a blocking factor of HIV infection by binding to the capsid that surrounds and protects the viral core (Stremlau et al., 2004). TRIM5 proteins share a common tripartite motif, which consists of RING, B-box 2, and coiled-coil domains, followed by SPRY, a C-terminal domain that is required for capsid recognition (Ganser-Pornillos and Pornillos, 2019). Interestingly, TRIM5 γ does not have a SPRY domain. Here, we further narrowed our investigation to TRIM5 γ anti-HBV function and showed that the BBox domain of TRIM5 γ is sufficient for interaction with HBx and for triggering the HBx-K48 ubiquitination and degradation. Furthermore, we showed that this process likely requires the E3 ubiquitin ligase activity of TRIM31, recruited by TRIM5 γ . More importantly, we found that high induction of TRIM5 γ in the IFN- α -treated HBV patients predicts a much better outcome. Our study, therefore, highlights a critical role for TRIM5 γ and TRIM31 in promoting HBx degradation and reveals another mechanism that might impact the effectiveness of IFN therapy in HBV-infected patients. In addition, our study suggests that TRIM5 γ may serve as a biomarker to predict the response to IFN- α therapy and that BBOX may be a potential candidate for the development of therapeutic agents that target HBx.

RESULTS

Identification of 26 ISG Proteins That Interact with the HBx Protein by BiFC and CoIP Assays

We hypothesized that ISGs can inhibit HBV viral infection and replication through interactions with individual viral proteins. A recent study researched how ISGs inhibit viruses by overexpression of about 300 individual ISGs (Liu et al., 2012). HBx is an indispensable transcriptional factor in the HBV life cycle, which interacts with multiple proteins and promotes HBV replication (Keasler et al., 2007). In the present study, we used a BiFC assay to screen 145 ISG proteins for interaction with HBx. This assay involves transient expression of HBx fused with the N-terminal portion of the yellow fluorescent protein (YFP) and cellular ISGs fused to the C terminus of YFP (Figure 1A). Interaction between viral proteins and ISGs in living cells results in the emission of fluorescent signals (Figure 1B) that can be detected by flow cytometric analysis. The BiFC assay identified 41 ISGs that potentially interact with the HBx protein (Figure 1C). To further validate the BiFC results, we conducted a coIP assay. DDB1 was used as a positive control (Hodgson et al., 2012) (Figure S1A), and two BiFC-negative ISGs (UBA7 and TRIM62) were used as negative controls (Figure S1B). Under conditions of overexpression, the BiFC assay may yield some false-positive results; therefore, 15 ISGs in the BiFC-positive group were not consistent with our coIP experiment. Finally, the coIP assay confirmed the results of the BiFC assay for 26 ISGs in 293T cells (Figure S1C). We further confirmed the interaction of HBx with seven ISGs (Figures S2F–S2K) that we found had an inhibitory effect on HBV replication (Figures S2A–S2D, gray bar; labeled in red in Figure 1C) in HepG2 cells.

High Induction of TRIM5 γ in IFN- α Treatment of HBV Patients Indicates a Better Therapeutic Effect

As multiple potent anti-HBV ISGs belonging to the TRIM family of proteins (including TRIM5 γ , TRIM38, and TRIM25) were identified in this study, we further investigated their anti-viral function. In a very recent study, we showed that TRIM25, which was induced by type I IFN in an interleukin-27 (IL-27)-dependent manner, might inhibit HBV replication by promoting IFN production (Tan et al., 2018a). Because TRIM5 γ was found to have the highest induction in response to IFN- α treatment among the 7 potent anti-HBV ISGs tested (Figure 2A), we asked if the induction of TRIM5 γ was important in IFN- α treatment of HBV patients. To answer this question, blood samples of 31 HBV-infected patients collected before or 17 weeks after IFN- α treatment were assessed for serum levels of ALT, AST, HBeAg and HBV DNA (Table S1). Our data indicated that IFN- α treatment significantly decreased ALT, AST, HBeAg, and HBV DNA levels (Figure 2B). Interestingly, after determining the TRIM5 γ expression levels in patient peripheral blood mononuclear cells (PBMCs) at 15 min before or 96 h after IFN- α treatment, we divided the patients into 2 groups; one group (15 patients) had low TRIM5 γ induction and the other (16 patients) had high induction (Figures 2C). Just as we expected, the high group responded well to the treatment and exhibited much better outcomes compared to the group with low TRIM5 γ induction (Figure 2E). As a control, we also detected the expression of

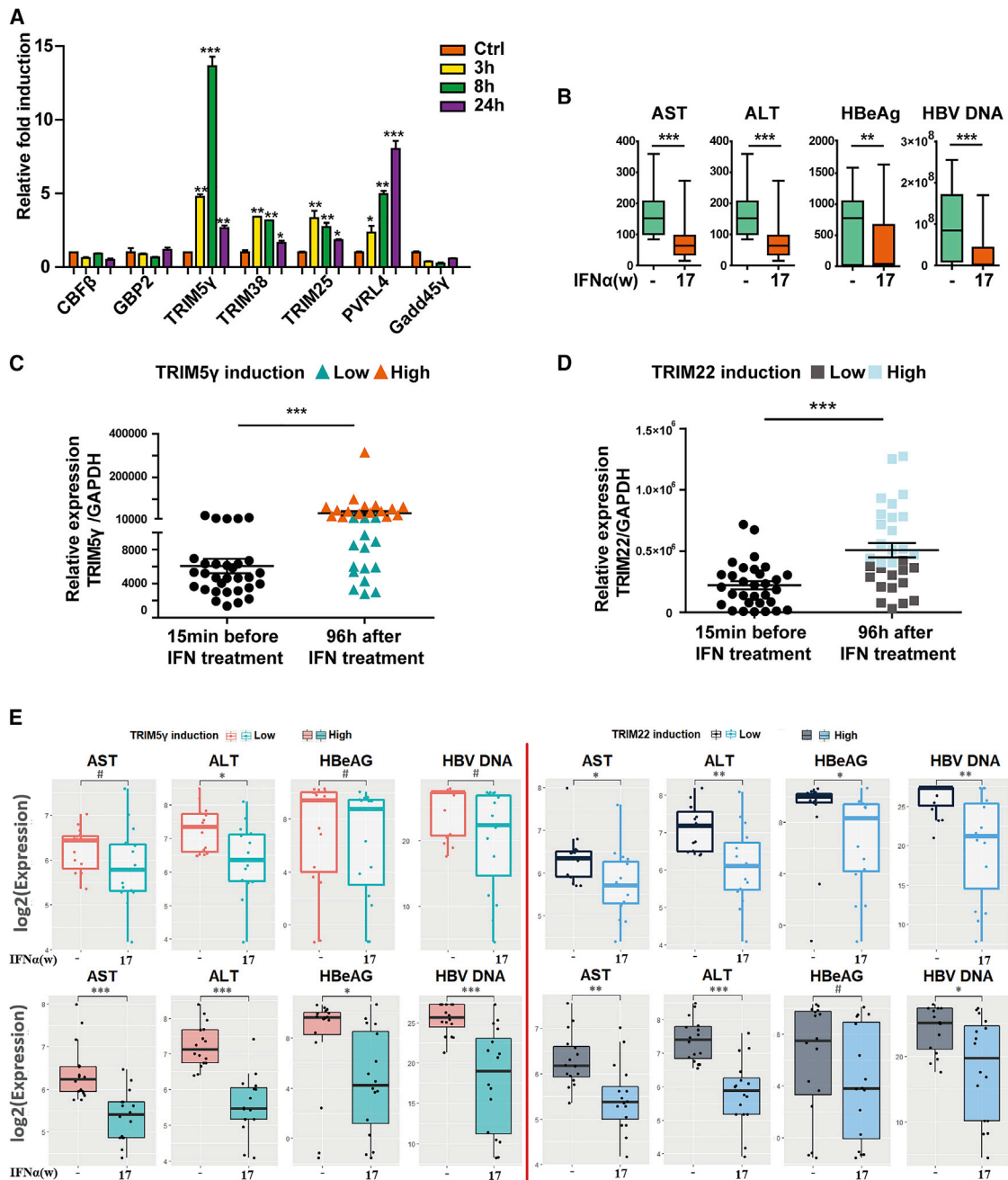


Figure 2. Higher TRIM5 γ Induction Indicates a Better Therapeutic Outcome of HBV Patients Undergoing IFN- α Treatment

(A) HepG2 cells were treated with IFN- α (10 ng/mL) or untreated; qPCR was performed to analyze the mRNA expression level of 7 ISGs, as indicated. (B) Blood samples of 31 HBV-infected patients before and 17 weeks after IFN- α treatment were collected for ALT, AST, HbeAg, and HBV DNA detection. (C and D) PBMCs from HBV patients before or after IFN treatment were isolated and subjected to RNA extraction; qPCR was performed to evaluate the TRIM5 γ (C) or TRIM22 (D) mRNA level. (E) Seventeen weeks after IFN treatment, the outcome of the two HBV-infected patients groups (TRIM5 γ high or low, TRIM22 high or low) were analyzed by comparing the AST, ALT, HbeAg, and HBV DNA levels. Mean \pm SD values from three independent experiments are shown. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, # $p > 0.05$.

was confirmed by immunofluorescence (Figure 4D). To further confirm the results, HepG2 cells with KO of both TRIM5 γ and TRIM5 α were generated using the CRISPR/Cas9 system (Figure 4E; Figure S4A). As expected, HBx expression was signifi-

cantly elevated in HepG2 cells that lacked TRIM5 α/γ after transfection of HBx (Figure 4E, line 1 and 5). In addition, degradation of HBx by IFN- α stimulation was almost blocked in TRIM5 α/γ KO HepG2 cells (Figure 4E). These results indicated that TRIM5 γ

