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ORIGINAL ARTICLE

TRIM27 Promotes Hepatitis C Virus Replication by Suppressing Type I Interferon Response

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Abstract— Type I interferon (IFN) response is central for host defense against viral infection. Tripartite motif 27 (TRIM27) is implicated in antiviral innate immune response; however, whether it affects the replication of hepatitis C virus (HCV) and the underlying mechanisms remain uncharacterized. Here, we show that TRIM27 expression is induced in Huh7.5 human hepatoma cells infected with HCV or stimulated with type I IFNs *in vitro*. In addition, TRIM27 overexpression increases and its knockdown decreases viral RNA and protein levels, suggesting that TRIM27 positively regulates HCV replication. Mechanistically, TRIM27 inhibits type I IFN response against HCV infection through inhibiting IRF3 and NF-κB pathways, since TRIM27 mutant unable to inhibit these two inflammatory pathways fails to promote HCV replication. Taken together, this study identifies TRIM27 as a novel positive regulator of HCV replication, and also implicates that targeting TRIM27 may serve as a therapeutic strategy for controlling HCV replication.

KEY WORDS: TRIM27; hepatitis C virus; type I interferon response; IRF3; NF-κB.

INTRODUCTION

Innate immune response is central for the host defense to combat viral infection, which is activated upon the recognition of pathogen-associated molecular patterns (PAMPs), such as viral nucleic acids and proteins [1–3]. Accordingly, PAMPs are recognized by a variety of pattern recognition receptors (PRRs), like Toll-like receptors (TLRs) [4], whereby stimulating a cascade of inflammatory signaling pathways for inducing the production of type I interferons (IFNs) and cytokines [5, 6]. Among these signaling pathways, the nuclear factor-κB (NF-κB) and IFN regulatory factor 3 (IRF3) play crucial roles in the transcriptional induction of type I IFNs [7], including IFN-α and IFN-β, which in turn signal through IFN-α receptor to induce the expression of several IFN-stimulated genes (ISGs) for restraining virus replication [8].

The hepatitis C virus (HCV) infects approximately 180 million people in the world [9, 10] and is one of the leading causes of chronic liver diseases, including cirrhosis, steatosis, and hepatocellular carcinoma. Currently, the mainstay treatment for HCV infection is a combination therapy consisting of peg-related IFN-α (Peg-IFN-α), ribavirin, and protease inhibitor [11]. However, due to strong pathogenicity of HCV and poor tolerance of drugs, new therapeutic strategies, and targets are urgently needed [12].

The ubiquitin ligase tripartite motif 27 (TRIM27) belongs to the TRIM family proteins that are involved in a wide-spectrum of cellular activities, including proliferation, differentiation, apoptosis, autophagy, and innate immune response [13]. In recent years, TRIM27 has been shown to regulate the innate antiviral immune response and production of type I IFNs against infection of vesicular stomatitis virus (VSV), herpes simplex virus (HSV), and

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Sendai virus (SeV) [14, 15]. However, its function and underlying mechanisms involved in HCV are unclear. In this study, we report the promotive role of TRIM27 in HCV replication with an *in vitro* infection model using hepatocellular carcinoma Huh-7.5 cells, in which the suppressed type I IFN immune response due to inhibition of NF-kB and IRF3 pathways plays a fundamental role.

MATERIALS AND METHODS

Cell Culture, Treatment, and HCV Infection

Hepatocellular carcinoma Huh-7.5 and HEK293 cells were purchased from ATCC and maintained in DMEM (Gibco) medium supplemented with 10% FBS (Gibco) and 0.1 mM nonessential amino acids (ThermoFisher Scientific) at 37 °C with an atmosphere of 5% CO_2. Huh-7.5 cells were treated with different concentrations of human recombinant IFN-α or IFN-β (R & D Systems) for 24 h. The clone FL-J6/JFH1 containing the full-length chimeric HCV genome was constructed by multiple steps to generate the infectious HCV (J6/JFH1) following a well-established protocol [16]. Huh-7.5 cells were then infected by HCV (J6/JFH1) as described in a previous study [17]. Infected cells were then passaged every 3 days and used for further analyses. All experimental protocols were approved by the Ethics Committee of Qilu Hospital of Shandong University.

