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TARBP2 inhibits IRF7 activation by suppressing TRAF6-mediated K63-linked ubiquitination of IRF7

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ABSTRACT

Interferon regulatory factor 7 (IRF7), a crucial regulator of type I interferons (IFNs), plays a crucial role in resistance to viral infection. The abnormal production of type I IFNs is associated with many types of disease, such as cancer and inflammatory disorders. Thus, understanding the post-translational modifications of IRF7 is essential to promoting an appropriate immune response. We have recently showed that the TAR RNA binding protein 2 (TARBP2) suppresses IFN-β production and the innate antiviral response by targeting MAVS. Here, we further identified TARBP2 as a novel inhibitor of IRF7, which inhibits IRF7-mediated IFN-β production triggered by the Sendai virus in 293 T cells. Overexpression of TARBP2 inhibits the phosphorylation as well as the K63-linked ubiquitination of IRF7, whilst TARBP2 also impairs the stability of endogenous TRAF6. Furthermore, TARBP2 participates in the interaction between IRF7 and TRAF6, thereby suppressing TRAF6-mediated K63-linked ubiquitination of IRF7, which is a prerequisite of IRF7 phosphorylation. Our findings further reveal the mechanism by which TARBP2 regulates the antiviral signaling pathways of the innate immune system.

1. Introduction

Interferons play a crucial role in fighting viral infections, and they constitute the first line of defense for host immune responses (Bonjardim et al., 2009; Honda and Taniguchi, 2006; Randall and Goodbourn, 2008). The expression of interferons is very finely regulated by interferon regulatory factors (IRFs), which are a family of transcription factors comprising of nine members in mammalian cells. Among them, IRF3 and IRF7 play significant roles in the regulation of interferons (Honda et al., 2005). IRF3 is most similar to IRF7 (Smith et al., 2001), with homologous primary protein structures and similar activation modes, but the two are not functionally redundant. In addition to its constitutive expression in various cells, IRF3 is also responsible for viral-mediated IFN production in the early stages of viral infection. In contrast, IRF7 has low levels of expression in most cells, which can be upregulated in a positive feedback loop mediated by IFN and viral infection (Honda and Taniguchi, 2006; Sato et al., 2000; Schirrmacher, 2009). However, IRF7 also participates in the production of the secondary, larger wave of type I IFN expression, as revealed using $IRF7^{-/-}$ mouse cells.

Besides its role in IFN-mediated immune responses, IRF7 is involved in cell growth and apoptosis, along with autoimmune diseases (Honda and Taniguchi, 2006; Kim et al., 2009; Pagano, 2002; Zhang et al., 2004). The regulatory mechanism of IRF7 has been widely investigated. Like other transcription factors, IRF7 undergoes multiple post-translation modifications (PTMs), including phosphorylation, ubiquitination, sumoylation, and acetylation, the most important of which is phosphorylation (Caillaud et al., 2002; Kubota et al., 2008; Liang et al., 2012; Smith et al., 2001). Kinases, including IKKε, TBK1, IRAK1, and IKKα, are responsible for the phosphorylation of IRF7 in a cell type-specific manner. Both IRAK1 and IKKα have been shown to play crucial roles in TLR7- and TLR9-mediated IRF7 signaling pathways, using IRAK1-deficient mice and IKKα-deficient plasmacytoid dendritic cells, respectively (Higgs et al., 2010; Hoshino et al., 2006; Kitagawa et al., 2013). Both IKKε and TBK1 phosphorylate serine residues at the C-terminus of IRF7 in response to viral infection (Fitzgerald et al., 2003; Sharma et al., 2003). IRF7 is also regulated by ubiquitination, which is one of the most important and prevalent regulatory mechanisms in the immune system (Bhoj and Chen, 2009; Davis and Gack, 2015; Gonzalez-Navajas et al., 2012; Shackelford and Pagano, 2004). Ubiquitin contains seven lysine residues, thus there can be seven different types of ubiquitin chain (Davis and Gack, 2015). The E3 ligases RAUL

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and Ro52 (HECT and RING-finger family, respectively) mediate the K48-linked ubiquitination of IRF7 and IRF3, leading to their degradation and, subsequently, suppressed type I IFN responses (Higgs et al., 2010; Yu and Hayward, 2010). Recent studies have shown that the K63-linked polyubiquitination of IRF7 by the TRAF6 E3 ligase and RIP leads to its activation (Huye et al., 2007; Konno et al., 2009; Ning et al., 2008), which is important in the LMP1 and IKKε pathways (Kawai et al., 2004).

TRAF6 is a member of the TRAF protein family; its N-terminal RING finger domain confers an E3 ubiquitin ligase catalytic activity, whilst the C-terminal β-sandwich domain mediates protein-protein interactions. This gives TRAF6 the ability to act as a receptor protein in the assembly of receptor-associated signaling complexes. In the RLR signaling pathway, TRAF proteins link the upstream receptor molecule MAVS to interferon regulatory factors (IRFs) downstream. Among these, TRAF6 is responsible for the RLR-induced activation of IRF7, however the activation of IRF3 is not impaired in TRAF6$^{-/-}$ cells (Konno et al., 2009).

Recently, we showed that TARBP2 inhibits IFN-β production by targeting MAVS (Ling et al., 2018). Here, we demonstrated that TARBP2 associated with IRF7 and TRAF6. TARBP2 inhibited the phosphorylation of IRF7 triggered by the Sendai virus or IKKε/TBK1, whilst impairing the stability of endogenous TRAF6. Further investigation revealed that TARBP2 suppressed the TRAF6-mediated K63-linked ubiquitination of IRF7, implying the intricate mechanism by which TARBP2 finely regulates IRF7-mediated signaling pathways. Our study provides an outline for the intricate mechanism by which TARBP2 regulates the RLR signaling pathway.

2. Materials and methods

2.1. Reagents

The monoclonal antibodies used in this study were obtained from the following manufacturers: mouse monoclonal antibodies to anti-Flag (F3165), anti-HA, and anti-Myc were purchased from Sigma (USA). The mouse antibodies against β-actin (sc-1616R), TRAF6 (sc-8409), and TARBP2 (sc-514124) were all purchased from Santa Cruz Biotechnology. The rabbit polyclonal affinity-purified antibodies against p-IRF7 (12390S) were purchased from Cell Signaling Technology. The dual-luciferase reporter assay system was obtained from Promega. Sendai virus was provided by Dr. Hong-Bing Shu (Wuhan University, Wuhan, China).