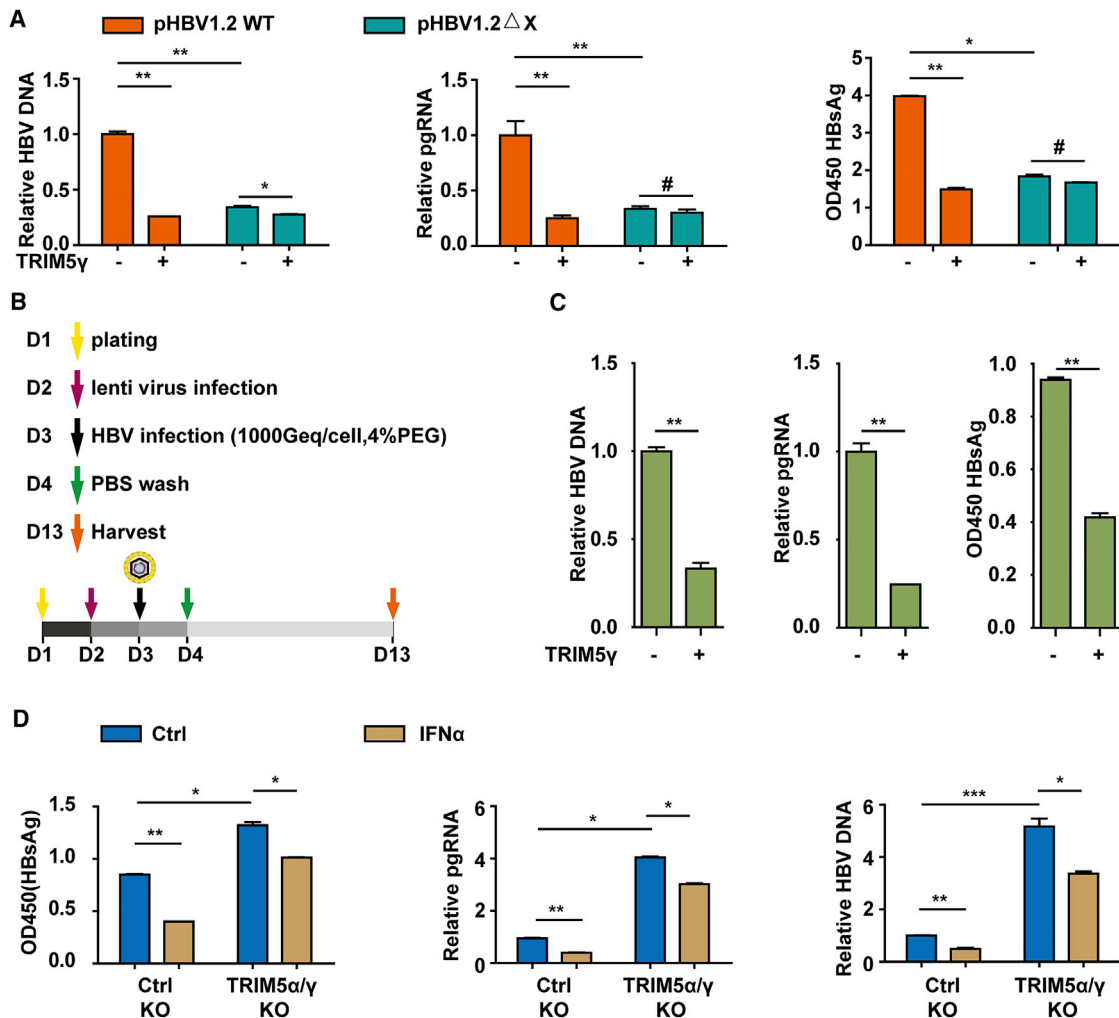


Figure 3. TRIM5 γ Inhibits HBV Infection and Replication by Targeting HBx

(A) HepG2 cells were co-transfected with constructs expressing TRIM5 γ proteins and either pHBV1.2 WT or Δ X, as indicated. After 72 h, cells and supernatant were harvested and subjected to qPCR or ELISA.

(B) Scheme of HBV infection.

(C) HepG2-NTCP cells were infected with lentiviral-ctrl or lentiviral-TRIM5 γ for 16 h, and later, cells were washed with PBS and inoculated for 24 h with HBV. After infection, cells were washed three times with PBS and were maintained for another 9 days; the medium was changed every 2 days. The supernatant and cells were collected for detection of HBV DNA, pgRNA, and HBsAg by qPCR or ELISA.

(D) HepG2-NTCP WT or TRIM5 α/γ KO cells were infected with HBV for 24 h with or without IFN- α treatment (10 ng/mL) and analyzed as in (C). Mean \pm SD values from three independent experiments are shown. * p < 0.05, ** p < 0.01, *** p < 0.001.

could specifically trigger HBx degradation and that it plays an important role in IFN-induced HBx degradation. TRIM5 was reported to be involved in autophagy (Keown et al., 2018); however, TRIM5 γ -mediated HBx degradation was not affected by 3-MA (3-Methyladenine), the autophagy inhibitor (Figure S4B). The TRIM family is a diverse family of RING-finger-domain-containing proteins that mediate post-translational modifications, such as protein ubiquitination, which are involved in a variety of cellular functions (Kawai and Akira, 2011). To further investigate the mechanisms involved in TRIM5 γ -mediated HBx degradation, we evaluated the HBx ubiquitination by TRIM5 γ . As expected, degradation of HBx by TRIM5 γ was rescued by the proteasome inhibitor MG132 but not by Z-VAD, the caspase in-

hibitor (Figure 4F); furthermore, the HBx mRNA level was not affected by TRIM5 γ (Figure S4C). In addition, the colP assay confirmed that TRIM5 γ promotes HBx ubiquitination; moreover, we found that enhanced TRIM5 γ -mediated HBx ubiquitination was linked to K48 but not to K63 in both 293T and HepG2 cells (Figures 4G and 4H; Figure S4D). These findings suggested that TRIM5 γ may promote HBx K48-linked ubiquitination and degradation by the proteasome pathway.

TRIM5 γ BBox Domain Is Responsible for the Interaction and Degradation of HBx

To identify the TRIM5 γ domain that interacts with HBx, individual plasmids encoding truncated TRIM5 γ (as shown in

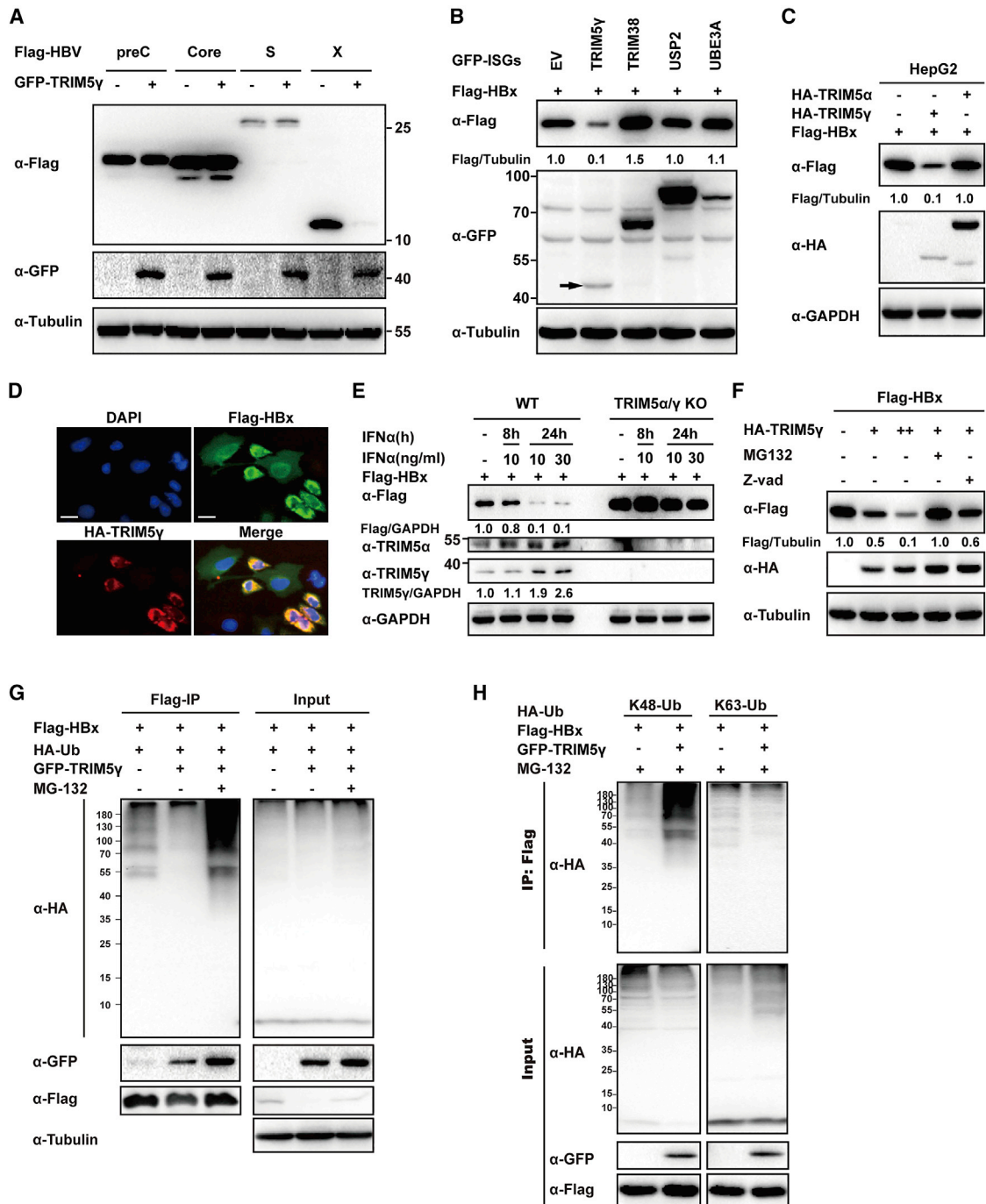


Figure 4. TRIM5 γ Specifically Promotes HBx Degradation

(A) 293T cells were co-transfected with constructs expressing FLAG-HBV proteins and either GFP-TRIM5 γ or EV as indicated. After 36 h, cells were harvested and whole-cell lysates were subjected to immunoblotting using anti-FLAG, anti-GFP, or anti-Tubulin antibody.

(B) 293T cells were co-transfected with FLAG-HBx and either GFP-TRIM5 γ , -USP2, -TRIM38, -UBE3A, or -EV expression plasmids as indicated and were analyzed as in (A).

(C) HepG2 cells were co-transfected with plasmids expressing FLAG-HBx and either HA-TRIM5 γ or TRIM5 α or hemagglutinin (HA) empty vector as indicated, and cells were collected 28 h after transfection. Whole-cell lysates were immunoblotted with antibodies as indicated.

(D) FLAG-HBx either HA-TRIM5 γ or HA empty vector was transfected into HepG2 cells as indicated; 48 h later, cells were fixed in acetone-methanol and subjected to immunofluorescence staining analysis using FLAG and HA antibodies. Scale bars represent 20 μ m.

(E) TRIM5 α and TRIM5 γ were both knocked out in HepG2 cells by CRISPR/Cas9 technology; WT or TRIM5 α/γ KO HepG2 cells were transfected with FLAG-HBx; and 24 h later, cells were treated with IFN- α as indicated. Post-treatment, whole-cell lysates were immunoblotted with the indicated antibodies.

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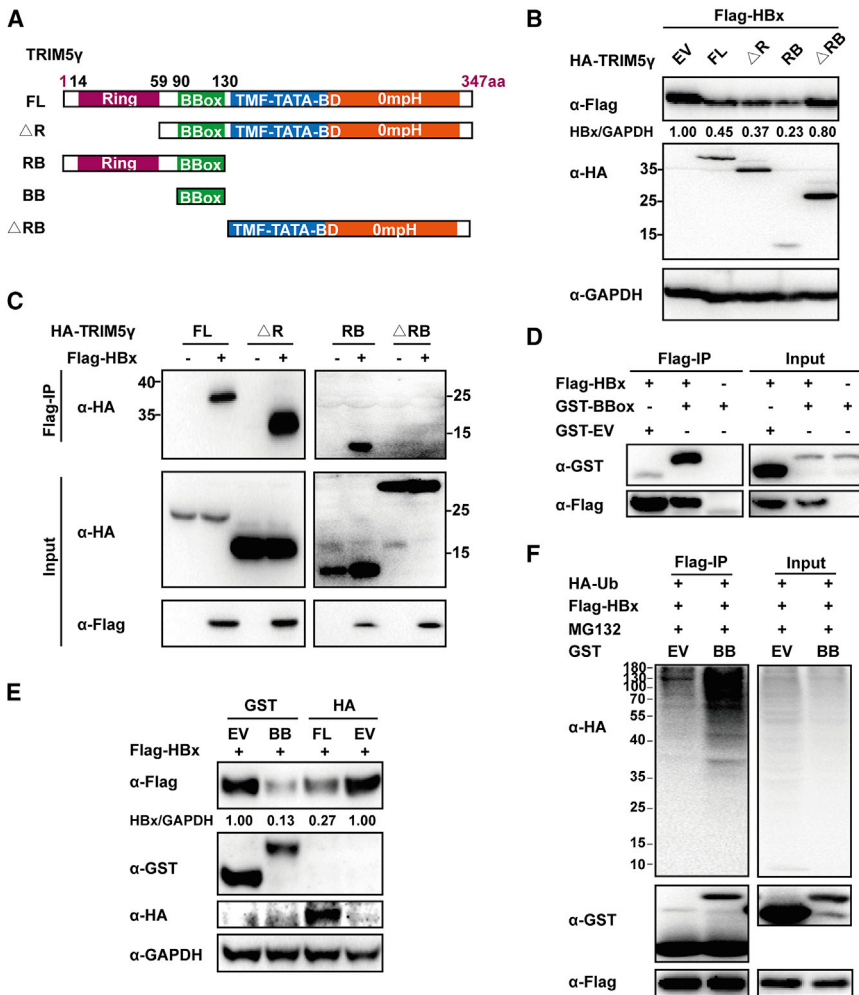


Figure 5. BBox Domain of TRIM5 γ Is Indispensable for TRIM5 γ -Induced HBx Degradation

(A) Diagrams of mutant TRIM5 γ constructs. (B) 293T cells were co-transfected with FLAG-HBx and TRIM5 γ -derived constructs or EV as indicated, and whole-cell lysates were immunoblotted with anti-FLAG, HA, or GAPDH antibodies. (C) 293T cells were transfected as indicated; 28 h later, coIP was carried out, and cell lysates and precipitated samples were analyzed by immunoblotting using an anti-FLAG or anti-HA antibody. (D) 293T cells were transfected with glutathione S-transferase (GST)-BBox, flag-HBx, or EV as indicated and were subjected to coIP analysis as in (C). (E) 293T cells were co-transfected with FLAG-HBx and GST-BBox or HA-TRIM5 γ plasmids and analyzed as in (B). (F) HepG2 cells were transfected with FLAG-HBx, HA-Ub, GST-Bbox, or GST-EV plasmids as indicated; 24 h later, cells were treated with MG132 for 8 h, collected, and subjected to coIP analysis. Data are representative of at least three independent experiments.

