# TRIM25 inhibits HBV replication by promoting HBx degradation and the RIG-I-mediated pgRNA recognition

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## Abstract

**Background:** The hepatitis B virus (HBV) vaccine has been efficiently used for decades. However, hepatocellular carcinoma caused by HBV is still prevalent globally. We previously reported that interferon (IFN)-induced tripartite motif-containing 25 (TRIM25) inhibited HBV replication by increasing the IFN expression, and this study aimed to further clarify the anti-HBV mechanism of TRIM25.

**Methods:** The TRIM25-mediated degradation of hepatitis B virus X (HBx) protein was determined by detecting the expression of HBx in TRIM25-overexpressed or knocked-out HepG2 or HepG2-NTCP cells via Western blotting. Co-immunoprecipitation was performed to confirm the interaction between TRIM25 and HBx, and colocalization of TRIM25 and HBx was identified via immunofluorescence; HBV e-antigen and HBV surface antigen were qualified by using an enzyme-linked immunosorbent assay (ELISA) kit from Kehua Biotech. TRIM25 mRNA, pregenomic RNA (pgRNA), and HBV DNA were detected by quantitative real-time polymerase chain reaction. The retinoic acid-inducible gene I (RIG-I) and pgRNA interaction was verified by RNA-binding protein immunoprecipitation assay.

**Results:** We found that TRIM25 promoted HBx degradation, and confirmed that TRIM25 could enhance the K90-site ubiquitination of HBx as well as promote HBx degradation by the proteasome pathway. Interestingly, apart from the Really Interesting New Gene (RING) domain, the SPRY domain of TRIM25 was also indispensable for HBx degradation. In addition, we found that the expression of TRIM25 increased the recognition of HBV pgRNA by interacting with RIG-I, which further increased the IFN production, and SPRY, but not the RING domain is critical in this process.

**Conclusions:** The study found that TRIM25 interacted with HBx and promoted HBx-K90-site ubiquitination, which led to HBx degradation. On the other hand, TRIM25 may function as an adaptor, which enhanced the recognition of pgRNA by RIG-I, thereby further promoting IFN production. Our study can contribute to a better understanding of host-virus interaction.

**Keywords:** HBx; Hepatitis B virus; pgRNA; RIG-I; TRIM25

## Introduction

Tripartite motif-containing 25 (TRIM25) family members play an important role in the immunological defense response to viral infections. The Really Interesting New Gene (RING) domain at the N-terminal region is the domain for the enzyme activity domain. The SPRY domain at the C-terminal region is the domain that interacts with other proteins.[1] In our previous study, we reported that TRIM5γ promoted hepatitis B virus X (HBx) protein ubiquitination and degradation. TRIM14 interacted with HBx and blocked the formation of the HBx–DNA damage-binding protein 1 (DDB1)–structural maintenance of chromosome (SMC) complex, which inhibited the replication of the hepatitis B virus (HBV). Interferon (IFN)-induced TRIM25 inhibited HBV replication by amplifying IFN signaling.[2-4] We recently found that severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) N protein inhibited IFN production, which reduced the TRIM25-mediated retinoic acid-inducible gene I (RIG-I) K63 ubiquitination.[5] This demonstrated the crucial role of TRIM25 in the antiviral response by regulating IFN signaling. Previous studies reported that TRIMs protein plays an important role in host–HBV interaction.[6] IFN therapy is an approved treatment

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modality for chronic HBV infection.[7] Interestingly, most of the TRIM members are IFN-inducible genes,[2-4,8,9] which should be up-regulated during the IFN therapy for patients infected with HBV. However, the interaction between the host and HBV by TRIM members is not clear.

HBV is a major threat to global public health. It is closely associated with the increase in the global incidence of hepatocellular carcinoma (HCC).[10] Even though the HBV vaccine has been used to prevent HBV infection for nearly 30 years, the mortality due to HBV-related complications is almost one million each year.[11] HBV is a partially double-stranded DNA virus. The covalently closed circular DNA (cccDNA) of HBV is located in the nucleus of the transcription template for all HBV viral RNA. Therefore, sustained cccDNA leads to the rebound of viral infection after treatment.[12] HBV-encoded regulatory protein, HBx, promotes the expression of the HBV gene from the cccDNA template.[13] HBx is a 154-amino acid protein with an N-terminal negative regulatory domain and C-terminal trans-activation or co-activation domain.[14] A previous study reported that an HBx–DDB1–cullin 4-regulator of cullins 1 E3 ligase complex targets the SMC5/6 complex to increase the expression of HBV gene from episomal cccDNA.[15] In addition, SMC5/6 degradation increases DNA damage, which contributes to HBx-mediated tumorigenesis. HBx plays an increasingly indispensable role in HBV replication and HCC progression.[16,17] Silencing the expression of HBx can be an important way toward the functional cure of HBV-related diseases.[18]

In the present study, by clarifying the interaction between TRIM25-HBx and TRIM25–pregenomic RNA (pgRNA)–RIG-I complexes, we aimed to further determine the role of TRIM25 in regulating HBV replication.