2.2. Cell culture and viral infection

The 293 T cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 10% fetal bovine serum (FBS; Gibco) at 37 °C in a 5% CO_2 incubator. Before infection with the virus, the cells were washed with PBS. One hour after exposure to Sendai virus (80 hemagglutination per mL) in serum-free DMEM, cells were washed with PBS three times and complete DMEM was then added. The viral titers were measured as described previously (Huang et al., 2005).

2.3. Plasmids

Mammalian expression plasmids for human HA- or Flag-tagged IRF7, TRAF6, IRF3, Ubiquitin, K48 and K63 Ubiquitin, an IFN-β promoter luciferase reporter plasmid, and an ISRE luciferase reporter construct were kindly provided by Dr. Hong-Bing Shu. Human HA-, Flag-or Myc-tagged TARBP2 and its truncated forms were amplified from HEK293 T cDNA and inserted into the PRK-HA-Neo-IRAK1 and PRK-Flag-Neo-IRAK1 expression vectors. The control shRNA constructs were kindly provided by Prof. Hong-Bing Shu. Human-specific shRNA-target TARBP2 was synthesized by Sangon Biotech (Shanghai, China). Double-stranded oligonucleotides corresponding to the target sequences were cloned into pSuper-Retro vectors (OligiEngine). The following TARBP2 sequences were targeted:

1) CCTGGGATTCTCTACGAAA;
2) GGAGCGTTTCATTGAGAT;
3) GCCTGGATGGTCTTCGAAA;

2.4. Transfection and luciferase reporter assay

293 T cells (~ 2.0×10^5) were seeded in 24-well plates and transfected using the calcium phosphate method. The cells were transfected with 100 ng of luciferase reporter plasmids and a pRL-TK Renilla luciferase reporter plasmid at 50 ng/well (to normalize the transfection efficiency), together with the indicated plasmids or an empty control vector (to ensure each well contained the same amount of total DNA). After transfection for 20–24 h, cells were collected and lysed with 1X passive lysis buffer (Promega). Subsequently, luciferase activity was measured using the dual-luciferase reporter assay system (Promega) with a GloMax™ 20/20 Luminometer (Promega) according to the manufacturer's protocols. All reporter gene assays were repeated at least three times.

2.5. Co-immunoprecipitation and immunoblotting

293 T or HEK293 T cells (~ 1×10^7) were seeded in 100 mm dishes and transfected with the appropriate expression plasmids or with the empty vector. At 20 h post-transfection, cells were collected and lysed with a lysis buffer containing 20 mM Tris (pH 7.5), 150 mM NaCl, 1% Triton, 1 mM EDTA, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM PMSF. Co-immunoprecipitation assays were performed as described previously (Xu et al., 2005) (30). Experiments were repeated three times and produced similar results.

2.6. Real-time fluorescent quantitative PCR

Total RNA was extracted using an RNA-extraction kit (Promega, Beijing) according to manufacturer's protocols, and then was reversed-transcribed using a Reverse Transcription Kit (Promega, USA) before performing quantitative RT-PCR analysis. An SYBR green PCR master kit (Promega, USA) was used for the real-time fluorescent quantitative PCR assays. The results were normalized to the levels of actin cDNA. The reverse transcription step was performed on 1 μg of total RNA. The primers used for the quantitative PCR were as follows:

hTARBP2 forward: CAGGAGTATGGGACCAGAATAGG;
hTARBP2 reverse: ACCCGGAAGGTGAAATTAGGC;
hIFN-β forward: CTAACTGCAACCTTTCGAAGC;
hIFN-β reverse: GGAAAGAGCTGTAGTGGAGAAG;
hβ-actin forward: GTCGTCGACAACGCTCCGGCATG;
hβ-actin reverse: ATTGTAGAAGGTGTGGTGCCAGAT.

2.7. Data collection and statistical analysis

Statistical analysis was performed using GraphPad Prism 4.0 (GraphPad Software, La Jolla California USA, www.graphpad.com). Data are presented as a mean ± SD. One-way analysis of variance (ANOVA) and Student's t-tests were performed. P-values < 0.05 were considered statistically significant.

3. Results

3.1. TARBP2 negatively regulates IRF7-mediated IFN-β signaling triggered by Sendai virus

TARBP2 was first identified as a binding protein of HIV TAR RNA (Gatignol et al., 1991). Subsequent studies have shown that TARBP2 acts as a cofactor of DICER, which is closely related to the occurrence of