TRIM5 γ Promotes HBx K95 Site Ubiquitination by Interacting with the C Terminus of HBx

It has been previously shown that the HBx region encompassing amino acid residues 88–100 and, particularly, Arg96 is critical for HBx interaction with some target proteins (Hodgson et al., 2012). To map the HBx region responsive for interaction with TRIM5 γ , we deleted the amino acid residues 88–100 or made a R96E mutation

(Figure 5A) were co-transfected with the HBx expression vector in 293T cells. The RING domain of TRIM proteins is known to confer E3 ligase activity by facilitating an interaction with E2 enzymes (Rajsbaum et al., 2014). Interestingly, deletion of the TRIM5 γ RING domain (Δ R) did not affect HBx degradation, whereas deletion of both the RING and BBox domains (Δ RB) abolished the ability of TRIM5 γ to degrade HBx (Figure 5B). Consistent with this finding, the interaction between HBx and TRIM5 γ was disrupted by RB deletion (Δ RB) but not by RING deletion (Δ R) (Figure 5C). In addition, we demonstrated that the BBox domain is sufficient to interact with HBx (Figure 5D) and induce HBx degradation by ubiquitination (Figures 5E and 5F). These results indicated that the TRIM5 γ BBox domain is indispensable for the interaction between TRIM5 γ and HBx.

(Figure 6A); surprisingly, we found that neither of these two mutations affect HBx interaction with TRIM5 γ (Figure 6B). Therefore, we generated three truncated HBx constructs and performed coIP of extracts from 293-T cells co-transfected with TRIM5 γ and a truncated HBx construct. The results indicated that X3 strongly and X2 partially interacts with TRIM5 γ (Figure 6C). To further map the TRIM5 γ interacting region of HBx, we truncated X2 by deleting amino acids 88–100 and divided X3 into two small peptides. CoIP results showed that X3b is likely responsible for interacting with TRIM5 γ (Figure 6D). To further confirm these results, the X2b, X3b, or both fragments were deleted from the full-length HBx protein. The results indicated that deleting the X3b but not the X2b fragment of HBx disrupts its interaction with TRIM5 γ (Figure 6E) and protects it from TRIM5 γ -mediated degradation (Figure 6F). Interestingly, we noticed that after

(F) HepG2 cells were co-transfected with HA-TRIM5 γ and FLAG-HBx as indicated; 24 h later, cells were treated with 10 μ M MG132 or Z-VAD for 8 h and collected for immunoblotting with antibodies as indicated. (G) 293T cells were co-transfected with HA-Ub and FLAG-HBx, with or without GFP-TRIM5 γ ; 24 h later, cells were treated with MG-132 as indicated for 8 h and subjected to coIP analysis. Cell lysates and precipitated samples were subjected to immunoblot analysis using the indicated antibodies. (H) 293T cells were co-transfected with K48 or K63-Ub and Flag-HBx, with or without GFP-TRIM5 γ ; 24 h later, cells were treated with MG132 (10 μ M) for 8 h; and cells were collected and analyzed as in (G). Data are representative of at least three independent experiments.

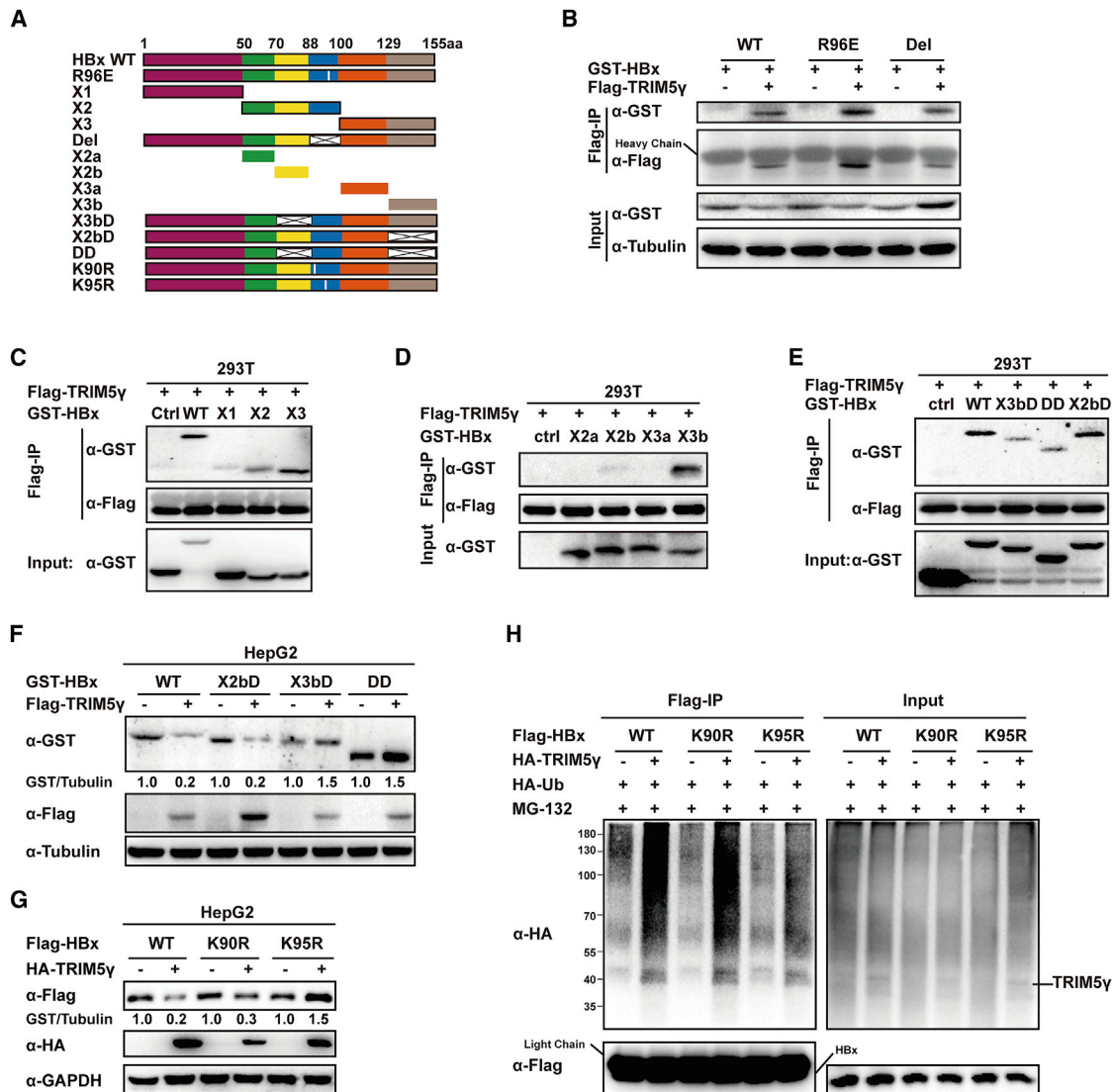


Figure 6. TRIM5 γ Interacts with the C Terminus of HBx and Promotes HBx K95 Site Ubiquitination

(A) Diagram of mutant HBx constructs. the numbers indicate the amino acids in the HBx constructs.
 (B–E) 293T cells were co-transfected with the expression vectors for Flag-TRIM5 γ and GST-HBx WT or mutants (B, R96E, Del; C, X1, X2, X3; D, X2a, X2b, X3a, X3b; E, X3bD, DD, X2bD) or EV as indicated. After 28 h, cells were harvested, colP with FLAG-HBx, and cell lysates and precipitated samples were analyzed by immunoblotting with the indicated antibodies.
 (F) HBx WT or truncations were co-transfected with TRIM5 γ or EV into HepG2 cells; 28h later, cell lysates were subjected to immunoblot analysis using the indicated antibodies.
 (G) Plasmids of expression of HBx WT, K90R, or K95R were co-transfected with TRIM5 γ or EV into HepG2 cells; 28h later, cell lysates were subjected to immunoblot analysis using the indicated antibodies.
 (H) HepG2 cells were co-transfected with the expression vectors for HA-TRIM5 γ , HA-Ub, and FLAG-HBx WT or mutants as indicated; 28h later, cells were harvested and colP with FLAG-HBx, and cell lysates and precipitated samples were analyzed by immunoblotting with the indicated antibodies. Data are representative of at least three independent experiments.

deletion of amino acid residues 88–100, HBx will not be degraded by TRIM5 γ (Figure 6B), which indicated that this region might be responsible for ubiquitination. Thus, mutation was performed to the two lysines in this region, and interestingly, HBx was no longer degraded by TRIM5 γ after K95 mutation but not K90 mutation (Figure 6G). In addition, TRIM5 γ will not promote the ubiquitination of HBx after K95R mutation (Figure 6H). Taken

together, these results clearly demonstrated that TRIM5 γ interacted with the C terminus (129–155) of HBx and triggered its degradation by promoting HBx K95 site ubiquitination.