## Methods

### Ethical approval

This study was approved by the First Hospital of Jilin University (No. 2018-093). All patients have signed informed consent.

### Cell culture, plasmids, and reagents

HepG2 and HEK293T cells from American Type Culture Collection (ATCC; Mansas City, Virginia, US) were maintained in Dulbecco's modified Eagle's medium (DMEM) (MilliporeSigma, Burlington, MA, USA) which was supplemented with 10% inactivated fetal bovine serum (Hyclone, UT, USA), including penicillin (100 IU/mL) and streptomycin (100 mg/mL), under a 5% $CO_2$ atmosphere at 37°C. The expression constructs were generated by cloning the sequence of the coding region into a VR1012 expression vector. Anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody, anti-influenza hemagglutinin epitope (HA)-tag, and glutathione S-transferase-tag antibody were sourced from Proteintech (IL, USA). Anti-tubulin antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and MG132 was obtained from Sigma (Danvers, MA, USA).

### HBV infection assay

The assay was performed as reported previously.[16] Briefly, the serum from HBV patients was collected, and the whole virus was concentrated using the PEG-it Virus Precipitation Solution (System Biosciences, CA, USA). HepG2-NTCP cells were inoculated with the serum-produced virus at a multiplicity of infection of 1000 genome equivalents (Geq) per cell; the cells were then cultured in the presence of 4% PEG8000 and 2% dimethyl sulfoxide for 24 h. After infection, the cells were washed thrice with phosphate-buffered saline (PBS) and maintained in the DMEM for another nine days; the medium was changed every two days. The supernatant and cells were collected for the detection of HBV DNA, pgRNA, and HBV surface antigen (HBsAg) by quantitative real-time polymerase chain reaction (q-RT-PCR) or enzyme-linked immunosorbent assay (ELISA).

### RNA extraction and q-RT-PCR

Total RNA was extracted from the cells by using the EasyPure RNA Kit (Transgen, Beijing, China) according to the manufacturer's instructions and then converted to first-strand complementary DNA (cDNA) using the TransScript First-Strand cDNA Synthesis SuperMix (Transgen). HBV DNA was isolated from the supernatants as per the manufacturer's instructions (Transgen). A housekeeping gene, *GAPDH*, was used as an internal control for quantitation, and the gene expression was quantified as described previously.[16] The gene-specific primer sequences used for q-RT-PCR in this study are listed in Supplementary Table 1, http://links.lww.com/CM9/B450.

### Co-immunoprecipitation and Western blotting

HepG2 or HEK293T cells were transfected with the ViaFect Transfection Reagent (Promega, WI, USA) between 24 and 48 h after the transfection of the expression plasmids; the cells were lysed with 50 mmol/L Tris–HCl (pH 8.0), 150 mmol/L NaCl, and 1% Nonidet P-40 (NP-40) containing cocktail inhibitors (Millipore, MA, USA). The cell lysates were immunoprecipitated and then incubated with the ANTI-FLAG® M2 Affinity Gel (Sigma, Burlington, MA, USA) overnight. Immunoblotting was performed as described elsewhere. Briefly, the cells were collected and lysed in ice-cold cell lysis buffer for 30 min, with the tubes tapped every 10 min. The protein concentration was quantified by using the Coomassie Plus™ Protein Assay Reagent (Thermo Scientific, Waltham, MA, USA). The band intensities were quantified with ChemiDoc™ XRS + Molecular Imager software (Bio-Rad, CA, USA). The samples were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes. The blots were blocked in Tris-buffered saline containing 0.1% Tween-20 and 5% skimmed milk and then probed with the relevant antibodies.

### RNA-binding protein immunoprecipitation assay (RIP)

Flag-TRIM25 or RIG-I was transfected into HepG2 cells together with or without HBV expression plasmid

(pHBV1.2), and the cell lysates were mixed with Flag-beads (Bimake, Shanghai, China) and incubated for 4 h with gentle shaking at 4°C. After washing thrice, the precipitated RNAs were analyzed by q-RT-PCR with primers to detect the target pgRNA. The number of immunoprecipitated RNAs was presented as the relative fold of the amount of input RNA.

### Immunofluorescence

Immunofluorescence was performed as reported in a past study.[19] Briefly, HepG2 cells were transfected with Flag-HBx plasmids or HA-TRIM25 plasmids. After 48 h, the cells were fixed in acetone–methanol (1:1) solution at 37°C for 10 min. Subsequently, the cells were washed with PBS, blocked with 5% bovine serum albumin in phosphate buffered saline tween (PBST) for 1 h, incubated with Flag (mouse) and HA (rabbit) antibodies at 37°C for 1 h, washed in PBS, and then incubated with goat anti-mouse IgG conjugated with fluorescein isothiocyanate isomer I (FITC) or both Cy3 (rabbit)- and FITC (mouse)-conjugated IgG (Proteintech). The cells were washed with PBS and observed by fluorescence microscopy.

### ELISA

The HepG2 cells were mock-transfected or transfected with TRIM25 expression plasmids together with the pHBV1.2-HBV expression plasmids. The supernatant was collected after 72 h and then subjected to ELISA to determine the levels of HBV e-antigen (HBeAg) and HBsAg (Kehua Biotech, Shanghai, China).