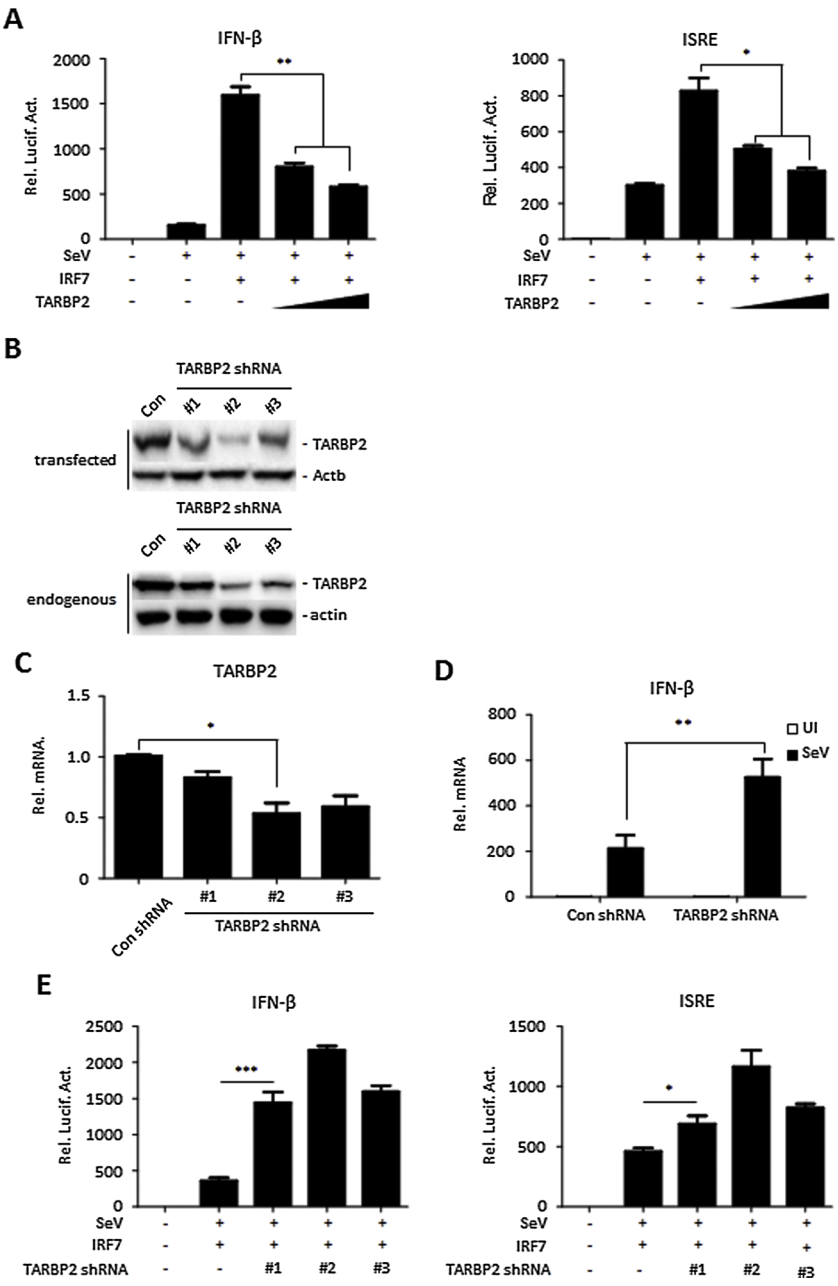

Fig. 1. TARBP2 negatively regulates IRF7-mediated IFN-β signaling triggered by SeV.
(A) TARBP2 inhibits IRF7-mediated activation of the IFN-β promoter or ISRE. 293 T cells (1×10^5) were transfected with IRF7 (0.5 μg each) alone with TARBP2 (0.2, 0.5 μg each) and the reporter plasmids (0.05 μg each). 12 h after transfection, cells were left uninfected or infected with SeV (80 hemagglutination per ml) for 12 h before luciferase assays were performed. Data shown are presented as mean ± SD, n = 3. **P < 0.01, *P < 0.05. (B) Effects of TARBP2 siRNA plasmid on the expression of transfected and endogenous TARBP2. In the upper panels, 293 T cells (2×10^5) were transfected with expression plasmids for Flag-TARBP2 (1.0 μg each) and HA-Actb (0.1 μg each) and the indicated RNAi plasmids (2 μg). 12 h after transfection, cell lysates were analyzed by immunoblot with anti-Flag and anti-HA. In the bottom panels, 293 T cells (2×10^5) were transfected with control or the indicated TARBP2-shRNA plasmids (2 μg each) for 24 h. Cell lysates were then analyzed by immunoblots with the indicated antibodies. (C) Effects of TARBP2-siRNA plasmids on the transcription of TARBP2. HEK293 T cells (2×10^5) were transfected with indicated plasmid (2 μg each). 24 h after transfection, qPCR assays were performed. We chose the best performing #2 to do the following experiments. (D) Knockdown of TARBP2 enhances SeV-induced transcription of endogenous IFN-β. HEK293 T cells (2×10^5) were transfected with indicated plasmid (2 μg each). 24 h after transfection, cells were left uninfected or infected with SeV (80 hemagglutination per ml) for 12 h before qPCR assays were performed while the actin was used for normalization. Data shown are presented as mean ± SD, n = 3. **P < 0.01. (E) Effects of TARBP2 siRNA plasmid on IRF7-mediated activation of the IFN-β promoter or ISRE. 293 T cells (1×10^5) were transfected with IRF7 (0.5 μg each) alone with TARBP2 siRNA (0.5 μg each) and the reporter plasmids (0.05 μg each). The data analysis was similarly performed as in A. Data shown are presented as mean ± SD, n = 3. ***P < 0.001. Similar results were obtained from three independent experiments (B).

biological shRNA (Daniels et al., 2009; Lee et al., 2013; Wilson et al., 2015), and that TARBP2 is also a component of the RNA-induced silencing complex (RISC) (Hammond, 2005; MacRae et al., 2008). Recently it was demonstrated that the TARBP2-LGP2 interaction is required to induce an innate immune response to Cardiovirus (Komuro et al., 2016). Our previous studies have indicated that TARBP2 negatively regulates IFN-β production and the antiviral response by targeting MAVS (Ling et al., 2018), and have shown an association between TARBP2 and IRF7, a molecule downstream of MAVS. A dual luciferase reporter assay was performed to test whether TARBP2 overexpression could inhibit the activation of the IFN-β promoter mediated by IRF7. We showed that TARBP2 overexpression inhibited the IRF7-mediated activation of the IFN-β promoter and ISRE upon Sendai virus stimulation (Fig. 1A), indicating that TARBP2 is a potential negative regulator of the IRF7 signaling pathway. To further determine the effects of TARBP2 on IRF7-mediated IFN-β production, we investigated its contribution to IRF7 signaling by knocking down TARBP2 in shRNA-mediated 293 T cells. We prepared three distinct shRNAs targeting the TARBP2 gene and confirmed that all three shRNAs suppressed TARBP2 expression using western blotting and qPCR analysis (Fig. 1B, C), whilst Sendai virus-induced IFN-β production was significantly enhanced (Fig. 1D). Correspondingly, the activation of the IRF7-mediated IFN-β promoter and ISRE were significantly promoted in TARBP2 knockdown cells (Fig. 1E).

3.2. TARBP2 interacts with IRF7

Next, we investigated the molecular mechanisms responsible for the role of TARBP2 in IRF7-mediated antiviral signaling. The TARBP2 protein is 366 amino acids in length, including three double-stranded RNA binding domains. It is generally believed that dsRBD1 and dsRBD2 are responsible for binding to dsRNA and the C-terminal basic domain, while dsRBD3 (also known as the Medipal domain) mediates protein-protein interactions (Gatignol et al., 1991; Bannwarth et al., 2001; St Johnston et al., 1992). To determine which domain is responsible for the interaction between TARBP2 and IRF7, we constructed truncated