TRIM5 γ Recruits TRIM31 for HBx Degradation

We showed that E3 ubiquitin ligase activity of TRIM5 γ is not indispensable for degradation of HBx. Therefore, another E3

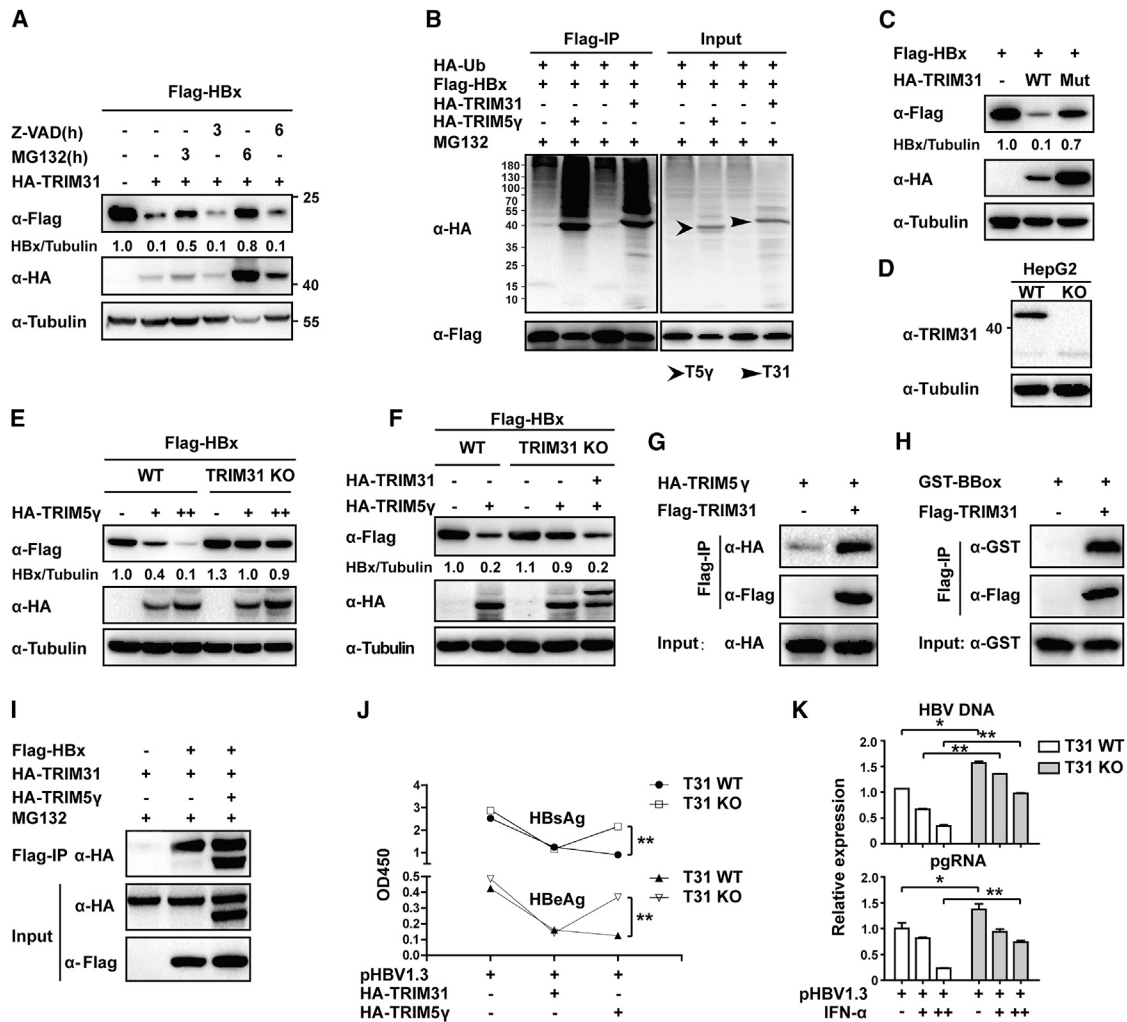


Figure 7. TRIM5 γ Recruits TRIM31 to Degrade HBx

(A) HepG2 cells were co-transfected with FLAG-HBx and HA-TRIM31 or HA empty vector plasmids; 36 h later, cells were left untreated or were treated with MG132 (10 μ M) or Z-VAD (10 μ M) as indicated. Cells were harvested after treatment, and whole-cell lysates were immunoblotted with the indicated antibodies. (B) HepG2 cells were transfected with FLAG-HBx, HA-Ub, HA-TRIM5 γ , or TRIM31 expression plasmids as indicated; 24 h later, cells were treated with MG132 for 6 h and were collected and subjected to coIP analysis. (C) HepG2 cells were transfected with FLAG-HBx or co-transfected with TRIM31 plasmids (WT or mutant); 36 h later, cells were harvested and analyzed as in (A). (D) HepG2 WT or TRIM31 KO cell lysates were immunoblotted with TRIM31 or Tubulin antibody. (E) HepG2 WT or TRIM31 KO cells were transfected with FLAG-HBx with or without the TRIM5 γ expression vector; 28 h later, cells were collected and analyzed as in (A). (F) WT HepG2 cells or TRIM31 KO cells were transfected as indicated; 28 h later, cells were collected and analyzed as in (A). (G and H) Expression vectors for HA-TRIM5 γ (G) or GST-BBox (H) were co-transfected into 293T cells with or without FLAG-TRIM31; 36 h later, cells were subjected to coIP using FLAG-TRIM31. Immunoblot analyses were carried out using anti-FLAG, anti-HA, or anti-GST antibody. (I) 293T cells were transfected with FLAG-HBx, HA-TRIM5 γ , or HA-TRIM31 expression plasmids as indicated; 24 h later, cells were treated with MG132 (10 μ M) for 8 h and subjected to coIP analysis. Immunoblotting was carried out using an anti-FLAG or anti-HA antibodies. Data are representative of at least three independent experiments. (J) HepG2 WT or TRIM31 KO cells were transfected with pHBV1.3 plasmids or together with TRIM5 γ or TRIM31 expression plasmids as indicated; 72 h later, the supernatant was tested for HBeAg and HBsAg content using ELISA. Mean \pm SD values from three independent experiments are shown. ** p < 0.01. (K) HepG2 WT or TRIM31 KO cells were transfected with pHBV1.3 plasmids; 24 h later, cells were treated with IFN- α (10ng/ml) or untreated. After another 48 h, qPCR was performed to evaluate the HBV pgRNA and DNA levels. Mean \pm SD values from three independent experiments are shown. * p < 0.05, ** p < 0.01.

protein may be responsible for HBx degradation and TRIM5 γ may possibly act as an adaptor in this process. Several TRIM proteins have been shown to inhibit HBV infection (Zhang et al., 2013), of which TRIM31 has recently emerged as an attractive candidate that possesses E3 ubiquitin ligase activity (Koz-

kova et al., 2015; Ra et al., 2016; Song et al., 2016). Consistent with this postulation, our data indicated that akin to TRIM5 γ , TRIM31 also promoted HBx degradation and that MG132 inhibited this process (Figure 7A); furthermore, both TRIM5 γ and TRIM31 promoted HBx ubiquitination in HepG2 cells

(Figure 7B). In addition, C31 and H33 double mutation in the RING domain has been shown to block the E3 ligase activity of TRIM31 (Kozakova et al., 2015). We found that this double mutation abolished TRIM31-mediated HBx degradation (Figure 7C). This indicated that unlike TRIM5 γ , TRIM31 functioned as an E3 ligase in HBx degradation. Therefore, TRIM31 could be the E3 ubiquitin ligase that causes the ubiquitination and degradation of HBx. To investigate the role of TRIM31 in TRIM5 γ -mediated HBx degradation, we used CRISPR/Cas9 technology and generated TRIM31-KO HepG2 cells (Figure 7D; Figure S5). Interestingly, we found that TRIM5 γ -mediated HBx degradation was impaired in TRIM31 KO cells (Figure 7E) but was restored upon reconstitution of TRIM31 in KO cells by using a plasmid expressing WT TRIM31 (Figure 7F). Therefore, it appeared that TRIM31 is indispensable for TRIM5 γ -mediated HBx degradation. Next, we investigated whether TRIM5 γ recruits TRIM31 to degrade HBx. Our data indicated that both TRIM5 γ and its BBox domain could interact with TRIM31 (Figures 7G and 7H) and form a TRIM31-TRIM5 γ -HBx complex (Figure 7I). In addition, TRIM31 KO disrupted TRIM5 γ -mediated inhibition of HBV (Figure 7J) and also inhibited the IFN- α -mediated inhibition of HBV replication (Figure 7K). Therefore, in response to HBV infection, TRIM5 γ might recruit TRIM31 and form a complex with HBx, which leads to proteasomal degradation of HBx.

DISCUSSION

Although IFN is widely used for treatment of HBV in clinical settings, the underlying mechanisms responsible for the antiviral effects of IFN against HBV are not fully understood. In the present study, we hypothesized that the anti-viral effect of IFN is at least partially dependent on the interaction between ISG products and HBV proteins. Therefore, we screened 145 ISGs that potentially interact with HBx to characterize the molecular mechanism of IFN-mediated inhibition of HBV through direct HBx-ISG protein-protein interactions. We identified 7 ISGs that significantly inhibited HBV replication by interacting with HBx. Moreover, we found that TRIM5 γ inhibited HBV replication, likely by directing TRIM31 toward HBx K48 chain ubiquitination and degradation on the K95 ubiquitin site. An important finding of this study was that induction of high expression levels of TRIM5 γ in HBV patients treated with IFN- α might indicate a better therapeutic effect and that BBOX may be a potential candidate for development of therapeutic agents that target HBx.

Activation of IFN production is one of the earliest transcriptional responses to viral infection. Interestingly, after HBV infection, INF signaling was reported to be impaired by HBx through upregulation of the suppressor of cytokine signaling 3 and protein phosphatase 2A (Tsunematsu et al., 2016). In addition, HBx was also shown to inhibit IFN-induced TRIM22 production by single CpG methylation in the 5' UTR of TRIM22 (Lim et al., 2017), which demonstrates the importance of HBx in combating IFN signaling. In the data shown in Figure S1, several ISG proteins (e.g., Bach1 and IFITs) were much less expressed in the HBx-transfected cells, although we transfected the same amount of plasmids, which indi-

cated that those proteins might be degraded by HBx. This finding is consistent with a report that showed lower expressions of IFIT families in HCC tissues than those in normal tissues (Yang et al., 2017). Moreover, HBx was recently shown to hijack the cellular DDB1-containing E3 ubiquitin ligase to target the Smc complex Smc5/6 for degradation, which, in turn, facilitates HBV replication (Murphy et al., 2016). As a key viral regulatory protein known to play a central role in HBV infection, replication, pathogenesis, and, possibly, carcinogenesis (Murakami, 2001), HBx is essential for maximal HBV replication and development of HCC (Berasain and Lechel, 2017; Keasler et al., 2007). HBV X region mutations were shown to be associated with clinical severity (Yeh et al., 2010). Direct interaction between ISGs and viral proteins plays an important role in restricting viral replication (Jäger et al., 2011; Stremlau et al., 2004). Therefore, identification of ISGs that directly interact with HBx may shed some light on the mechanism by which type I IFN inhibits HBV. We identified 7 ISGs (GBP2, PVRL4, CBF β , TRIM38, TRIM5 γ , TRIM25, and Gadd45 γ) that interact with HBx and inhibit HBV replication. GBP2 has been previously shown to inhibit the replication of vesicular stomatitis virus (VSV) and encephalomyocarditis virus (EMCV) (Carter et al., 2005). The V domain of PVRL4 has been shown to be critical for cell entry and intercellular spread of canine distemper virus (CDV) (Delpout et al., 2014). Interestingly, CBF β was shown to be hijacked by HIV viral infectivity factor (VIF) to degrade APOBEC3G and promote HIV-1 infection (Jäger et al., 2011); conversely, it was found to play an opposite role with respect to HBV replication in the present study, and our further study on CBF β suggested CBF β inhibited HBV replication by blocking the HBx-Smc6 interaction (Xu et al., 2019). Gadd45 γ acts as a tumor suppressor gene by blocking S and G2/M cell cycle checkpoints (Vairapandi et al., 2002). To the best of our knowledge, there are only a few reports on the role of the above five proteins in HBV inhibition. Therefore, characterization of the mechanisms by which these ISGs combat HBV replication may facilitate the development of therapeutic strategies.