### Statistical analysis

GraphPad Prism 8 software (GraphPad Software, San Diego, CA) was used for data analyses; the results are presented as mean ± standard deviation and analyzed using the Student's *t*-test. $P < 0.05$ was considered statistically significant.

## Results

### TRIM25 specifically promoted HBx degradation

TRIM family members act as the E3 ligase which binds to the target proteins and promotes the ubiquitination of the targets, especially in the response to viral infections.[20] TRIMs are always induced and they interact with the virus proteins, which might induce the degradation of the virus structure or non-structure proteins and block the virus infection or replication in the host cells.[21] In the present study, we investigated TRIM25 as an E3 ligase to specifically promote HBx degradation [Figure 1A].[22] TRIM25 did not statistically significantly affect the HBx mRNA [Supplementary Figure 1A, http://links.lww.com/CM9/B450]. As a negative control, SARS-CoV-2M protein was not degraded by TRIM25 [Supplementary Figure 1B, http://links.lww.com/CM9/B450]. We further demonstrated that HBx was a stronger partner of TRIM25 than Core or S protein in the co-immunoprecipitation assay (Co-IP) [Supplementary Figure 1C, http://links.lww.com/CM9/B450] and that the immunofluorescence assay indicated that TRIM25 and HBx were located in the same region in the HepG2 cells [Figure 1B]. In addition, the expression of HBx protein was significantly enhanced in TRIM25 Knockout (KO) HepG2 cells [Figure 1C], and this result was also confirmed in the HepG2-NTCP infection systems [Supplementary Figure 2, http://links.lww.com/CM9/B450]. We then determined whether HBx was degraded by TRIM25 via the proteasome pathway. TRIM25-induced HBx degradation was rescued by MG132 (proteasome inhibitor) treatment but not by bafilomycin A1 (Baf, lysosome degradation inhibitor) or Z-VAD (caspase inhibitor) [Figure 1D]. We finally confirmed that TRIM25 enhanced the ubiquitination of HBx [Figure 1E]. All these results collectively suggested that TRIM25 specifically promotes HBx degradation by the proteasome pathway.

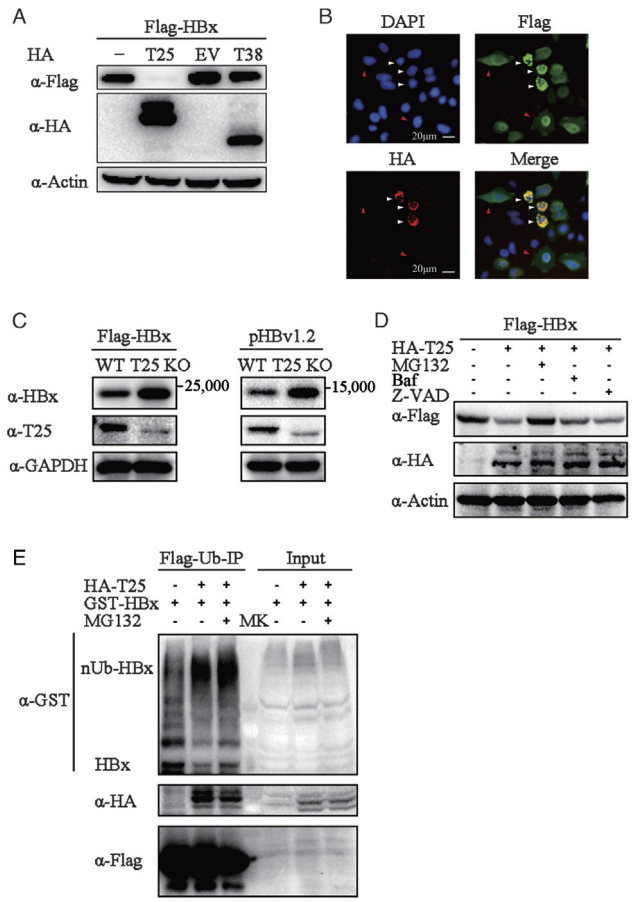


**Figure 1:** TRIM25 specifically promotes HBx degradation. (A) Western blot analysis of the HepG2 cells transfected with the plasmids as indicated showed that TRIM25 (T25) but not TRIM38 (T38) promoted HBx degradation. (B) Immunofluorescence assay analysis of the HepG2 cells transfected with Flag-HBx and HA-T25 expression plasmids indicated the colocalization of TRIM25 and HBx. (C) Western blot analysis of the HepG2 cells (WT or T25 KO) transfected with Flag-HBx or pHBV1.2 expression plasmids indicated HBx expression was enhanced in the T25 KO HepG2 cells. (D) Western blot analysis of the HepG2 cells transfected with the plasmids as indicated, which showed proteasome pathway inhibitor MG132 rescued the T25-mediated HBx degradation but not the other inhibitors. (E) Co-IP analysis of the HEK293T cells transfected with the plasmids as indicated showed that T25 promoted HBx ubiquitination. α: Antibody; Co-IP: Co-immunoprecipitation; DAPI: 4′,6-diamidino-2-phenylindole; EV: Empty vector; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; GST: Glutathione S-transferase; HA: Hemagglutinin epitope; HBV: Hepatitis B virus; HBx: Hepatitis B virus X; KO: Knockout; LP: Leupeptin; MK: Marker; nUb: Ubiquitin (Ub)>1; pHBV1.2 WT: Wild-type HBV expression plasmid with 1.2-fold HBV genome; TRIM25: Tripartite motif-containing 25; WT: Wild type; Z-VAD: Pan-caspase Inhibitor Z-VAD-FMK.