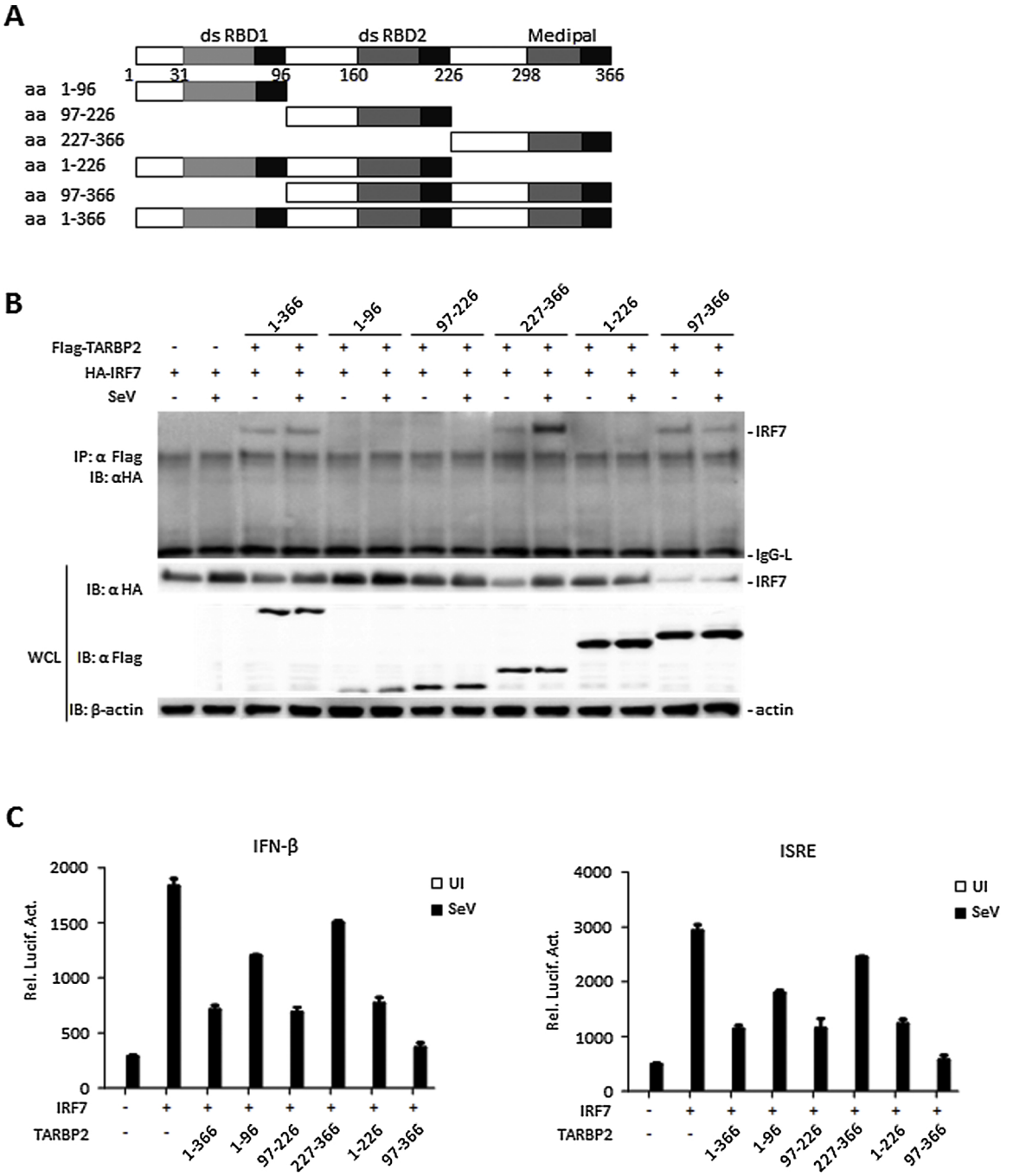

Fig. 2. TARBP2 interacts with IRF7.
(A) The scheme of TARBP2 and its truncated forms. (B) Domain mapping of TARBP2-IRF7 interaction. 293 T cells (1×10^7) were transfected with the indicated truncations before co-immunoprecipitation and immunoblot analysis with the indicated antibodies. (C) TARBP2 and its s effects on IRF7-mediated activation of the IFN-β promoter or ISRE. The experiments were similarly performed as in 1 A. Data shown are presented as mean ± SD, n = 3. Similar results were obtained from three independent experiments (B).

forms of TARBP2 (Fig. 2A). The interactions between IRF7 and the truncated forms of TARBP2 were investigated using co-immunoprecipitation assays with the cell lysate prepared from 293 T cells overexpressing HA-tagged IRF7 and Flag-tagged truncated forms of TARBP2. Immunoblot analysis using anti-HA antibodies revealed that truncated Flag-tagged TARBP2 (aa227-366 and aa977-366), as well as full-length Flag-tagged TARBP2, co-precipitated with HA-tagged IRF7, indicating that TARBP2 interacts with IRF7 and that aa277-366 (the third dsRNA-binding domain) is required for this interaction (Fig. 2B), consistent with reports that the Medipal domain is responsible for protein-protein interactions (Haase et al., 2005). To determine the effects of the truncated forms of TARBP2 on IRF7-mediated IFN-β production, we again performed a dual luciferase reporter assay. All the truncated forms of TARBP2 suppressed the IRF7-mediated activation of the IFN-β promoter and ISRE to varying degrees (Fig. 2C), indicating that aa97-226 (the second dsRNA-binding domain) is the most important domain for inhibiting the IRF7-mediated activation of the IFN-β promoter and ISRE. Since dsRBD2 is responsible for binding RNA, it is possible that TARBP2 forms a link between pattern-recognition receptors and RNA viruses. Our findings suggest that the Medipal domain not only mediates the interaction between TARBP2 and Merlin, Dicer, and PACT, but also mediates the interaction between TARBP2 and IRF7.