Interestingly, in addition to the four abovementioned ISGs, the rest of the most potent anti-HBV ISGs were Tripartite motif (TRIM) proteins, including TRIM5 γ , TRIM38, and TRIM25. TRIM proteins are involved in multiple cellular functions, including antiviral activity (Nisole et al., 2005). TRIM E3 ubiquitin ligases, a subset of the TRIM family, have been shown to restrict viral replication by targeting viral proteins directly (Rajsbaum et al., 2014). TRIM25 has been shown to inhibit HBV replication (Zhang et al., 2013). In our recent study, TRIM25, which was induced by type I IFN in an IL-27-dependent manner, was shown to inhibit HBV replication by promoting IFN production (Tan et al., 2018a). In contrast, TRIM38 was shown to downregulate TLR-mediated immune responses by targeting TRIF and TNF-receptor-associated factor 6 (Zhao et al., 2012), which explains its adverse role in counteracting viral replication. In the present study, we found that TRIM5 γ can specifically promote HBx degradation by ubiquitination on the K95 ubiquitin site; we also found that the Ring domain responsible for the ligase activity is not essential for degradation of HBx by TRIM5 γ and that only the BBox of TRIM5 γ , which is a 40 amino acid peptide, is enough

to trigger HBx degradation. HBx has been shown to interact with multiple proteins, including the ubiquitin proteasome system (UPS) components (Minor and Slagle, 2014), and some deubiquitinases (DUBs) target HBx and protect it from proteasome degradation. It is possible that the interaction of TRIM5 γ with other E3 ligase proteins results in HBx degradation. TRIM31, which is also reported to inhibit HBV replication (Zhang et al., 2013), has been shown to possess ubiquitin ligase activity (Liu et al., 2016; Song et al., 2016), and TRIM31 RING-finger mutations (C31A and H33A) have been shown to abolish the ligase activity of TRIM31 (Sugiura and Miyamoto, 2008). We have shown that overexpression of TRIM31 leads to HBx degradation. In addition, knocking out TRIM31 blocked the degradation of HBx by TRIM5 γ , which indicated that TRIM31 was indispensable for TRIM5 γ -induced HBx degradation. We further confirmed that TRIM31 interacts with TRIM5 γ and HBx and forms a TRIM31-TRIM5 γ -HBx complex. Our findings are consistent with a very recent report that showed that TRIM31 promotes NLRP3 K48-linked polyubiquitination and proteasomal degradation (Song et al., 2016). Nevertheless, further investigations are required to explore the exact mechanism underlying the HBx degradation by TRIM31.

In addition, we have shown that TRIM5 γ but not TRIM5 α can promote HBx degradation. TRIM5 γ has a shorter and distinct C terminus than its isoform alpha, which may support the distinct molecular structure required for interaction with HBx. Interestingly, overexpression of TRIM5 γ has been shown to be devoid of antiviral activity against N-MLV and HIV-1 by inhibiting the activity of TRIM5 α (Stremlau et al., 2004) (Battivelli et al., 2011). Similarly, it will be easy to understand that the formation of the TRIM5 α/γ dimer might inhibit the degradation of HBx by TRIM5 γ and that overexpression of TRIM5 α might promote HBV replication by offsetting the inhibitory effects of TRIM5 γ . With sgRNA targeting the N terminus of TRIM5 γ , the BBox region that we verified as the interaction domain with HBx could be destroyed, but TRIM5 α will be also knocked out; thus it is difficult to generate a cell line with TRIM5 γ KO only. Nevertheless, overexpression of WT TRIM5 γ but not TRIM5 α in HepG2 cells resulted in HBx degradation and HBV replication inhibition, which confirmed the critical role of TRIM5 γ in inhibiting HBV replication. It would be very interesting to fully understand the mechanism responsible for the differential function of these two molecules.

Collectively, we have demonstrated a crucial role for TRIM5 γ and TRIM31 in promoting HBx degradation and have shown that a short peptide is sufficient to trigger HBx degradation. In addition, induction of high expression levels of TRIM5 γ in HBV patients undergoing IFN- α therapy was associated with better outcomes. These findings may facilitate the development of therapeutic agents for the treatment of patients with IFN-resistant HBV infection, and TRIM5 γ may serve as a useful biomarker to predict IFN- α therapeutic response. Nevertheless, because upregulation of the downstream ISGs is a key driver of the efficacy of IFN therapy, it will be interesting to investigate if HBV could regulate TRIM5 γ and TRIM31 expression in response to IFN treatment, which may explain the mechanism of IFN resistance. Further investigation of the molecular mechanisms involved in the IFN-HBV

interaction is required to fully resolve the problem of IFN resistance in HBV-infected patients.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at <https://doi.org/10.1016/j.celrep.2019.11.041>.

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AUTHOR CONTRIBUTIONS

G.T. planned, designed, and performed the experiments and wrote the paper; Z.Y. performed BiFC experiments; H.S. developed experiments for identification of ISGs that inhibit HBV replication; F.X. and F.L. did the infusion cloning experiments; H.Z. and Y.D. collected and provided the HBV patients samples; P.D., J.N., and X.W. provided technical assistance and facility; R.A., L.S., and F.X.-F.Q. gave suggestions and revised the manuscript; and G.C. conceived the research project and wrote the paper.

DECLARATION OF INTERESTS

The authors declare that they have no conflict of interest.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-TRIM5 gamma	Abcam	Cat# ab17203; RRID:AB_2208592
Anti-TRIM5 alpha	Abcam	Cat# ab59000; RRID:AB_2208591
Anti-TRIM31	Abcam	Abcam Cat# ab82680; RRID:AB_1860041
Anti-GFP Tag Rabbit Polyclonal Antibody	Abbkine	Cat#A02021-2
HA-tag Antibody	proteintech	Cat# 51064-2-AP; RRID:AB_11042321
GST Tag Antibody	proteintech	Cat# 10000-0-AP; RRID:AB_11042316
Monoclonal ANTI-FLAG® M2 antibody produced in mouse	Sigma	Cat# F1804; RRID:AB_262044
GAPDH Antibody	proteintech	Cat# 10494-1-AP; RRID:AB_2263076
β Tubulin Antibody (SAP.4G5)	Santa Cruz Biotechnology	Cat# sc-58884; RRID:AB_793548
NTCP Polyclonal Antibody	Signalway Antibody	Cat#41759
HBsAg antibody	lifespan biosciences	Cat# LS-C67520-1000; RRID:AB_10634869
HRP-Goat Anti-Rabbit IgG(H+L)	proteintech	Cat# SA00001-2; RRID:AB_2722564
HRP-Goat Anti-Mouse IgG(H+L)	proteintech	Cat# SA00001-1; RRID:AB_2722565
Goat anti-mouse IgG (H+L), Cy3 conjugate	proteintech	Cat# SA00009-1; RRID:AB_2814746
Goat anti-rabbit IgG (H+L), Cy3 conjugate	proteintech	Cat# SA00009-2
Goat anti-rabbit IgG (H+L), FITC conjugate	proteintech	Cat#SA00003-2
Goat anti-mouse IgG (H+L), FITC conjugate	proteintech	Cat#SA00003-1
Biological Samples		
HBV patient venous blood samples	The First Hospital of Jilin University	N/A
Chemicals, Peptides, and Recombinant Proteins		
ANTI-FLAG M2 Affinity Gel	Sigma	A2220
Z-VAD-FMK	promega	G7232
Protease inhibitor	Roche	11697498001
MG132	Sigma	M7449
BMS-200475	MedChemExprss	HY-13623A
3-MA	MedChemExprss	HY-19312
Recombinant Human IFN-α	biolegend	592706
Critical Commercial Assays		
HBeAg detection Kit	Kehua shengwu	N/A
HBsAg detection Kit	Kehua shengwu	N/A
Viafect transfection reagent	promega	E4982
PEG-it Virus Precipitation Solution	System Biosciences	BY-LV810A-1
lentiviral-ctrl	Genecopoeia	Lot#: GC12182K1701
lentiviral-TRIM5γ	Genecopoeia	Lot#: GC05212K1804
EasyPure® Viral DNA/ RNA Kit	Transgen	ER201-01
EasyPure RNA Kit	Transgen	ER101-01
TransScript® All-in-One First-Strand cDNA Synthesis SuperMix for qPCR(One-Step gDNA Removal)	Transgen	AT341-02
E.Z.N.A. Endo-Free Plasmid Mini Kit I	Omega	D6948-02
pEASY-Blunt Cloning Kit	Transgen	CB101-02
Faststart Universal SYBR Green Master(ROX)	Roche	#04913914001
Cell Counting Kit-8 (CCK-8)	Bimake	B34302

(Continued on next page)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Protease Inhibitor Cocktail (EDTA-Free, mini-Tablet)	Bimake	B14012
FastDigest Xbal	Thermo Sci	FD0684
Experimental Models: Cell Lines		
HepG2	ATCC	HB-8065
HepG2-NTCP	This paper	
293T	ATCC	ACS-4500
Oligonucleotides		
See Tables S2 and S3	N/A	N/A
Recombinant DNA		
HBx wt expression plasmid	This paper	N/A
HBx K90R expression plasmid	This paper	N/A
HBx K95R expression plasmid	This paper	N/A
HBx R96E expression plasmid	This paper	N/A
HBx 88-100 deletion expression plasmid	This paper	N/A
TRIM5 γ and the truncations expression plasmids	This paper	N/A
pHBV1.3 plasmid	Lishan Su lab of University of North Carolina at Chapel Hill	N/A
pHBV1.2 WT and pHBV1.2 Δ X plasmids	Qiang Deng lab of Fudan University	N/A
GFP-ISGs expression plasmids	Cheng lab in UCLA	N/A
Software and Algorithms		
Prism version 5	GraphPad Software	https://www.graphpad.com

LEAD CONTACT AND MATERIALS AVAILABILITY

Plasmids and cell lines generated in this study will be made available under a standard material transfer agreement. Requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Genhong Cheng (GCheng@mednet.ucla.edu).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Thirty one HBV-infected patients were enrolled in this study ([Table S1](#)). Naive chronic hepatitis B patients (age: 18-65, serum HBsAg detectable for > 6 months) undergoing clinical IFN α 2a treatment were enrolled into this study. Venous blood samples (5ml) were collected to obtain serum and PBMCs. HBV DNA level was detected using Roche's COBAS TaqMan kit. Liver function, as well as biochemical parameters, were tested by an auto-matic biochemical instrument. Tests were conducted at the Hepatology Department of the First Hospital of Jilin University, Changchun, China. The study protocol was approved by the IRB of Jilin University, the First Hospital.

Human Embryonic Kidney 293T (HEK293T) cells, HepG2 cells, and HepG2-NTCP cells were maintained in DMEM containing 10% inactivated fetal bovine serum, penicillin (100 IU/mL) and streptomycin (100 mg/mL) and incubated at 37°C with 5% CO₂.

METHOD DETAILS

Infusion cloning

The expression construct of TRIM5 γ , TRIM5 α , TRIM31, HBx, and TRIM5 γ truncations were generated by cloning the coding region sequence of relative human genes into VR1012 vector with Flag, HA or GST tag according to the instruction of *EASY-Uni Seamless Cloning kit* (TransGen, Beijing, China). The YFP constructs were generated by inserting half of the YFP coding region sequence into the C-terminal of ISGs expression plasmids and the other half into the N-terminal of the HBx expression plasmid. Site directed mutagenesis of TRIM5 γ and TRIM31 was generated by Quik-Change PCR (TransGen, Beijing, China). Primers are listed in [Table S2](#).

Flow cytometry

Cells were collected by trypsinization, fixed in 2% paraformaldehyde, and analyzed on BD FACS Calibur flow cytometer (BD Biosciences, USA). HBV-YFP was quantified as the product of percent GFP-positive population and geometric mean of the fluorescence index (MFI), and normalized to that of the vector.