### RING and SPRY domains are important in the TRIM25-induced HBx degradation

We investigated the TRIM25 region responsible for HBx interaction and the inhibition of HBV replication. Generally, the RING domain is decisive for the E3 ligase activity, and SPRY is the domain for the interaction with the target protein.[23] Thus, the RING or SPRY domain of TRIM25 was deleted, respectively. RING deletion rescued the TRIM25-mediated HBx degradation. SPRY deletion showed the same result, which indicated that both the RING and SPRY domains are important in the HBx degradation process [Figure 2A]. The Co-IP assay was performed to determine the interaction of HBx with the truncated sites. The result indicated that the mutants with both RING or SPRY domain deletion showed a weaker interaction with HBx protein, especially the mutants with SPRY deletion [Figure 2B]. In addition, we further determined the inhibitory effect of truncations in HBV replication. Interestingly, the RING domain or SPRY domain-deletion mutant demonstrated a significantly weaker inhibition compared with the wild-type TRIM25. Nevertheless, the mutants continued to indicate a significant inhibition of HBV replication [Figures 2C and 2D], suggesting the involvement of other mechanisms. These results cumulatively indicated that the RING domain and the SPRY domain are important in the TRIM25-mediated HBx degradation and HBV replication.

### TRIM25 promotes the K90-site ubiquitination of HBx

We further continued to explore the critical region or site of HBx that is involved in the interaction with TRIM25. Truncations and mutants as shown in Figure 3A were used. After 88 to 100 amino acid deletion (Del2), which was important in HBx interaction with some target proteins,[24] we found that the interaction between HBx and TRIM25 was inhibited, suggesting that the 88 to 100 amino acid region of HBx was the target of TRIM25 [Figure 3B]. Moreover, unlike TRIM5γ or TRIM31, TRIM25 continued to show no statistically significant activity in the degradation of K95R-mutated HBx but not K90R, thereby indicating that TRIM25 promoted HBx ubiquitination on the K90 site [Figure 3C], and the ubiquitin assay further confirmed our results [Figure 3D]. Altogether, these data suggested that TRIM25 binds to the 88 to 100 region of HBx and promotes K90-site ubiquitination. Further, to check if TRIM25 could inhibit HBV replication when HBx was not expressed, pHBV1.2 ΔX plasmids were used, and interestingly, similar to that in the pHBV1.2 WT group, pgRNA, HBsAg, and HBeAg were significantly inhibited [Figure 3E–3G], thereby suggesting the involvement of other mechanisms in the TRIM25-mediated HBV inhibition.

### TRIM25 promotes the recognition of pgRNA by RIG-I

As shown in Supplementary Figures 2C and 2D (http://links.lww.com/CM9/B450), we noticed that after RING or SPRY deletion, TRIM25 still played a role in inhibiting HBV replication. Furthermore, HBV without HBx expression was still inhibited by TRIM25 [Figures 3E–G], and TRIM25 was reported to be essential for RIG-I-mediated antiviral activity by promoting IFN production. RIG-I recognized HBV pgRNA, which further induced type III IFN.[22,25] We implied that TRIM25 might promote the recognition of pgRNA by RIG-I; hence, we performed an RNA-binding protein immunoprecipitation assay (RIP) assay to verify our speculation. As we expected, pgRNA expression was significantly inhibited by TRIM25 overexpression in the input sample, and significantly more pgRNA was pulled down by TRIM25 in the RIP sample [Figure 4A], which indicated the interaction of TRIM25 with pgRNA. Over-expression of TRIM25 in the HepG2 cells further enhanced the interaction of RIG-I and pgRNA. A primer used to detect HBs RNA indicated significant enhancement in the TRIM25 co-transfection sample, thereby indicating that the RIG-I-TRIM25 dimer might be targeted to the HBs RNA region [Figures 4B and 4C]. In addition, RIG-I-mediated pgRNA recognition was significantly inhibited in the TRIM25 KO HepG2 cells [Figures 4D, 4E and 4G]. The interaction between TRIM25 and RIG-I was confirmed by the Co-IP assay [Figure 4F]. Collectively, the results indicated that TRIM25 might promote the recognition of pgRNA by RIG-I.