Plasmids, Overexpression, and siRNA Transfection

Plasmids of pcDNA-vector and pcDNA-TRIM27 were purchased from Origene. TRIM27 mutant with a truncated B-Box domain was constructed using QuikChange Site-Directed Mutagenesis kit (Stratagene) and cloned into pcDNA-vector to generate pcDNA-TRIM27 (ΔB) plasmid. For overexpression, Huh-7.5 cells were cultured in six-well plates. When cell density reached nearly 50% confluency, 2 μg or 4 μg plasmids of pcDNA-vector, pcDNA-TRIM27, or pcDNA-TRIM27 (ΔB) were transfected using FuGENE(R) 6 Transfection Reagent (Promega) according to manufacture's instructions. After 2 days of transfection, cells were harvested for further analyses. For siRNA transfection, Huh-7.5 cells were transiently transfected with siRNA control or two different specific siRNAs targeting TRIM27 synthesized by GenePharma (Shanghai, China) using Lipofectamine RNAiMAX reagent (ThermoFisher Scientific) according to manufacture's protocols. The final concentration of siRNA is 25 nM. After 3 days of transfection, cells were harvested for further analyses. The efficacy of overexpression and knockdown was validated by analyses of qRT-PCR and Western blot as described below.

qRT-PCR Analysis

The total RNA was isolated from Huh-7.5 cells with the TRIzol reagent (ThermoFisher Scientific) and then reversely transcribed using the kit of RevertAid First Strand cDNA Synthesis (ThermoFisher Scientific) according to manufacturer's instructions. qRT-PCR analysis was conducted using SYBR green reagent (Takara) and CFX96 PCR System (Bio-Rad). The primer sequences are listed as follows: TRIM27 sense 5′-TTGGGAAGGAATCAGCAGGT-3′ and antisense 5′-ATCCCTGGAAAGAAGCCTCC-3′; HCV sense 5′-CTTCACGCAGAAAGCGTCTA-3′ and antisense 5′-CAAGCACCCTATCAGGCAGT-3′; β-actin sense 5′-AGGCTGTGCTATCCCTGTAC-3′ and antisense 5′-AATGTCACGCACGATTTCCC-3′. β-Actin was used as a normalization control. Data are expressed as relative to control treatment. Experiments were conducted in triplicates.

Western Blotting Analysis

Huh-7.5 cells were collected after treatment and lysed using RIPA lysis buffer (Beyotime) for extracting total protein. Samples containing equal amount of proteins were resolved by SDS-PAGE and then transferred to nitrocellulose (NC) membranes [18]. Membranes were then sequentially probed with primary antibodies and secondary antibodies. Antibodies were purchased from the following sources: anti-TRIM27 (1:500, Novus Biologicals), anti-β-actin (1:5000, Santa Cruz), anti-NS5A (1:1000, Abcam), anti-p-IRF3 (1:500, Cell Signaling), anti-IRF3 (1:1000, Cell Signaling), anti-p-NF-κB (1:1000, Abcam), anti-NF-κB (1:1000, Abcam), and HRP-conjugated goat-anti-rabbit and goat-anti-mouse secondary antibodies (1:5000, Santa Cruz). After rinse with TBST, membranes were incubated with ECL Western blot regent (Pierce) for visualizing protein bands. The quantification of band intensity was analyzed by ImageJ.

Luciferase Reporter Assay

The procedures of luciferase reporter assay were conducted according to a previous study [19]. Briefly, HEK293 cells were cultured in 24-well plates. When cell density reached nearly 50% confluency, cells were then cotransfected pcDNA-vector or pcDNA-TRIM27

expression plasmid with the luciferase reporter plasmid of NF-κB, IFN-β, or ISRE using FuGENE(R) 6 Transfection Reagent (Promega). HEK293 cells were collected and lysed at 48 h after transfection. The luciferase activity was evaluated using the Dual Luciferase Reporter Assay system (Promega) following manufacturer's protocols. The luciferase activity was calibrated to Renilla luciferase control and expressed as relative to vector control.

ELISA Assay

For determining IFN-β level in cell-free supernatants, the enzyme-linked immunosorbent assay (ELISA) was performed (eBioscience) according to the manufacturer's instructions.

Statistical Analysis

All data are presented as the mean ± SD. The statistical analysis comparing two sets of data was performed using the unpaired Student's *t* test. *P* values less than 0.05 were defined as statistically significant.