Total RNA and HBV DNA Extraction and Quantitative Real-time PCR

Total RNA was extracted using TRIzol (Invitrogen, San Diego, CA, USA) and then converted to first-strand cDNA using Superscript III transcriptase (Invitrogen San Diego, CA, USA). HBV DNA was isolated from the whole cell-lysate or culture supernatant according to the kit instructions (TransGen, Beijing, China). GAPDH was used as an internal control; quantitative real-time PCR was carried out as described previously (Tan et al., 2018a). The sequences of gene-specific primer used for qPCR are shown in Table S3.

Co-immunoprecipitation and Western Blot Analysis

Cells were lysed 24–48 h after transfection of expression plasmids using 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% NP-40 containing cocktail inhibitors (Roche, USA). For immunoprecipitation, lysates were incubated overnight with ANTI-FLAG® M2 Affinity Gel (Sigma, USA). Immunoblotting was carried out as described previously (Tan et al., 2018a). Briefly, cells were collected and lysed by adding RIPA lysis buffer together with Protease/Phosphatase Inhibitor Cocktail on ice for 30 min and by tapping tubes every 10 min. Protein concentration was quantified by Coomassie Plus protein assay Reagent (Thermo Scientific, Rockford, IL, USA). The quantification of immunoblotting band intensity was carried out with ChemiDoc XRS⁺ Molecular Imager software (Bio-Rad, Philadelphia, PA, USA). Samples were separated by SDS-PAGE and transferred to PVDF membranes. After blocking in TBS containing 0.1% Tween-20 and 5% skim-milk, the blots were probed with relative antibodies.

Immunofluorescence

HepG2 cells were transfected with Flag-HBx plasmids or together with HA-TRIM5 γ plasmids; 48 hours later, cells were fixed in acetone-methanol (1:1) at 37°C for 10 min. Subsequently, cells were washed with PBS and blocked with 5% BSA in PBST for 1 h, incubated with Flag(Mouse) and HA(Rabbit) antibody at 37°C for 1 h, washed in PBS, and then incubated with goat anti-mouse IgG conjugated with FITC or both Cy3(rabbit) and FITC(mouse) conjugated IgG (Proteintech). Cells were washed with PBS and observed under a fluorescence microscope.

ELISA

HepG2 Cells were mock transfected or transfected with ISG-expression plasmids together with pHBV1.3-HBV expression plasmids. Supernatant was collected after 72 hours, and the supernatant from HBV infected HepG2-NTCP cells was collected after 9 days for analysis by ELISA to detect the levels of HBeAg and HBsAg (Kehua Shengwu, China).

CRISPR / Cas9 Knockout

HepG2 or 293T cells were seeded in 24 well plates; 16 h later, two plasmids, one expressing Cas9 with TRIM5 α/γ or TRIM31 sgRNA (FG-EH-Cas9-2F-PPW) and the other carrying a puromycin resistant gene (PL-GFP-IP), were co-transfected into HepG2 cells using ViaFect transfection reagent (Promega). At 36 h after transfection, cells were either selected by adding puromycin at a concentration of 2 μ g/mL or collected for immunoblotting with specific TRIM5 γ , TRIM5 α or TRIM31 antibodies. Two days later, live cells were sorted into a 96-well plate at a density of 1 cell per well using a BD FACSAria. Immunoblotting was performed again to determine the level of gene editing efficiency after the clonal expansion and DNA sequencing was performed to verify the edited genes. SgRNAs are listed in the Table S2.

HBV infection assay

Serum from HBV patients with different virus strains (HBV DNA copies $> 10^7$) was collected and whole virus were concentrated using a PEG-it Virus Precipitation Solution (System Biosciences, USA). HepG2-NTCP cells were infected with lentiviral-ctrl (Lot#: GC12182K1701) or lentiviral-TRIM5 γ (Lot#: GC05212K1804) for 16 hours, and later, cells were inoculated with the serum produced virus at an MOI (multiplicity of infection) of 1000 equivalents (Geq) per cell and were cultured in the presence of 4% PEG8000 and 2% DMSO for 24 h. After infection, cells were washed three times with PBS, and were maintained in DMEM medium for another 9 days; the medium was changed every two days. The supernatant and cells were collected for detection of HBV DNA, pgRNA and HBsAg by Q-PCR or Elisa; Expressions of TRIM5 γ were detected by Q-PCR.

QUANTIFICATION AND STATISTICAL ANALYSIS

GraphPad Prism 5 software (GraphPad Software, San Diego, CA) was used for data analysis; a two-tail unpaired t test was used to assess between-group differences. *p* value < 0.05 was considered statistically significant.

DATA AND CODE AVAILABILITY

The published article includes all the datasets generated or analyzed during this study.

Cell Reports, Volume 29

Supplemental Information

Type-I-IFN-Stimulated Gene TRIM5 γ

Inhibits HBV Replication

by Promoting HBx Degradation

Guangyun Tan, Zhaohong Yi, Hongxiao Song, Fengchao Xu, Feng Li, Roghiyh Aliyari, Hong Zhang, Peishuang Du, Yanhua Ding, Junqi Niu, Xiaosong Wang, Lishan Su, F. Xiao-Feng Qin, and Genhong Cheng

Supplementary figures and figure legends

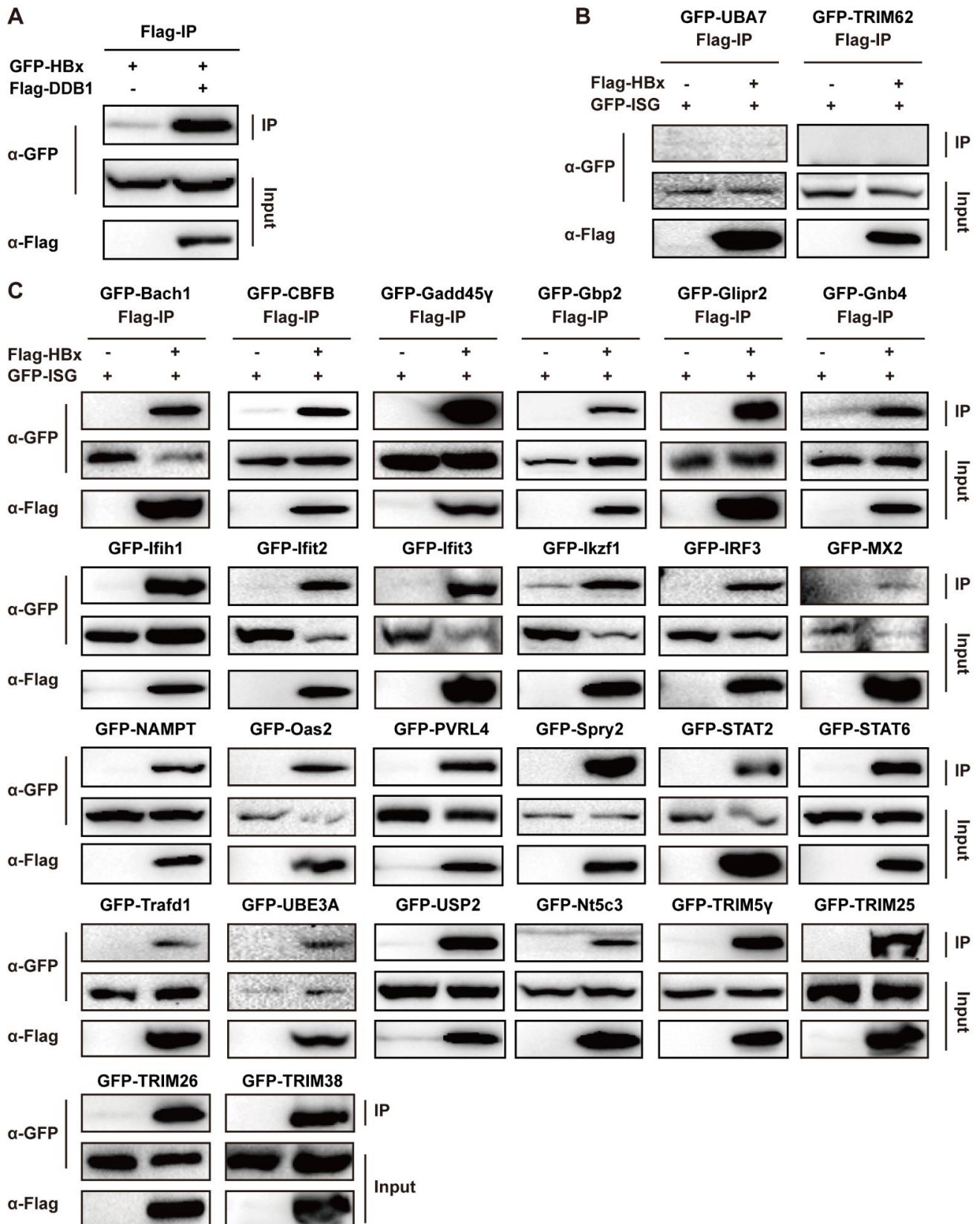


Figure S1. Confirming the ISGs-HBx interaction by Co-immunoprecipitation assay in 293T cells, Related to Figure 1.

(A) The GFP-HBx expression plasmid was co-transfected with Flag-DDB1 or an empty Flag reporter plasmid (Flag-EV) into 293T cells as indicated; 28 h later, cells were harvested and lysed. 1/20 of the whole cell lysates were used as input, and the rest were incubated overnight with Flag-tagged beads. After washing, the beads were boiled with SDS loading buffer and subjected to immunoblotting using anti-GFP or anti-Flag antibodies. **(B)** Plasmids expressing two ISGs (UBA7 or TRIM62) negative for HBx interaction in the BIFC assay were co-transfected with Flag-HBx or Flag-EV plasmids into 293T cells as indicated, and analyzed as in **A**. **(C)** Plasmids expressing ISGs positive for HBx interaction by BIFC assay were co-transfected with Flag-HBx or Flag-EV plasmids into 293T cells as indicated, and analyzed as in **A**.