### TRIM25 SPRY domain is critical in the enhancement of the RIG-I-mediated recognition of HBV pgRNA

TRIM25 is an RNA binding protein. Similar to other TRIM family proteins, TRIM25 consists of an N-terminal

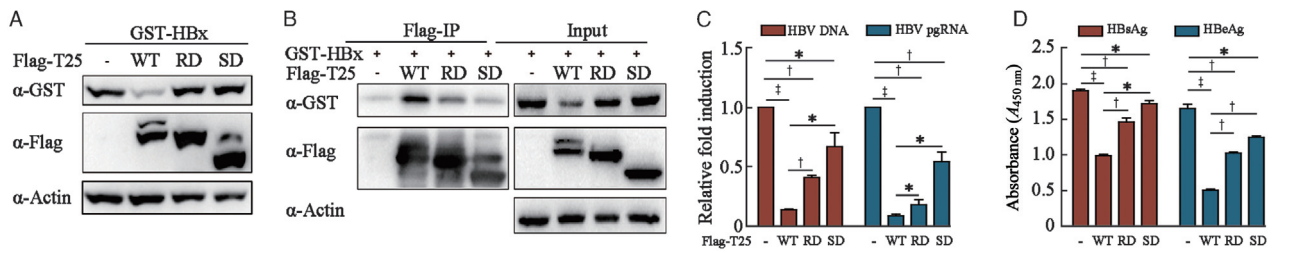


**Figure 2:** Both RING and SPRY domains are important in TRIM25 (T25)-induced HBx degradation. (A) Western blot analysis of the HepG2 cells transfected with the plasmids as indicated that T25 lost the ability to promote HBx degradation after the loss of RING or SPRY domains. (B) Co-IP analysis of the HEK293T cells transfected with the plasmids as indicated showed that HBx interacted with WT T25 but not RD or SD. (C) q-RT-PCR or (D) ELISA analysis of HepG2 cells (C) or supernatants (D) transfected with pHBV1.2 plasmids or plasmids as indicated that RING or SPRY deleted mutant had weaker inhibition of HBV compared to the WT T25. $^{*}P < 0.05$; $^{\dagger}P < 0.01$; $^{\ddagger}P < 0.001$. α: Antibody; Co-IP: Co-immunoprecipitation; ELISA: Enzyme-linked immunosorbent assay; GST: Glutathione S-transferase; HBeAg: HBV e-antigen; HBsAg: HBV surface antigen; HBV: Hepatitis B virus; HBx: Hepatitis B virus X; pgRNA: Pregenomic RNA; pHBV1.2 WT: Wild-type HBV expression plasmid with 1.2-fold HBV genome; q-RT-PCR: Quantitative real-time PCR; RD: RING domain deletion; RING: Really Interesting New Gene; SD: SPRY domain deletion; WT: Wild type.