RESULTS

TRIM27 Expression Is Induced by HCV Infection or Stimulation of Type I IFNs

In primary peritoneal macrophages, infection of VSV or SeV results in upregulated expression of TRIM27 [15], implying that cellular TRIM27 expression varies in response to viral infection. However, at present, whether HCV infection affects TRIM27 expression is still unknown. To address it, we utilized Huh-7.5 cell line cultured *in vitro*, one naturally HCV-permissive human hepatocellular carcinoma [20], and infected it with HCV (J6/JFH1), and then determined TRIM27 expression by qRT-PCR and Western blotting analyses. The result showed that compared with mock infection (HCV−), HCV infection (HCV+) induced a significant upregulation in TRIM27 mRNA in Huh-7.5 cells (Fig. 1a). Moreover, a similar trend was observed in TRIM27 protein level in Huh-7.5 cells which were infected by HCV (Fig. 1b). HCV infection triggers the production of type I IFNs in host cells, including IFN-α and IFN-β, which activate the transcription of ISGs so as to restrict virus replication [21, 22]. Interestingly, we found that both the mRNA level (Fig. 1c) and protein level (Fig. 1d) of TRIM27 were dose-dependently induced in Huh-7.5 cells by the stimulation with either IFN-α or IFN-β. Together, these results show that HCV infection and stimulation of type I IFNs upregulate TRIM27 expression, at least in Huh-7.5 cells *in vitro*.

TRIM27 Positively Regulates HCV Replication

TRIM27 upregulation in response to HCV infection hints that it may have a functional role during this pathological process. To test this possibility, TRIM27 was overexpressed in HCV-infected Huh-7.5 cells through transfecting pcDNA-TRIM27 plasmid. As a result, compared with pcDNA-vector transfection, TRIM27 overexpression led to a remarkable increase in the protein level of HCV nonstructural protein 5 A (NS5A) (Fig. 2a), which belongs to the C-terminal portion of the polyprotein of HCV [23]. Likewise, the mRNA level of NS5A was also elevated in TRIM27-overexpressing Huh-7.5 cells infected with HCV (Fig. 2b). Therefore, these findings suggest that TRIM27 may promote HCV replication. To consolidate this conclusion, we depleted TRIM27 in HCV-infected Huh-7.5 cells through siRNA transfection. As shown, contrary to TRIM27 overexpression, siRNA-mediated knockdown of TRIM27 decreased protein level (Fig. 2c) as well as mRNA level (Fig. 2d) of NS5A in HCV-infected Huh-7.5 cells, further supporting a promotive role of TRIM27 in HCV replication.

TRIM27 Suppresses Type I IFN Response Against HCV Infection

Type I IFN immune response plays a fundamental role in restricting HCV infection [24]. To clarify how TRIM27 promotes HCV replication, we focused on investigating whether TRIM27 affects type I IFN response. We found that TRIM27 transient overexpression in HEK293 cells dose-dependently suppressed the activity of IFN-β-responsive promoter, as analyzed by luciferase reporter assay (Fig. 3a). Next, we examined whether TRIM27 regulates IRF3 pathway and NF-κB pathway, which coordinately determine IFN-β production [25]. The results showed that similar to the effect on IFN-β-responsive promoter, TRIM27 transient overexpression dose-dependently inhibited the activity of both IRF3-dependent promoter of IFN-sensitive response element (ISRE) (Fig. 3b) and NF-κB (Fig. 3c). Moreover, TRIM27 overexpression reduced the phosphorylation level of IRF3 and NF-κB in HCV-infected Huh-7.5 cells (Fig. 3d), the hallmark of activated IRF3 and NF-κB [26]. Furthermore, in HCV-infected Huh-7.5 cells, the mRNA level of IFN-stimulated genes, including IFNB1, ISG56, and ISG15, was also decreased by TRIM27 overexpression (Fig. 3e). In concert with these results, the production of IFN-β was