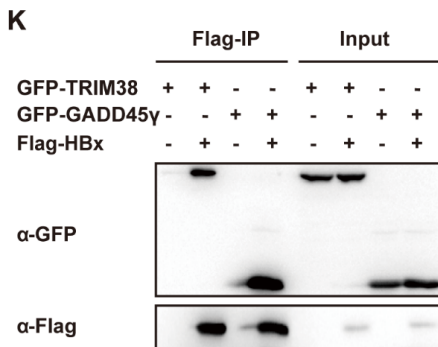
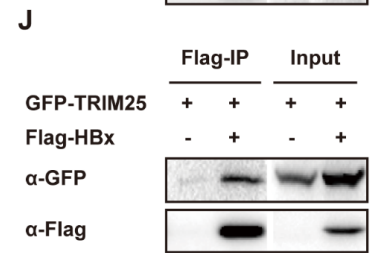
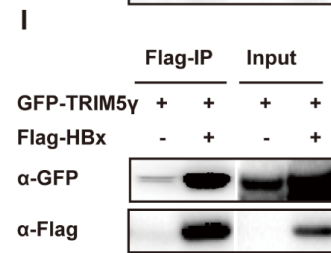
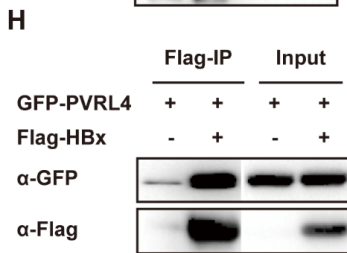
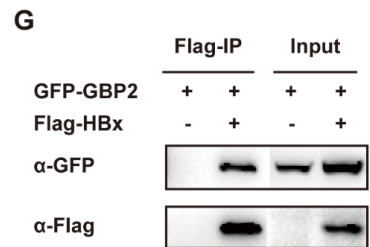
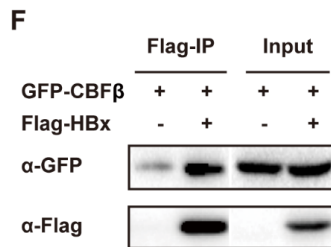
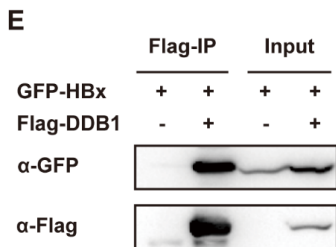
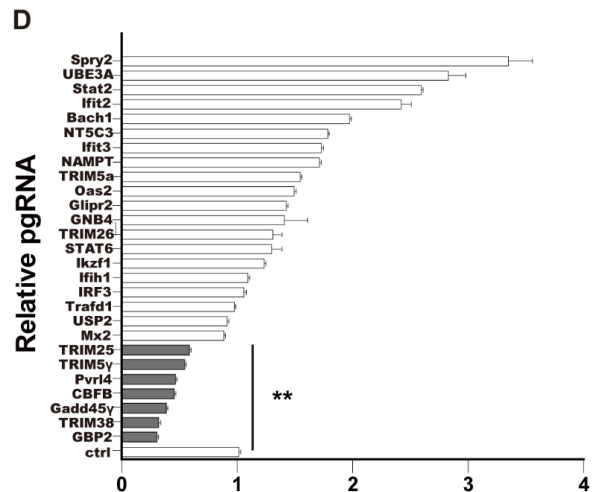
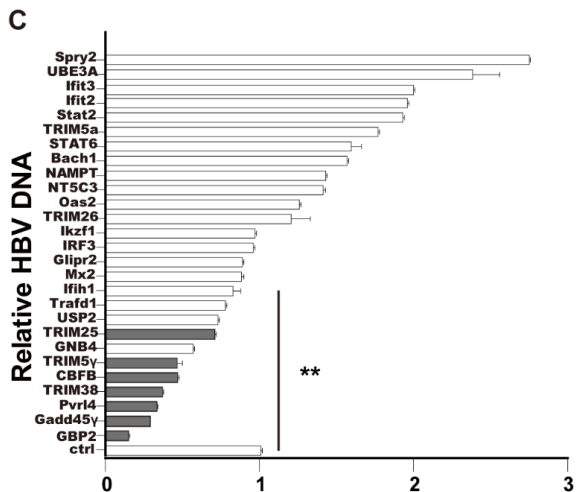
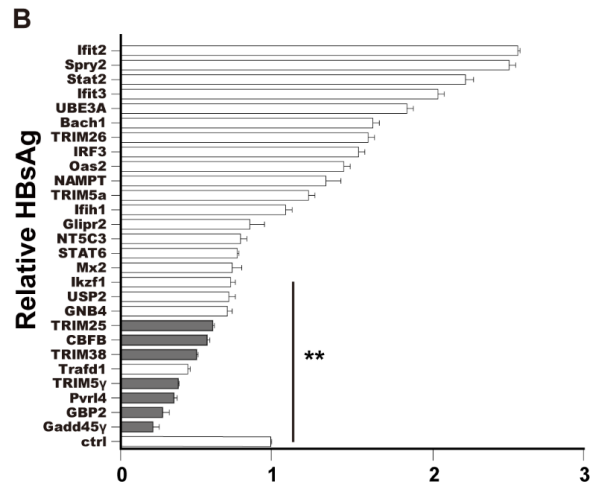
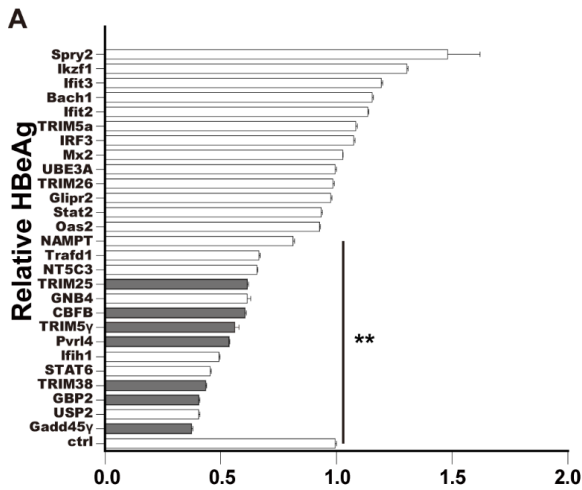


Figure S2. Evaluation of anti-HBV replication and HBx interaction activity of 7 ISG products in HepG2 cells, Related to Figure 1.

(A,B) pHBV1.3 plasmid DNA was co-transfected into HepG2 cells with either an empty GFP reporter plasmid (GFP-EV) or GFP reporter plasmids encoding ISG products positive for interaction with HBx by both BiFC and Co-IP assays. After 72 hours, culture media was collected and screened for HBeAg, HBsAg expression by ELISA. (C,D) The supernatant and cell lysates were subjected to HBV DNA and RNA isolation; HBV DNA and pgRNA were analyzed by Q-PCR. Mean (\pm SD) values from three independent experiments are shown. $**p < 0.01$. (E) The GFP-HBx expression plasmid was co-transfected with Flag-DDB1 or an empty Flag reporter plasmid (Flag-EV) plasmids into HepG2 cells as indicated; 24 hours later, cells were harvested and lysed. 1/20 of the whole cell lysates were used as input, and the rest were incubated overnight with Flag-tagged beads. After washing, the beads were boiled with SDS loading buffer and subjected to immunoblotting using anti-GFP or -Flag antibodies. (F-K) 7 GFP-tagged ISGs expression plasmids were co-transfected with Flag-HBx or an Flag-EV plasmids as indicated, and cells were collected after 24 hours and analyzed as in E.

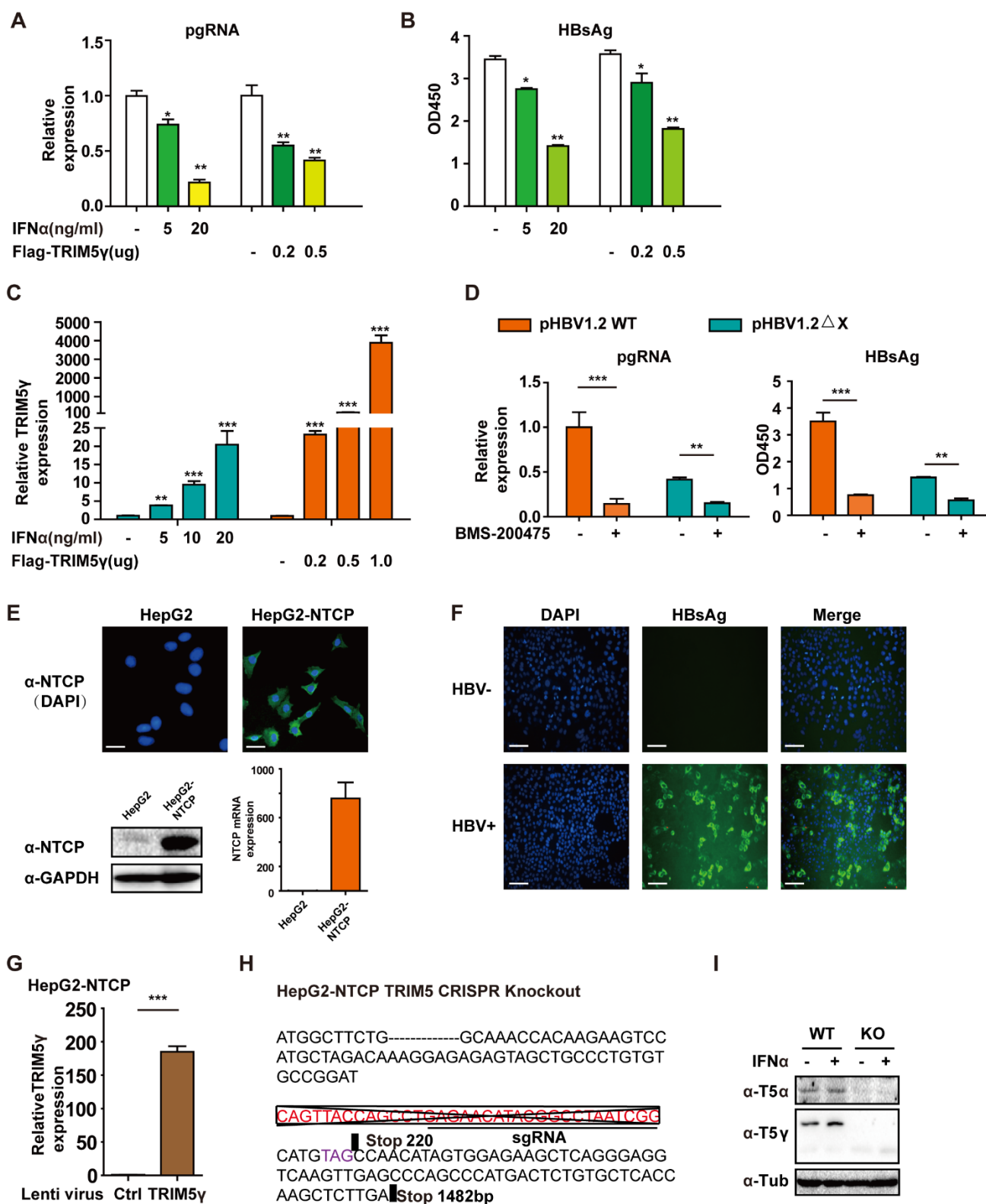


Figure S3. Related to Figure 3. (A, B) pHBV1.3 plasmid DNA was transfected into HepG2 cells alone or co-transfected with constructs expressing TRIM5 γ proteins. 24 h later, cells transfected with pHBV1.3 plasmid alone were treated with IFN- α as indicated, after 48 h, cells and supernatant were harvested and subjected to Q-PCR or ELISA. (C) HepG2 cell were transfected and treated as in A, and subjected to Q-PCR to analyze TRIM5 γ mRNA expression. (D) HepG2 cells were transfected

with constructs expressing pHBV1.2 WT or Δ X as indicated. 24 h later, cells were treated with BMS-200475 (1 μ M) for 24 h or left untreated, cells and supernatant were harvested and subjected to Q-PCR or ELISA. **(E)** Expression of NTCP in HepG2 or HepG2-NTCP cells were analyzed by Immunofluorescence, Western Blot or Q-PCR. Scale bar represents 20 μ m. Mean (\pm SD) values from three independent experiments are shown. * p <0.05, ** p <0.01, *** p <0.001. **(F)** HBsAg expression in HepG2-NTCP cells with or without HBV infection were analyzed by Immunofluorescence. Scale bar represents 100 μ m. **(G)** Samples from Figure 3C were subjected to Q-PCR to analyse the expression of TRIM5 γ mRNA. Mean (\pm SD) values from three independent experiments are shown. *** p <0.001. **(H)** Diagram of HepG2-NTCP cells TRIM5 α/γ KO results, bases from 180 to 213 were cut off in the coding region of TRIM5 and make a shorter transcript stopped at 220 bp. **(I)** Samples from Figure 3D were analyzed by immunoblotting using antibodies as indicated.