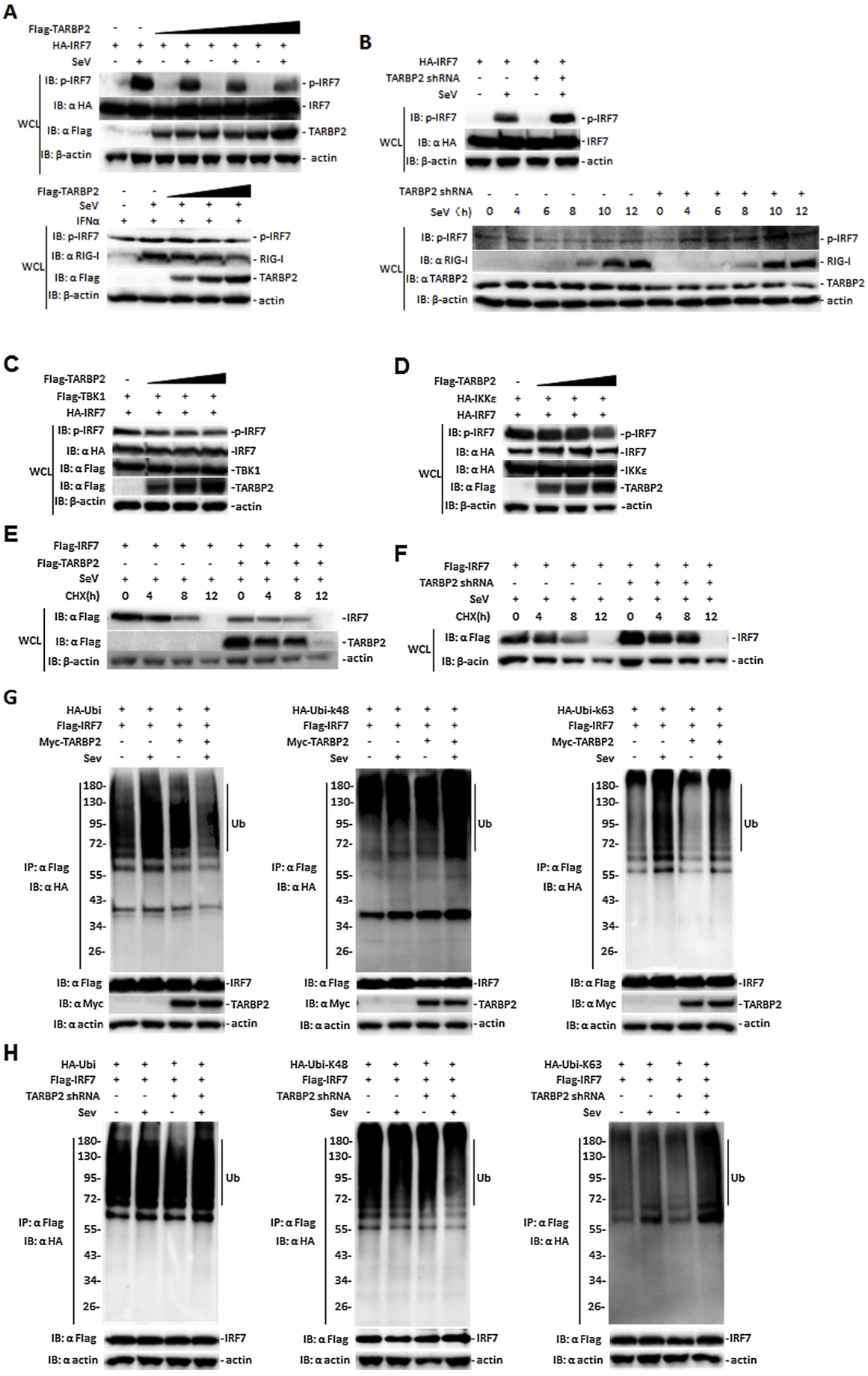

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Fig. 3. TARBP2 inhibits phosphorylation of IRF7 and enhances its degradation in SeV-dependent manner.
(A) TARBP2 inhibits virus-induced phosphorylation of transfected and endogenous IRF7. In the upper panels, 293 T cells (2×10^5) were transfected with IRF7 (1.0 μg each), alone with TARBP2 (0.1, 0.5, 2.0 μg each). 12 h after transfection, cells were left uninfected or infected with SeV for 12 h before immunoblot analysis with anti-p-IRF7 and other indicated antibodies. In the bottom panels, HEK293 T cells (2×10^5) were transfected with TARBP2 (0, 0.1, 0.5, 1.0 μg each) for 12 h. Before infected with SeV for 10 h, cells were treated with IFNα (500 units per ml) for 12 h. The immunoblot analysis with anti-p-IRF7 and other indicated antibodies. (B) Knockdown of TARBP2 increases virus-induced phosphorylation of transfected and endogenous IRF7. In the upper panels, the experiments were similarly performed as in A. In the bottom panels, HEK293 T cells (2×10^5) were transfected with TARBP2 shRNA (2.0 μg each). 12 h after transfection, cells were left uninfected or infected with SeV for indicated time before immunoblot analysis with anti-p-IRF7 and other indicated antibodies. (C) TARBP2 inhibits TBK1-mediated phosphorylation of IRF7. 293 T cells (2×10^5) were transfected with IRF7 (1.0 μg each), TBK1 (1.0 μg each), alone with TARBP2 (0.1, 0.5, 2.0 μg each) for 24 h. Immunoblot analysis were similarly performed as in A. (D) TARBP2 inhibits IKKε-mediated phosphorylation of IRF7. The experiments were similarly performed as in C. (E) TARBP2 reduces IRF7 protein stability in SeV-dependent manner. 293 T cells co-transfected with empty vector or TARBP2 and IRF7 (1.0 μg each) .12 h later, cells were left infected with SeV for 12 h before treated with/with-out CHX (20 μM) for different time course, and then cells were lysed immunoblotting assay with indicated antibodies. (F) Knockdown of TARBP2 enhances IRF7 protein stability. The experiments were similarly performed as in E. (G) TARBP2 increases K48-linked but decreased K63-linked polyubiquitination of IRF7. 293 T cells (2×10^6) were transfected with the indicated plasmids. 12 h after transfection, cell lysates were immunoprecipitated with anti-Flag. The immunoprecipitates were analyzed by immunoblots with anti-HA, anti-Flag or anti-Myc as indicated. (H) Knockdown of TARBP2 decreases K48-linked but increased K63-linked polyubiquitination of IRF7. Co-immunoprecipitation and immunoblot analyses were similarly performed as in G. Similar results were obtained from three independent experiments (A–H).

3.3. TARBP2 inhibits the phosphorylation of IRF7, and promotes the K48-linked ubiquitination and degradation of IRF7 in infected cells

IRF7 phosphorylation is required for the induction of IFN gene expression in virus-infected cells (Marie et al., 2000; Smith et al., 2001). Vaccinia virus E3L protein and TARBP2 are both dsRNA-binding proteins, capable of suppressing the role of PKR in the immune response. Interestingly, Vaccinia virus E3L is an effective IRF7 phosphorylation inhibitor (Smith et al., 2001). To investigate the effect of TARBP2 on IRF7 phosphorylation, we examined the p-IRF7 expression level induced by virus as well as kinases, including TBK1 and IKKε, using western blotting. We found that TARBP2 attenuated exogenous and IFN- α induced endogenous IRF7 phosphorylation upon viral stimulation in a dose-dependent manner (Fig. 3A), and that knockdown of endogenous TARBP2 using TARBP2-specific shRNA promoted SeV-induced phosphorylation of both exogenous and endogenous IRF7 (Fig. 3B). The kinases IKKε and TBK1, which induce IRF7 phosphorylation, were consistently inhibited in cells overexpressing TARBP2 (Fig. 3C, D). We then investigated whether the effect of TARBP2 on IRF7 involves ubiquitination using western blotting. To directly examine TARBP2-mediated IRF7 ubiquitination, Flag-tagged IRF7, HA-tagged ubiquitin, and Myc-tagged TARBP2 were co-transfected into 293 T cells. The results suggest that the overexpression of TARBP2 significantly inhibits IRF7-linked linear ubiquitination as well as K63-linked ubiquitination in virus-infected cells (Fig. 3G). In contrast, IRF7 showed increased linear ubiquitination and K63-linked ubiquitination in TARBP2-knockdown virus-infected cells (Fig. 3H). Taken together, these results show that TARBP2 only affects IRF7 signaling in virus-infected cells or in IRF7-activated cells.