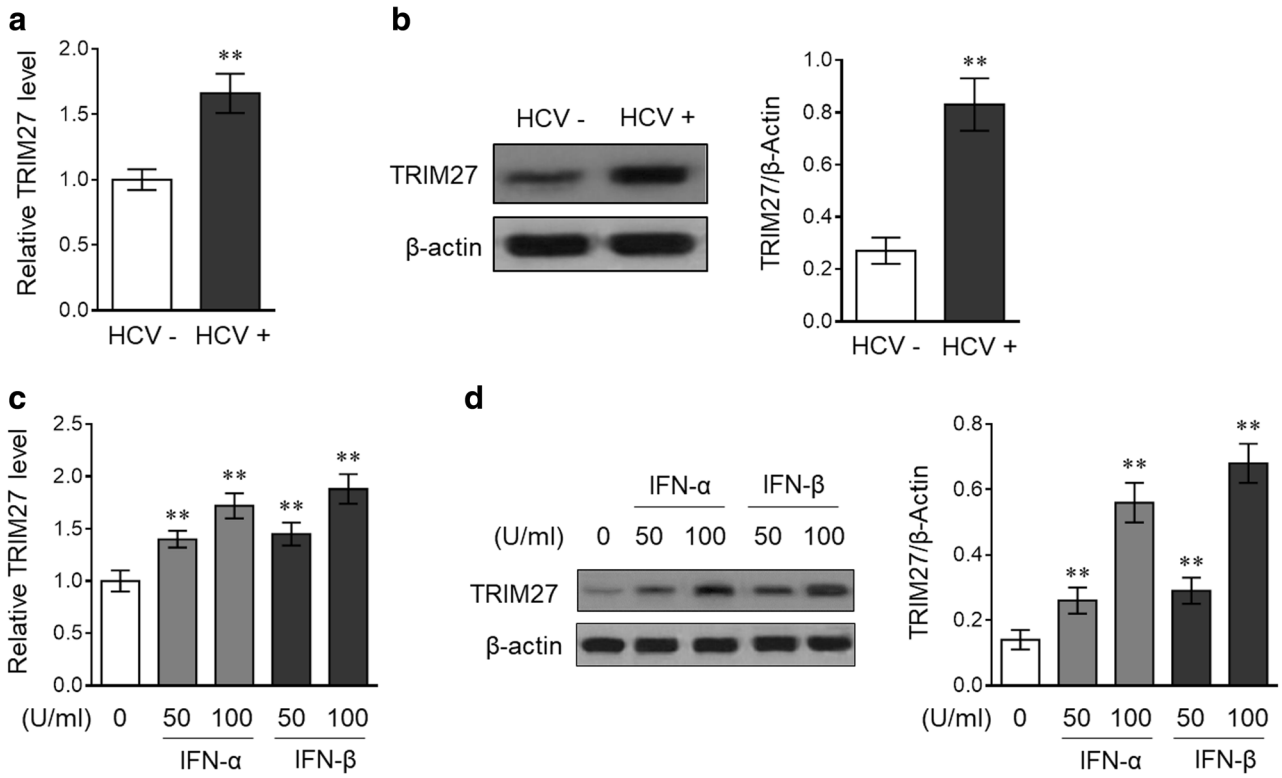

Fig. 1. TRIM27 is induced in response to HCV infection or stimulation of type I IFNs. **a** and **b** The mRNA level (**a**) and protein level (**b**) of TRIM27 in hepatocellular carcinoma Huh-7.5 cells infected with mock (HCV−) or HCV JFH-1 (HCV+). β-actin was used as a reference or loading control. Results are representative of three independent experiments. Data are mean ± SD. $n = 3$. Data were compared with HCV− group using Student's t test. $^{**}P < 0.01$. **c** and **d** The mRNA level (**c**) and protein level (**d**) of TRIM27 in hepatocellular carcinoma Huh-7.5 cells stimulated with 50 U/ml or 100 U/ml IFN-α or IFN-β as indicated for 24 h. β-actin was used as a reference or loading control. Results are representative of three independent experiments. Data are mean ± SD. $n = 3$. Data were compared with control using Student's t test. $^{**}P < 0.01$.

significantly inhibited in HCV-infected Huh-7.5 cells (Fig. 3f). Collectively, these data illustrate that TRIM27 suppresses type I IFN response against HCV infection, which may be attributed to the suppressed IRF3 and NF-κB pathways.

TRIM27 Promotes HCV Replication by Inhibiting IRF3 and NF-kB Pathways

Lastly, to clarify the contribution of suppressed IRF3 and NF-κB pathways to TRIM27-promoted HCV replication, a mutant form of TRIM27 with a truncated B-Box domain (ΔB) was constructed, since B-Box domain contains zinc-binding motifs that are critical for mediating its normal function [14]. We found that in contrast to wild-type TRIM27, the overexpression of mutant TRIM27 (ΔB) could no longer suppress the activity of IFN-β-responsive promoter (Fig. 4a), ISRE promoter (Fig. 4b), and NF-κB promoter (Fig. 4c), indicating that mutant TRIM27 (ΔB) loses the ability to inhibit NF-kB and IRF3 pathways. Further, the overexpression of mutant TRIM27 (ΔB) did not inhibit the production of IFN-β as compared with the wild-type TRIM27 (Fig. 4d). Consistently, the overexpression of wild-type TRIM27 increased the protein (Fig. 4e) and mRNA (Fig. 4f) levels of NS5A in HCV-infected Huh-7.5 cells, whereas, the overexpression of mutant TRIM27 (ΔB) had no similar effects (Fig. 4e, f). Thus, these lines of evidence suggest that TRIM27-promoted HCV replication relies on its ability to suppress IRF3 and NF-κB pathways, whereby inhibiting type I IFN response against HCV infection.