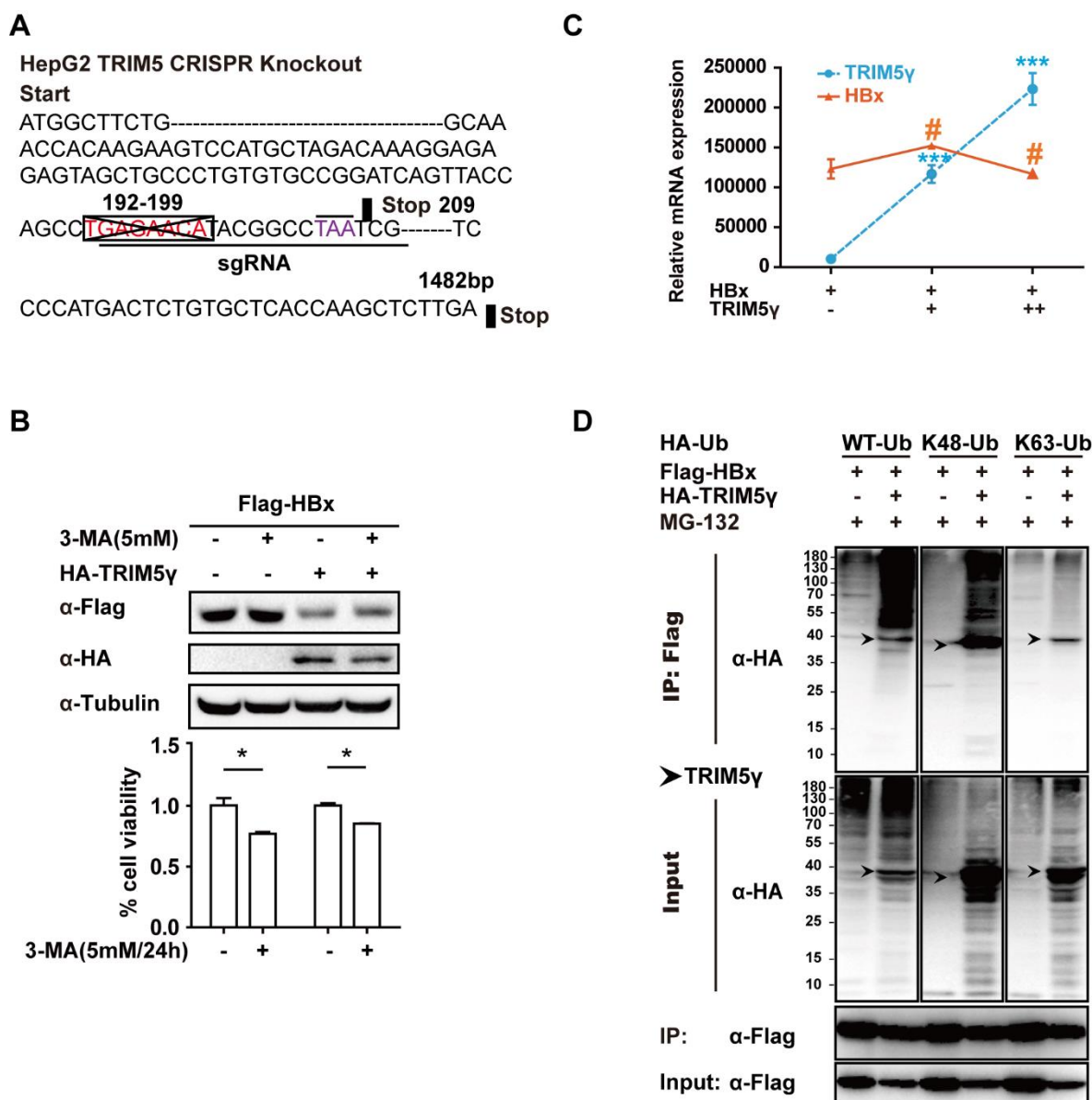


Figure S4. Related to Figure 4. (A) Diagram of HepG2 cells TRIM5 α/γ KO results; bases from 192 to 199 were cut off in the coding region of TRIM5 and make a shorter transcript stopped at 209bp. (B) HepG2 cells were co-transfected with HA-TRIM5 γ and Flag-HBx as indicated; 24 h later, cells were treated with 3-MA (5mM, autophagy inhibitor) for another 24 h or left untreated, cell viability was measured by a CCK8 kit, and whole cell lysates were subjected to immunoblotting analysis using the indicated antibodies. (C) 293T cells were co-transfected with HA-TRIM5 γ and Flag-HBx as indicated; 36 h later, cells were subjected to RNA extraction and Q-PCR was performed to evaluate the expression levels of TRIM5 γ and HBx transcripts. Mean (\pm SD) values from three independent experiments are shown. * p <0.05, ** p <0.01, # p >0.05. (D) HepG2 cells were co-transfected with HA-Ub and Flag-HBx, with or without HA-TRIM5 γ expression plasmids; 24 h later, cells were treated with MG-132 as indicated for 6 h, and subjected to Co-IP analysis. Cell lysates and co-IP samples were subjected to immunoblotting analysis using the indicated antibodies.

Supplementary Table 1. HBV infected patients with IFN treatment, Related to Figure 2.

NO	Sex	Age	before treatment				17 weeks after treatment				Gene induction	
			AST 0	ALT 0	HBeAG 0	HBV DNA 0	AST 17	ALT 17	HBeAG 17	HBV DNA 17	TRIM5 γ fold	TRIM22 fold
1	M	28	188	280	963.762	170000000	63	64	13.754	119000	10.29	5.86
2	F	22	60	106	861.489	170000000	59	96	782.74	170000000	1.82	1.23
3	F	22	130	162	903.773	124000000	104	141	657.992	14500000	0.91	2.54
5	F	36	78	152	1368.318	170000000	21	17	35.267	165000	6.63	0.71
6	M	55	111	293	1240	170000000	55	117	667.605	170000000	0.47	1.4
7	M	43	95	219	675.52	170000000	73	135	434.09	170000000	1.11	10.56
8	F	24	54	108	0.455	2560000	44	65	0.349	296	10.18	17.1
9	M	38	89	145	1028.726	170000000	80	82	20.289	1310000	0.78	0.99
10	M	39	89	91	0.433	25400000	193	273	0.415	224	1.67	1.35
11	M	24	86	227	12.351	498000	39	68	4.455	1120	2.4	20.13
12	M	26	64	188	1134.673	255000000	42	73	1071.894	142000000	2.03	15.03
13	M	31	52	89	1293.93	165000000	45	54	803.594	5120000	2.73	2.33
14	M	49	88	206	1045.839	170000000	83	154	686.078	56200000	0.96	1.95
15	F	31	62	172	840.371	170000000	30	36	2.837	1390	12.71	2.1
16	M	23	52	89	9.171	2080000	40	51	2.771	3170	3.36	1.71
17	M	42	62	128	1256.277	38000000	29	36	1.202	1130	10.34	7.37
18	M	22	101	360	117.151	1440000	18	15	81.417	196000	3.21	3.11
19	F	34	144	337	202.293	31300000	49	54	23.13	42800000	6.52	3.48
20	M	32	255	247	684.086	9570000	74	69	15.661	2720	10.18	1.16
21	M	22	92	108	1011.575	84600000	52	45	559.859	2330000	3.74	1.86
22	M	31	58	125	5.325	9950000	29	36	0.469	306	44.28	15.26
23	M	25	54	84	1145.428	45000000	38	31	582.172	3120000	5.89	1.23
24	M	35	79	117	732.383	170000000	24	18	742.352	20600000	7.64	3.01
25	F	33	81	164	776.892	170000000	29	43	318.496	31800000	4.78	0.23
26	M	41	73	206	824.12	35100000	49	87	12.839	45600	31.03	3.88
27	F	31	64	101	0.345	65900000	41	44	0.41	6690000	3.96	5.86
28	M	56	72	177	0.399	198000	129	194	0.408	23.1	2.78	44.23
29	F	26	52	93	20.685	170000000	39	52	14.56	102000000	1.79	60.15
30	M	20	60	86	344.962	10100000	52	72	69.589	1740000	4.88	0.26
31	F	33	94	205	1589.529	170000000	88	171	1639.966	170000000	4.40	1.98
32	F	33	41	101	158.344	767000	33	36	713.4	4040000	1	3.12

Supplementary Table 2. SgRNAs and Primers for infusion cloning, Related to STAR Methods

TRIM5 primers

GENE	PRIMER
T5a cloning F	GAAGAATTCATGGGTTACCCTTATGATGTGCCAGATTATGCCATG GCTTCTGGAATC
T5a cloning R	GAACTCGAGTCA AGA GCTTGGTGAG
T5γ cloning F	GAAGAATTCATGGGTTACCCTTATGATGTGCCAGATTATGCCATGGCTTCTGGA ATC
T5γ cloning R	GAACTCGAGTTATAAGGAGGGGTAAG
ΔRB F	GAATCTAGAATGGGTTACCCTTATGATGTGCCAGATT ATGCCATGACAGAGGAGGTTGCC
ΔR F	GAA TCTAGA ATGGGTTACCCTTATGATGTGCCAGATTATGCC ATGATCAGTTACCAGCCT G
RB R	GAA GGATCC TTA GAG GAA CGT GTG GTG
BB F	GAA GAATTC ATG CAT TGT GCA CGC CAT G
BB R	GAA GTCGAC TTA GAG GAA CGT GTG GTG

HBx primers

GENE	PRIMER
HBx FORWARD	TCGCGGCCGCTCTAGA ATGGCTGCTCGGGTGTGCTGC
HBx REVERSE	AGGCGCCTGGTCTAGA TTAGGCAGAGGTGAAAAAG

TRIM31 primers

GENE	PRIMER
TRIM31C31H33A F	GTCACCATCGACGCTGGGGCCAATTTCTGCCTC
TRIM31C31H33A R	GAGGCAGAAATTGGCCCCAGCGTCGATGGTGAC
TRIM31cloning F	TCGCGGCCGCTCTAGAATGGCCAGTGGGCAGTTT
TRIM31cloning R	AGGCGCCTGGTCTAGATTAGCTTGAAGGAACCTCACAA

sgRNAs

GENE	PRIMER
TRIM5α sgRNA1F	CACCgTGGTAACTGATCCGGCACAC
TRIM5α sgRNA1R	AAACGTGTGCCGGATCAGTTACCAc
TRIM5α sgRNA2F	CACCgCGATTAGGCCGTATGTTCTC
TRIM5α sgRNA2R	AAACGAGAACATACGGCCTAATCGc
TRIM31 sgRNAF	CACC CAA CTC GCT GTT GCG GAA TC
TRIM31 sgRNAR	AAAC GA TTC CGC AAC AGC GAG TTG

Supplementary Table 3. Q-PCR primers, Related to STAR Methods

Gene	Forward	Reverse
GAPDH	CGGATTTGGTCGTATTGGG	TCTCGCTCCTGGAAGATGG
HBVDNA	GAGTGTGGATTTCGCACTCC	GAGGCGAGGGAGTTCTTCT
pgRNA	TCTTGCCTTACTTTTGGAAG	AGTTCTTCTTCTAGGGGACC
HBx	ACGTCCTTTGTTTACGTCCCGT	CCCAACTCCTCCCAGTCCTTAA
TRIM5 γ	TATCATAAGCCACCCTGCGG	TGTGTGTCCTTGGAAGGAGAATCA
CBFB	AGTTTGATGAGGAGCGAGCC	TCTTCTTGCCTCCATTCCTCC
GBP2	CATCCGAAAGTTCTTCCCAA	CTCTAGGTGAGCAAGGTACTTCT
GADD45G	CAGATCCATTTTACGCTGATCCA	TCCTCGCAAACAGGCTGAG
PVRL4	AGGACGCAAAACTGCCCTG	TGAAGCCCGTATTTGGAGTGC
TRIM25	AGGGATGAGTTCGAGTTTCTGG	GTTTTTGAGGTCTATGGTGCTCT
TRIM38	ACACGGAGAGCAGTTCCAC	GGCATACTCTTCAACAAGAGC