We next sought to determine the role of TARBP2 in the proteasome-dependent proteolysis of IRF7. The co-expression of TARBP2 and IRF7 led to decreased levels of the IRF7 protein upon Sendai virus stimulation (Fig. 3E). To verify this result, we transfected TARBP2-specific shRNA and IRF7 expressing plasmids into cells infected with Sendai virus before CHX treatment. The data showed increased IRF7 expression in TARBP2-knockdown cells (Fig. 3F). We then investigated whether TARBP2-mediated IRF7 degradation occurs through ubiquitination. The level of K48-linked ubiquitination on IRF7 was consistently increased in the presence of TARBP2 (Fig. 3G). In contrast, IRF7 showed reduced K48-linked ubiquitination in TARBP2 knockdown cells (Fig. 3H).

3.4. TARBP2 interacts with TRAF6 and enhances the K48-linked ubiquitination and degradation of TRAF6

TRAF6 is a member of the tumor necrosis factor receptor (TNF-R)-associated factor (TRAF) family, linking upstream receptors to downstream effector proteins by serving as an adaptor protein in receptor-associated signaling complexes. Besides directly interacting with MAVS, TRAF6 is required for the RLR-induced activation of IRF7 but not IRF3, as revealed using TRAF6$^{-/-}$ fibroblasts, whilst TRAF6 also mediates the K63-linked ubiquitination of IRF7 (Funakoshi-Tago et al., 2008; Yoshida et al., 2008). Intriguingly, recent studies have shown that TRAF6 affects the replication of HIV-1 (Sirois et al., 2011). To investigate the relationships between TARBP2 and TRAF6, we transfected 293 T cells with HA-tagged TARBP2 and Flag-tagged TRAF6 plasmids for co-immunoprecipitation assays using the anti-HA antibody. These assays revealed that TRAF6 interacts with TARBP2 (Fig. 4A). Intriguingly, the level of endogenous TRAF6 was reduced in the presence of TARBP2 in a dose-dependent manner (Fig. 4B), yet endogenous TRAF6 exhibited enhanced stability in TARBP2 knockdown cells (Fig. 4C). TRAF6 has the ability to auto-ubiquitinate upon viral stimulation (Wang et al., 2010a), which allows it to maintain an activated conformation and activate downstream signaling. To determine the mechanisms involved in TRAF6 degradation, we co-transfected Myc-tagged TARBP2, Flag-tagged TRAF6, WT HA-tagged ubiquitin, and a K48 or K63 mutant which has one ubiquitinated lysine at either the 48 or 63 position, respectively. TARBP2 overexpression in 293 T cells decreased TRAF6-linked linear ubiquitination but enhanced K48-linked ubiquitination (Fig. 4D). In contrast, TARBP2 knockdown increased TRAF6-linked linear ubiquitination but decreased K48-linked ubiquitination (Fig. 4E). Taken together, these findings suggest that TARBP2 facilitates the K48-linked polyubiquitination and proteasome-dependent degradation of TRAF6.

3.5. TARBP2 attenuates the relationship between TRAF6 and IRF7

Considering the relationship between IRF7 and TRAF6 in the RLR pathway and the effect of TARBP2 on the stability of IRF7 and TRAF6, we have reason to speculate that TARBP2 would play a significant inhibitory role in the formation of signal complexes between TRAF6 and IRF7. To test this hypothesis, HA-IRF7, Flag-TRAF6, and Myc-TARBP2 were constructed to examine the role of TARBP2 during the association of IRF7 and TRAF6. Co-immunoprecipitation analyses showed that the correlation between IRF7 and TRAF6 was attenuated when overexpressing TARBP2 (Fig. 5A), whilst a stronger interaction between IRF7 and TRAF6 was observed in TARBP2 knockdown cells (Fig. 5B). Previous studies have shown that TRAF6 is responsible for the K63-linked polyubiquitination of IRF7 (Ning et al., 2008; Ning et al., 2011), thus to investigate the effect of TARBP2 on the TRAF6-mediated K63-linked polyubiquitination of IRF7, Flag-tagged IRF3, HA-tagged ubiquitin K63 mutant, pCMV-tagged TRAF6, and Myc-tagged TARBP2 were co-transfected into 293 T cells. Co-immunoprecipitation analyses suggested that the K63-linked ubiquitination of IRF7 was significantly decreased in the presence of TARBP2 (Fig. 5C). In contrast, IRF7 showed enhanced TRAF6-mediated K63-linked ubiquitination in