DISCUSSION

In previous studies, TRIM27 has been reported to cooperate with the lectin family member Siglec1 to

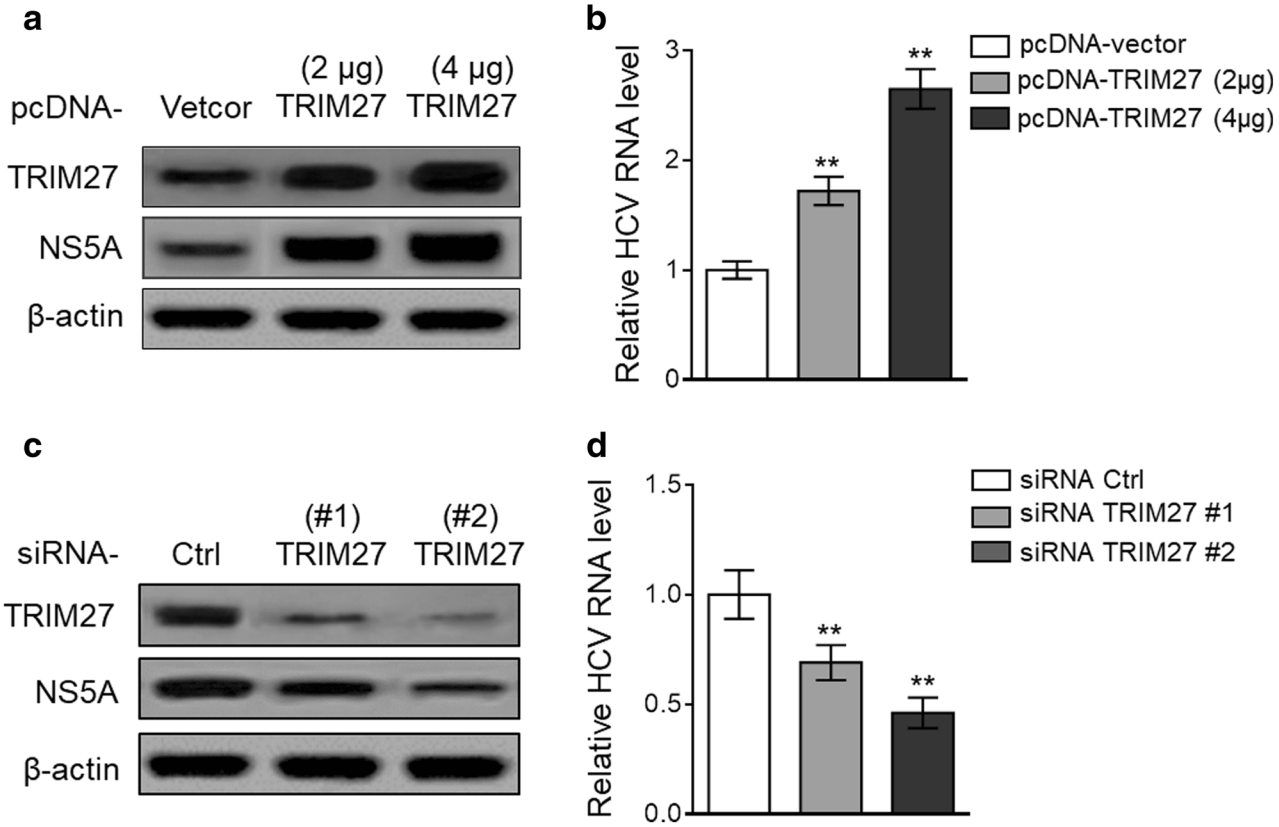

Fig. 2. TRIM27 promotes HCV replication. **a** and **b** HCV-infected Huh-7.5 cells were transfected with pcDNA-vector or 2 μg/well or 4 μg/well pcDNA-TRIM27, and further cultured for 3 days. **a** The protein level of TRIM27 and NS5A was determined by Western blot analysis. β-actin was used as a loading control. Results are representative of three independent experiments. **b** The HCV RNA level was determined by qRT-PCR analysis. β-actin was used as a reference control. Data are mean ± SD. $n = 3$. Data were compared with vector group using Student's t test. $^{**}P < 0.01$. **c** and **d** HCV-infected Huh-7.5 cells were transfected with siRNA control, siRNA TRIM27 #1 or siRNA TRIM27 #2, and further cultured for 3 days. **c** The protein level of TRIM27 and NS5A was determined by Western blot analysis. β-actin was used as a loading control. Results are representative of three independent experiments. **d** The HCV RNA level was determined by qRT-PCR analysis. β-actin was used as a reference control. Data are mean ± SD. $n = 3$. Data were compared with control group using Student's t test. $^{**}P < 0.01$.