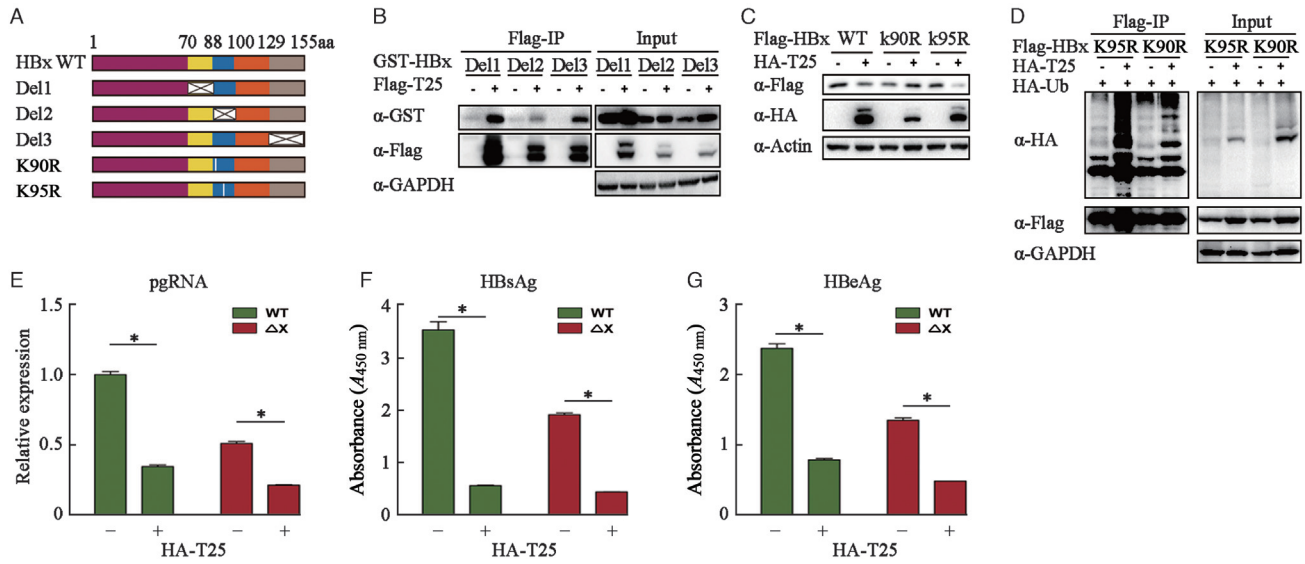


**Figure 3:** TRIM25 (T25) promotes the K90 site ubiquitination of HBx. (A) Diagrams depicting mutant HBx constructs. (B) Co-IP analysis of the HEK293T cells transfected with the plasmids as indicated showed that 88 to 100aa region of HBx was critical in the interaction with T25. (C) Western blot analysis of the HepG2 cells transfected with the plasmids as indicated showed that K90 of HBx was the major ubiquitin site by T25. (D) Co-IP analysis of the HEK293T cells transfected with the plasmids as indicated showed K90 of HBx was the ubiquitin site caused by T25. (E–G) q-RT-PCR or ELISA analysis of HepG2 cells transfected with pHBV1.2 WT or ΔX or co-transfected with T25 expression plasmid as indicated, which showed T25 inhibited both WT or ΔX HBV replication. $^{*}P < 0.01$. α: Antibody; aa: Amino acid; Co-IP: Co-immunoprecipitation; Del: Deletion; ELISA: Enzyme-linked immunosorbent assay; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; GST: Glutathione S-transferase; HA: Hemagglutinin epitope; HA-Ub: HA-tag ubiquitin; HBeAg: HBV e-antigen; HBsAg: HBV surface antigen; HBx: Hepatitis B virus X; K/R: Lys to Arg mutation; pgRNA: Pregenomic RNA; pHBV1.2 WT: Wild-type HBV expression plasmid with 1.2-fold HBV genome; q-RT-PCR: Quantitative real-time polymerase chain reaction; TRIM25: Tripartite motif-containing 25.

zinc-finger RING domain that is responsible for its E3 ubiquitin ligase activity, two B-box zinc-finger domains, a coiled-coil domain (CCD), and a linker domain leading to a C-terminal-associated SPRY domain.[26] The CCD domain was critical in TRIM25 and RNA binding. In our study, TRIM25 could bind to HBV pgRNA, and TRIM25 could significantly promote RIG-I and RNA binding. Interestingly, after SRPY but not RING domain deletion, TRIM25 could not promote the binding of pgRNA to RIG-I [Figures 5A–D], which indicated that the SPRY domain is critical in this process. We further found that the TRIM25 SPRY deletion mutant could not interact with RIG-I [Figure 5E]. This result confirmed that TRIM25 might promote the RNA recognition of RIG-I by directly binding to RIG-I [Supplementary Figure 3, http://links.lww.com/CM9/B450].

## Discussion

TRIM25 acts as an E3 ubiquitin ligase protein and plays multiple roles in response to virus infection by co-operating with RIG-I.[22,27-29] In this study, we found that TRIM25 interacted with HBx and promoted HBx-K90-site ubiquitination, which resulted in HBx degradation. On the other hand, TRIM25 might function as an adaptor, which enhanced the recognition of pgRNA by RIG-I, thereby further promoting IFN production. Our study showed the direct and indirect role of TRIM25 in regulating HBV replication, which will contribute to a better understanding of host–virus interaction.

TRIM25 is an important molecule among the TRIM family members; it was first found to be an E3 ubiquitin ligase that induces Lys 63-associated RIG-I ubiquitination, which is crucial for the cytosolic RIG-I signaling pathway to induce IFN production.[22] In addition, in response to HBV infection, RIG-I-mediated sensing of the 5′-ε region of HBV pgRNA resulted in the induction of type-III IFN.[25] TRIM25 was reported to recognize RNA through the CCD together with the SPRY domain.[26] According to these studies, TRIM25 might be an enhancer of the RIG-I-mediated pgRNA recognition. In our study, we noticed that SPRY domain deletion can more significantly rescue HBV replication compared with that of RING, although both of them cannot promote HBx degradation, thereby suggesting that SPRY might play a role in some other mechanisms. We found that SPRY deletion blocked the interaction between TRIM25 and RIG-I and also disrupted pgRNA binding. Our study indicated that TRIM25 can bind to pgRNA, which is dependent on the SPRY domain.

HBx is a small transactivator that might activate several signaling pathways by interacting with host factors; several studies showed that HBx plays an indispensable role in HBV replication and also in HCC development.[30] The blocking of HBx expression might cure human HBV infection.[18] Dicoumarol, an inhibitor of NAD(P)H: quinone oxidoreductase 1, promoted HBx degradation, and significantly inhibited HBV replication by rescuing SMC5/6 complex expression[31]; in addition, HBx-mediated SMC5/6 degradation also inhibited DNA damage response and was beneficial to HCC development.[17] We have reported two TRIM members, TRIM5γ and TRIM31, that worked together to promote HBx degradation.[3,8,9] Interestingly, HBx could be also degraded by another TRIM member TRIM21.[32] Here, we determined that TRIM25 could also promote HBx