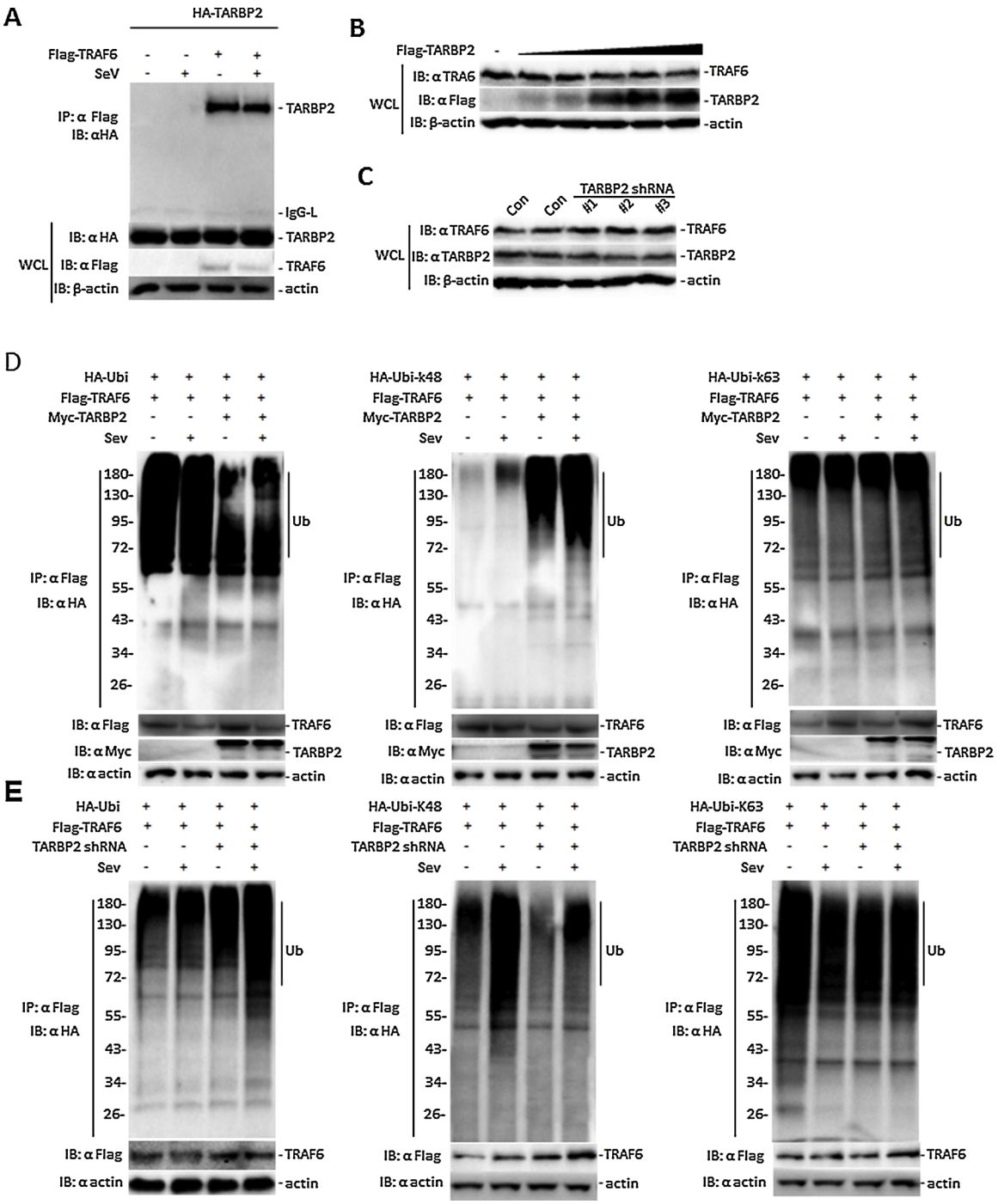

Fig. 4. TARBP2 interacts with TRAF6 and promotes K48-linked ubiquitination and degradation of TRAF6.
(A) The interaction between TARBP2 and TRAF6. 293 T (1×10^7) were transfected with the indicated plasmid (5 μg each). 12 h after transfection, cells were left uninfected or infected with SeV before harvested and lysed to perform co-immunoprecipitation assays with anti-Flag, following immunoblotting assay with anti-HA (upper panel). Expression levels of TARBP2 and TRAF6 were analyzed by immunoblotting analysis (lower panels) with indicated antibodies. (B) TARBP2 reduces endogenous TRAF6 protein stability. 293 T cells were transfected with empty vector or TARBP2 (0.1, 0.2, 0.5, 1.0, 1.5 μg). 24 h later, cells were lysed immunoblotting assay with indicated antibodies. (C) Knockdown of TARBP2 enhances endogenous TRAF6 protein stability. The experiments were similarly performed as in B. (D) TARBP2 increases K48-linked but not K63-linked polyubiquitination of TRAF6. Co-immunoprecipitation and immunoblot analyses were similarly performed as in 3 G. (E) Knockdown of TARBP2 decreases K48-linked but not K63-linked polyubiquitination of TRAF6. Co-immunoprecipitation and immunoblot analyses were similarly performed as in 3H. Similar results were obtained from three independent experiments (A–E).

TARBP2 knockdown cells (Fig. 5E). These results suggest that TARBP2 attenuates the interaction between TRAF6 and IRF7 and thus negatively regulates the TRAF6-mediated K63-linked ubiquitination of IRF7.

4. Discussion

In this study, we showed that TARBP2 inhibited IRF7-mediated IFN-β production in virus-infected cells. Whilst inactive IRF7 is predominantly located in the cytoplasm, p-IRF7 is translocated to the nucleus, and K63-linked ubiquitination is a prerequisite for IRF7