negatively regulate the production of type I IFNs and inhibit antiviral innate response against VSV and SeV [14, 15]. Besides, TRIM27 depletion was also found to decrease the yield of HSV-1 [27]. These observations possibly point out that TRIM27 may have a broadly positive effect on viral infection. While, it has been reported that TRIM27 reduces mycobacteria survival in macrophages *via* the promotion of innate immune response [28], which renders it controversial on how TRIM27 affects innate immune response against infection of different pathogens. For combating with HCV infection, type I IFN response is a pivotal mechanism for the host defense; however, to our knowledge, little is known about whether TRIM27 plays a role in this process.

In the current study, by utilizing the HCV-infected Huh-7.5 cells cultured *in vitro*, we show that TRIM27 expression is increased by HCV infection or stimulation of type I IFNs, including IFN-α and IFN-β, thus connecting TRIM27 to HCV infection-induced type I IFN response. Subsequent functional studies *via* applying the tactic of manipulation of TRIM27 expression describe that TRIM27 expression is positively correlated with the mRNA and protein levels of HCV nonstructural protein NS5A, which suggests that TRIM27 acts to positively regulate HCV replication. Moreover, TRIM27 inhibits the activation of IRF3 and NF-κB pathways and downstream type I IFN immune response in Huh-7.5 cells infected by HCV. We further demonstrate that the suppressed IRF3 and