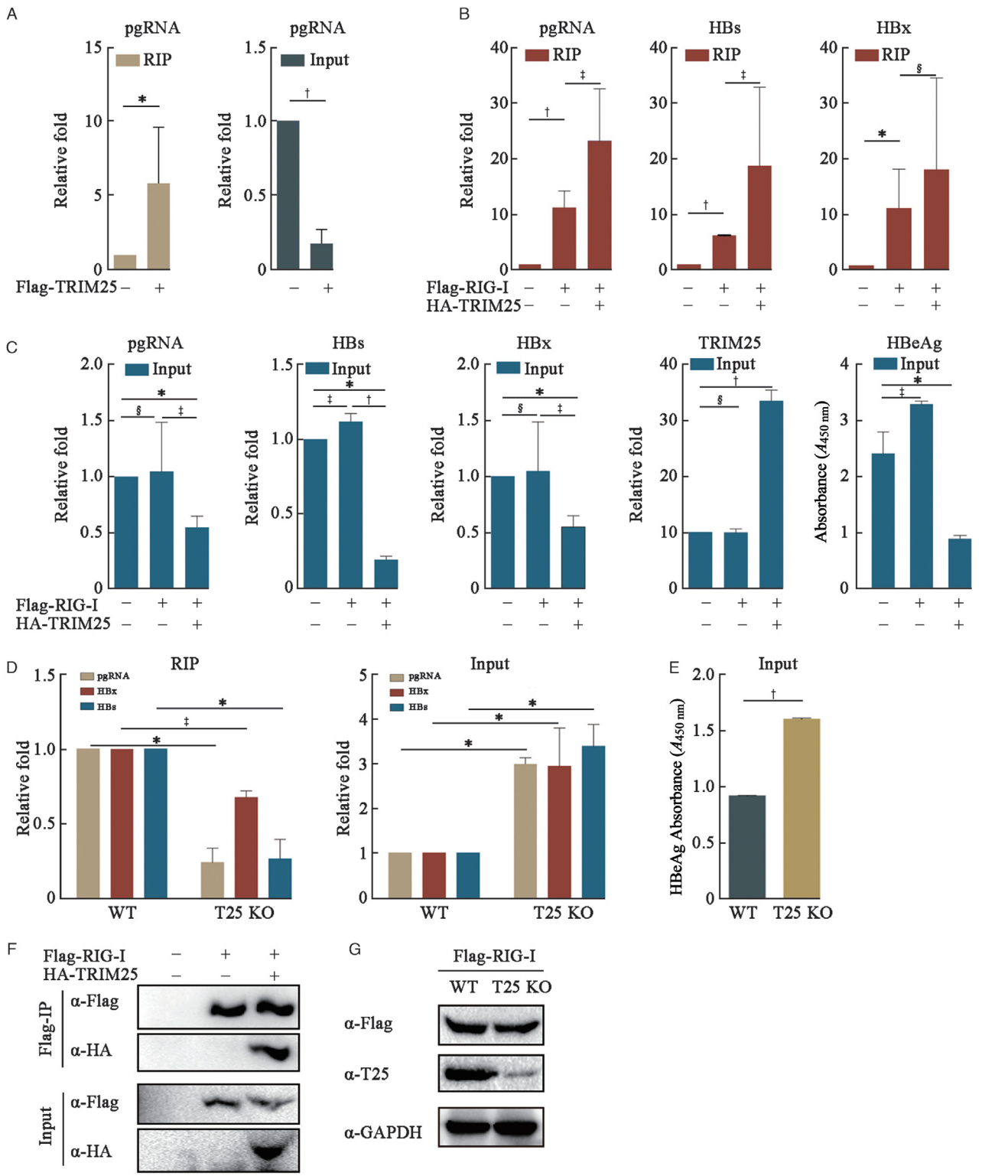


**Figure 4:** TRIM25 (T25) promotes the RIG-I-mediated recognition of pgRNA. (A) RIP analysis of HepG2 cells transfected with T25 and pHBV1.2 plasmids indicated T25 interacted with HBV pgRNA. (B, C) RIP analysis of HepG2 cells transfected with T25 and pHBV1.2 plasmids or co-transfected with RIG-I plasmids indicated T25 enhanced the interaction of RIG-I and HBV pgRNA, HBx or HBs. (D, E) RIP analysis of HepG2 WT or T25 KO cells transfected with Flag-RIG-I and pHBV1.2 plasmids indicated T25 KO reduced the RIG-I-interacted HBV pgRNA, HBx or HBs. (F) The cells were transfected as (B) and subjected to Co-IP analysis, which indicated the interaction between T25 and RIG-I. (G) Western blot analysis of the HepG2 cells transfected with the plasmids as (D) showed the expression of RIG-I and T25. $^{*}P < 0.01$; $^{\dagger}P < 0.001$; $^{\ddagger}P < 0.05$; $^{\S}P > 0.05$. α: Antibody; α-GAPDH: Antibody of glyceraldehyde-3-phosphate dehydrogenase; Co-IP: Co-immunoprecipitation; ELISA: Enzyme-linked immunosorbent assay; HA: Hemagglutinin epitope; HBeAg: HBV e-antigen; HBs: Hepatitis B virus surface gene; HBV: Hepatitis B virus; HBx: Hepatitis B virus X; pgRNA: Pregenomic RNA; KO: Knockout; pHBV1.2: HBV expression plasmid with 1.2-fold HBV genome; RIG-I: Retinoic acid-inducible gene I; RIP: RNA immunoprecipitation; TRIM5: Tripartite motif-containing 25; WT: Wild type.