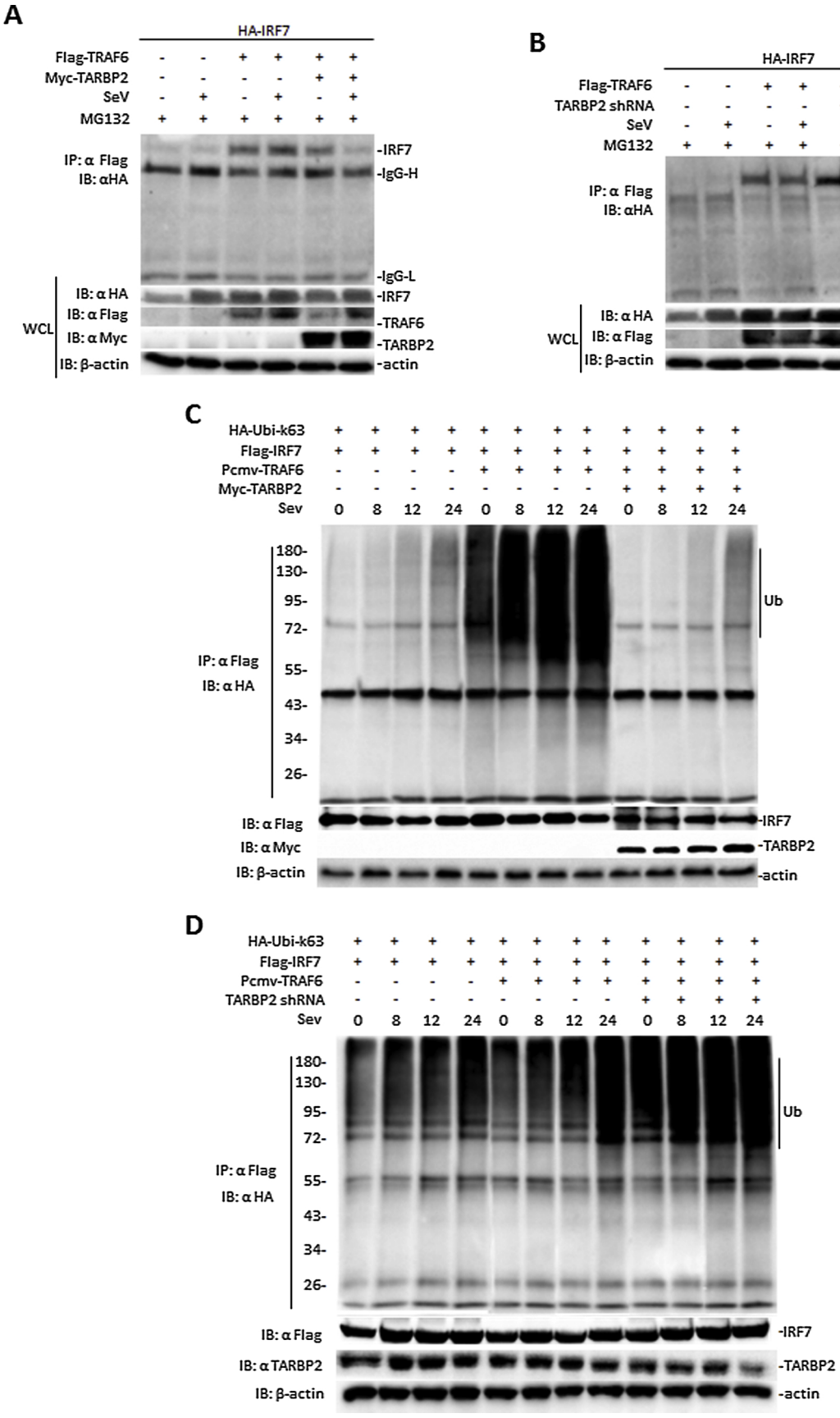

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Fig. 5. TARBP2 impairs the interaction between IRF7 and TRAF6.
(A) TARBP2 weakens the interaction between IRF7 and TRAF6. 293 T (1×10^7) were transfected with the indicated plasmid (5 μg each). 5 h after transfection, cells were treated with MG132 (20 μM), and 12 h after transfection, cells were left uninfected or infected with SeV before harvested and lysed to perform co-immunoprecipitation assays with anti-Flag, following immunoblotting assay with anti-HA (upper panel). Expression levels of IRF7, TRAF6 and TARBP2 were analyzed by immunoblotting analysis (lower panels) with indicated antibodies. (B) Knockdown of TARBP2 enhances the interaction between IRF7 and TRAF6. Co-immunoprecipitation and immunoblot analyses were similarly performed as in A. (C) TARBP2 decreases TRAF6-mediated K63-linked polyubiquitination of IRF7. 293 T cells (2×10^6) were transfected with the indicated plasmids. Cells were left uninfected or infected with SeV for indicated time before harvested and lysed to perform co-immunoprecipitation assays with with anti-Flag. The immunoprecipitates were analyzed by immunoblots with anti-HA, anti-Flag, or anti-Myc as indicated. (D) Knockdown of TARBP2 increases TRAF6-mediated K63-linked polyubiquitination of IRF7. Co-immunoprecipitation and immunoblot analyses were similarly performed as in C. Similar results were obtained from three independent experiments (A-E).

phosphorylation (Ning et al., 2011). We showed that TARBP2 negatively regulates IRF7 phosphorylation. Our findings suggested that TARBP2 blocked the K63-linked ubiquitination of IRF7, mainly via its effect on TRAF6 protein stability. Analysis of TARBP2 mutants indicated that the Medipal domain of TARBP2 was responsible for its interaction with IRF7 and its RNA-binding domain played a role in IRF7-mediated IFN-β production.

In addition to viral infection, the kinases IKKε, TBK1, IRAK1, and IKKα, are responsible for the phosphorylation of IRF7 in cell type-specific manner. Among these, IKKε and TBK1 are the primary kinases involved in IRF7 phosphorylation and activation (Fitzgerald et al., 2003; Sharma et al., 2003). A recent study has reported that the E3L protein, like TARBP2, is a dsRNA-binding protein capable of inhibiting PKR, which acts as an effective inhibitor of IRF7 phosphorylation (Smith et al., 2001). Consistent with these findings, we demonstrated that TARBP2 negatively regulates IKKε/TBK1-mediated and virus-induced IRF7 phosphorylation. Whether TARBP2 can inhibit the phosphorylation of IRF7 induced by other kinases, requires further experimental investigation.

TRAF6 is self-ubiquitinated upon viral infection via its RZ and MATH domains (Wang et al., 2010a). In the RLR/TLR signaling pathway, upstream receptor proteins (such as MAVS/MDA5) could directly activate the ubiquitination of natural TRAF6 upon viral infection. Recent studies have shown that the deubiquitinating enzyme 0TUB1/OTUB2 were responsible for the deubiquitination of TRAF6 upon viral infection (Li et al., 2010). In addition, USP2a can also deubiquitinats TRAF6 upon viral stimulation (He et al., 2013). Determining whether TARBP2 suppresses TRAF6 ubiquitination by recruiting OTUB1/OTUB2 or other molecular requires further investigation.

The IRF7 regulatory mechanism is complicated, and TRAF6 promotes its activation via the innate immune signaling pathway. However, during HIV-1 replication, the downregulation of TRAF6 upregulates IRF7 expression, which promotes HIV-1 replication (Funakoshi-Tago et al., 2008). This illustrates the functional polymorphism of immune molecules. In our investigation, the regulation of IRF7 and TRAF6 by TARBP2 appears to be consistent within the innate immune pathway, however the roles of IRF7 and TRAF6 in HIV-1 replication conflict with these findings. HIV-1 infection is associated with robust activation of the immune response, however the level of immune response caused by HIV replication *in vivo* varies among individuals and the underlying mechanisms are not completely understood (Chang et al., 2011). One of the primary functions of TARBP2 is to promote HIV-1 replication in combination with HIV-1 LTR (Gatignol et al., 1991). Recent studies have also reported that IRF7 and TRAF6 directly affect HIV-1 replication (Schwartz et al., 2017; Wang et al., 2010b). The overexpression of IRF7 has been shown to lead to a remarkable increase in HIV-1 replication in primary human macrophages, whilst the downregulation of TRAF6 resulted in enhanced viral replication in HIV-1 infected macrophages (Sirois et al., 2011). The TRAF family is in fact closely associated with the replication of HIV-1. It has been reported that the HIV-1 Nef protein binds to the HCV core, subsequently recruiting TRAF family members, including TRAF2, TRAF5, and TRAF6, and promoting HIV-1 replication in macrophages (Khan et al., 2013). Intriguingly, our recent study shows that TARBP2 interacts with TRAF3 as well (Ling et al., 2018). Since we are still carrying out preliminary research at this stage, it is unknown whether there is crosstalk between TARBP2, IRF7 and other TRAFs, however their role in the process of HIV replication is worth further exploration. Therefore, further studies should focus on the mechanism of IRF7/TRAF6/TARBP2 interactions in HIV-1 replication, which may help to elucidate the role of immune molecules in the interaction between antiviral signaling pathways and viral replication.

Conflict of interest

The authors declare no competing financial interests.

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