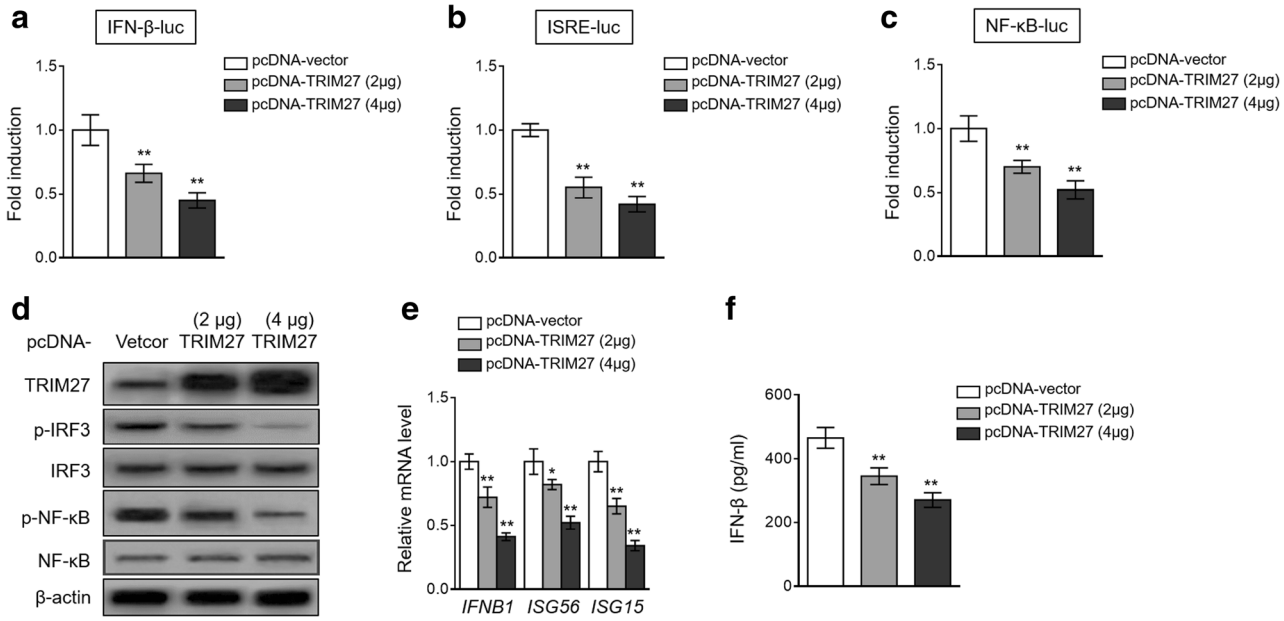

Fig. 3. TRIM27 inhibits type I IFN response against HCV infection. **a**–**c** HEK293 cells were cotransfected IFN-β (**a**), ISRE (**b**), or NF-κB (**c**) reporter plasmid with pcDNA-vector or 2 μg/well or 4 μg/well pcDNA-TRIM27, and further cultured for 2 days. The luciferase activity of reporter plasmid of IFN-β, ISRE, and NF-κB was measured by Dual Luciferase Reporter Assay system. The results are expressed as relative to pcDNA-vector transfection. Data are mean ± SD. $n = 3$. Data were compared with vector group using Student's t test. $^{**}P < 0.01$. **d**–**f** HCV-infected Huh-7.5 cells were transfected with pcDNA-vector or 2 μg/well or 4 μg/well pcDNA-TRIM27, and further cultured for 3 days. **d** The protein level of TRIM27, p-IRF3, IRF3, p-NF-κB, and NF-κB was determined by Western blot analysis. β-actin was used as a loading control. Results are representative of three independent experiments. **e** The mRNA level of IFNB1, ISG56, and ISG15 was by qRT-PCR analysis. β-actin was used as a reference control. **f** The level of IFN-β in the supernatants was determined by ELISA assay. Data are mean ± SD. $n = 3$. Data were compared with vector group using Student's t test. $^{**}P < 0.01$; $^{*}P < 0.05$.

NF-κB pathways are critical for mediating the promotive role of TRIM27 in affecting HCV replication, since a TRIM27 mutant with a truncated B-Box domain fails to suppress the activation of IRF3 or NF-κB pathway or inhibit the production of IFN-β, and accordingly is unable to promote HCV replication. In sum, our study may reveal a positive feed-back loop between TRIM27 and HCV replication, in which HCV infection upregulates the expression of TRIM27, which in turn suppresses type I IFN response by inhibiting IRF3 and NF-κB pathways, whereby impairing the antiviral innate response of the host and enhancing HCV replication. Therefore, based on these findings, we propose that targeting TRIM27 might be of clinical significance in restricting HCV replication.

The production of type I IFNs downstream of PRRs further stimulates the expression of ISGs, which function to restrict the replication of invading virus [29]. We found that IFN-α and IFN-β treatment induced the expression of TRIM27 in Huh-7.5 cells at both mRNA level and protein level, which implies that TRIM27 may be a novel candidate of ISGs. Coincidely, previous studies have shown that there are some TRIM proteins which can be recognized as ISGs and involved in mediating antiviral response [30], such as TRIM79α [31], TRIM14 [19], TRIM5α [32], *etc.*, Nonetheless, due to the limited cell type utilized in this study, whether TRIM27 expression could be induced by the stimulation of type I IFNs in other cell types is uncertain. One study has reported that TRIM27 expression is also upregulated in macrophages infected with VSV or SeV [15]. Therefore, it would be of interest to test whether TRIM27 is induced as an ISG in other types of viral infection.

We demonstrate that TRIM27 promotes HCV replication by suppressing NF-kB and IRF3 pathways and limiting type I IFN response against HCV infection, as evidenced by inhibited activity of IFN-β-responsive promoter, ISRE promoter, and NF-κB promoter, and reduced the phosphorylation level of both IRF3 and NF-κB, and decreased expression of IFNB1, ISG56, and ISG15, and inhibited production of IFN-β in HCV-infected Huh-7.5 cells. TRIM proteins have been found to affect viral infection through multiple mechanisms. For instance, TRIM14 inhibits HCV infection by promoting viral NS5A protein degradation [33], and