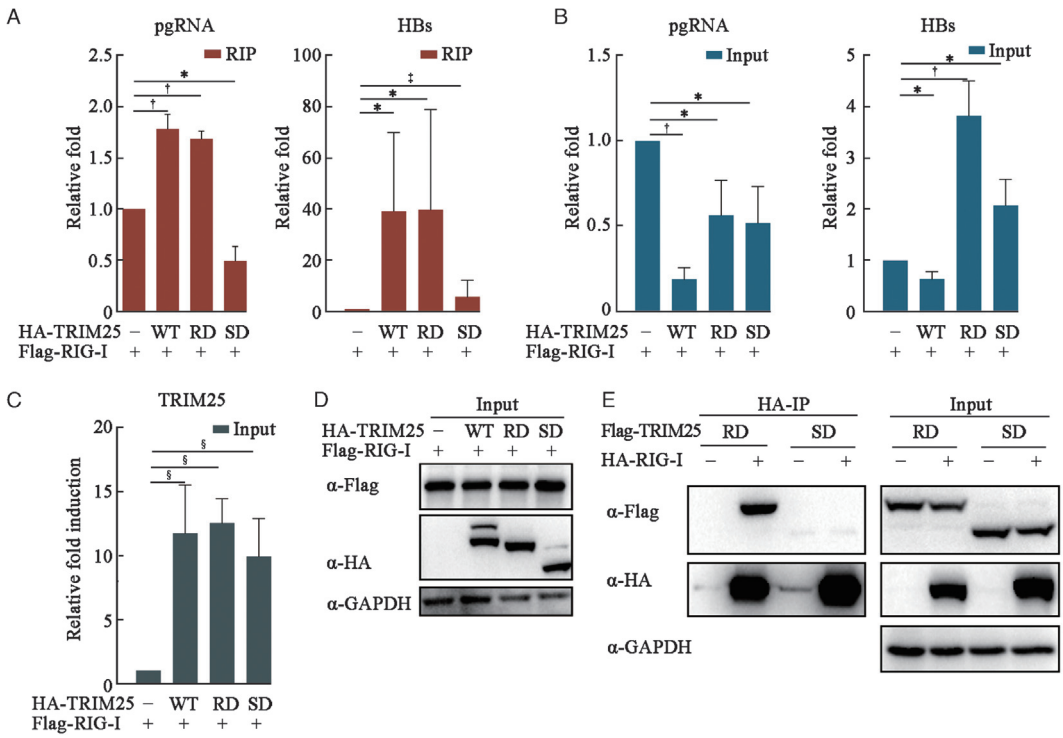


**Figure 5:** TRIM25 SPRY domain is critical for the enhancement of RIG-I-mediated recognition of HBV pgRNA. (A) RIP analysis of HepG2 cells transfected with TRIM25 (or mutants) and pHBV1.2 plasmids indicated TRIM25 (WT and RD) interacted with HBV pgRNA, but not SD. (B) q-RT-PCR analysis of input samples as in (A) showed both RING and SPRY domain were important in the TRIM25-mediated HBV inhibition. (C) q-RT-PCR analysis of HepG2 cells transfected with the plasmids as indicated showed TRIM25 was highly expressed in WT, RD and SD samples. (D) Western blot analysis of the HepG2 cells as in (A) showed the TRIM25 and RIG-I protein expression. (E) Co-IP analysis of the HEK293T cells transfected with the plasmids as indicated showed SPRY domain was critical in the TRIM25 and RIG-I interaction, but not RING domain. $^{*}P < 0.05$; $^{\dagger}P < 0.01$; $^{\ddagger}P > 0.05$; $^{\S}P < 0.001$. α: Antibody; Co-IP: Co-immunoprecipitation; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; HA: Hemagglutinin epitope; HBV: Hepatitis B virus; HBs: Hepatitis B virus surface gene; pgRNA: Pregenomic RNA; pHBV1.2: HBV expression plasmid with 1.2-fold HBV genome; q-RT-PCR: Quantitative real-time PCR; RD: RING domain deletion; RIG-I: Retinoic acid-inducible gene I; RING: Really Interesting New Gene; RIP: RNA immunoprecipitation; SD: SPRY domain deletion; TRIM25: Tripartite motif-containing 25; WT: Wild type.

degradation. These studies indicated the importance of the E3 ligase activity of TRIM family members. Most TRIMs are IFN-stimulated genes; therefore, TRIMs might be critical in the treatment of HBV-infected patients by inhibiting HBx in IFN therapy.

Finally, we proposed a working model for TRIM25 that inhibited HBV replication by direct and indirect mechanisms.

However, there are also limitations in our study. Whether the E3 ligase activity of TRIM25 is indispensable in the HBx degradation needs to be further confirmed.

To conclude, we found that TRIM25 interacts with HBx and promotes its proteasome degradation by K90-site ubiquitination. Moreover, TRIM25 also plays a critical role in the RIG-I-mediated recognition of pgRNA via the SPRY domain. Our study helps to better understand the mechanism involved in the cross-talk of HBV and the host.

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## Conflicts of interest

None.

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