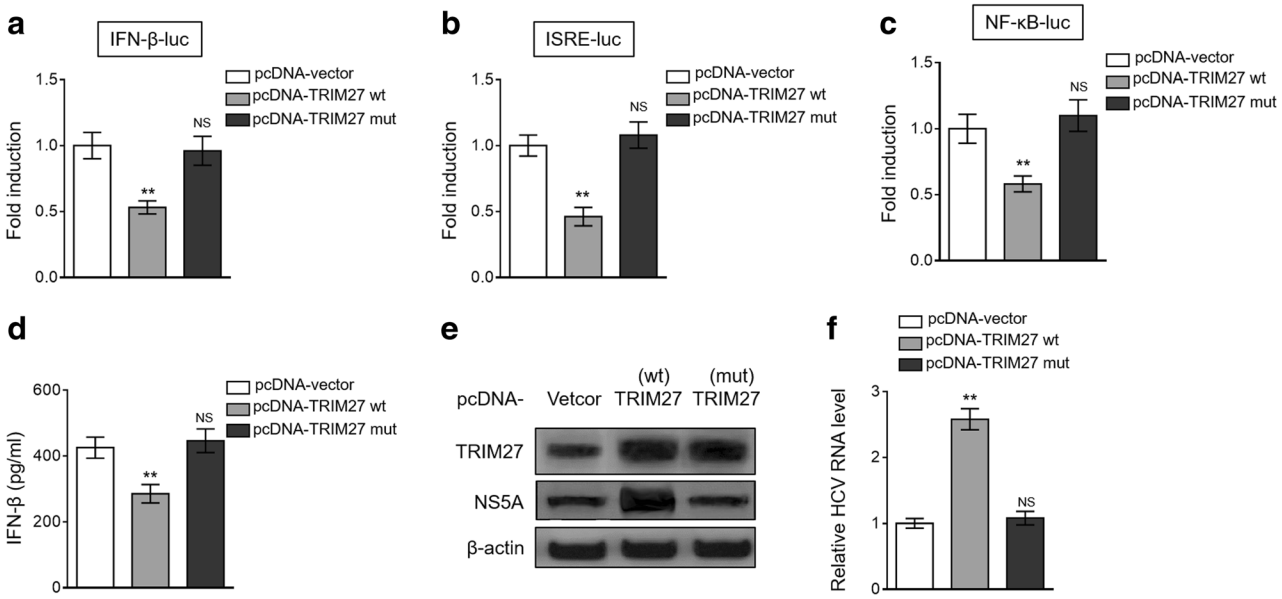

Fig. 4. Mutant TRIM27 unable to inhibit NF-kB and IRF3 pathways fails to promote HCV replication. **a**–**c** HEK293 cells were cotransfected the IFN-β (**a**), ISRE (**b**), or NF-κB (**c**) reporter plasmid with pcDNA-vector or 4 μg/well pcDNA-TRIM27-wt or pcDNA-TRIM27-mut, and further cultured for 2 days. The luciferase activity of reporter plasmid of IFN-β, ISRE, and NF-κB was measured by Dual Luciferase Reporter Assay system. The results are expressed as relative to pcDNA-vector transfection. Data are mean ± SD. $n = 3$. Data were compared with vector group using Student's t test. $^{**}P < 0.01$; NS, not significant. **d**–**f** HCV-infected Huh-7.5 cells were transfected with pcDNA-vector or 4 μg/well pcDNA-TRIM27-wt or pcDNA-TRIM27-mut, and further cultured for 3 days. **d** The level of IFN-β in the supernatants was determined by ELISA assay. **e** The protein level of TRIM27 and NS5A was determined by Western blot analysis. β-actin was used as a loading control. Results are representative of three independent experiments. **f** The HCV RNA level was determined by qRT-PCR analysis. β-actin was used as a reference control. Data are mean ± SD. $n = 3$. Data were compared with vector group using Student's t test. $^{**}P < 0.01$; NS not significant.

intriguingly, TRIM14 also facilitates RLR-mediated antiviral innate response [19]. One study has also related TRIM27 function to the suppressed type I IFN antiviral innate response against infection of VSV, HSV, and SeV *via* mechanism of inducing TBK1 degradation mediated by K48-linked ubiquitination [14]. Another report associates the antiviral function of TRIM27 with microRNA-27a downregulation [15]. We provide further evidence extending TRIM27 as a negative regulator of type I IFN antiviral response against HCV infection, and highlighting the suppressed NF-kB pathway and IRF3 pathway as critical underlying mechanisms that predominantly account for TRIM27-promoted HCV replication. However, whether the suppression of NF-kB and IRF3 by TRIM27 is associated with the changes of TBK1 or microRNA-27a is unknown at present, and whether TRIM27 promotes HCV replication through other mechanisms cannot be ruled out. More studies in the future are needed to address these issues. Fully understanding the molecular mechanisms through which TRIM27 promotes HCV replication may help us to better exploit it as a drug target in interfering HCV infection.

COMPLIANCE WITH ETHICAL STANDARDS

All experimental protocols were approved by the Ethics Committee of Qilu Hospital of Shandong University.

Conflict of Interest. The authors declare that they have no conflict of interest.

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