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Ubiquitin-Specific Protease 2b Negatively Regulates IFN-β Production and Antiviral Activity by Targeting TANK-Binding Kinase 1

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TANK-binding kinase 1 (TBK1) is essential for IFN regulatory factor 3 activation and IFN-β production downstream of various innate receptors. However, how TBK1 activation is terminated is not well defined. In this study, we identified ubiquitin-specific protease (USP) 2b as a new negative regulator for TBK1 activation. Overexpression of USP2b inhibited retinoic acid–inducible gene-I–mediated IFN-β signaling; in contrast, knockdown of USP2b expression by small interfering RNA enhanced retinoic acid–inducible gene-I–mediated IFN-β signaling. Coimmunoprecipitation experiments demonstrated that USP2b interacted with TBK1. As a deubiquitinating enzyme, USP2b was demonstrated to cleave K63-linked polyubiquitin chains from TBK1 to inhibit TBK1 kinase activity. Consistent with the inhibitory roles of USP2b on TBK1 activation, knockdown of USP2b significantly inhibited the replication of vesicular stomatitis virus, whereas overexpression of USP2b resulted in enhanced replication of vesicular stomatitis virus. Therefore, our findings demonstrated that USP2b deubiquitinates K63-linked polyubiquitin chains from TBK1 to terminate TBK1 activation and negatively regulate IFN-β signaling and antiviral immune response. The Journal of Immunology, 2014, 193: 2230–2237.

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Abbreviations used in this article: cGAS, cyclic GMP–AMP synthase; DUB, deubiquitinating enzyme; HA, hemagglutinin; IP, immunoprecipitation; IRF3, IFN regulatory factor 3; ISD, IFN stimulatory DNA; MAVS, mitochondrial antiviral signaling protein; MDA5, melanoma differentiation-associated gene 5; NLR, NOD-like receptor; polyI:C, polyinosinic:polycytidylic acid; PRR, pattern-recognition receptor; RIG-I, retinoic acid–inducible gene-I; RLR, RIG-I–like receptor; Sendai virus; siRNA, small interfering RNA; TBK1, TANK-binding kinase 1; TRAF, TNFR-associated factor; TRIF, Toll/IL-1R domain–containing adaptor that induces IFN-β; USP, ubiquitin-specific protease; VSV, vesicular stomatitis virus; WT, wild type.

Copyright © 2014 by The American Association of Immunologists, Inc. 0022-1767/14/$16.00 are present in the cytosol. Upon viral infection, the C-terminal domain and RNA helicase domain of RIG-I and MDA5 serve as intracellular viral RNA receptors, whereas their caspase activation recruitment domain modules are associated with the downstream caspase activation recruitment domain–containing adapter protein mitochondrial antiviral signaling protein (MAVS; also known as VISA, IPS-1, and Cardif) (4–7). Recently, several DNA sensors have been identified as capable of sensing DNA from various microbes (8–10). Although the nature of DNA sensors needs further investigation, the adaptor protein STING (also called MPYS, MITA, and ERIS) in DNA-sensing pathway and downstream signaling molecules, including TANK-binding kinase 1 (TBK1) and IRF3, are well defined (3, 11, 12). After viral infection, these key adaptors, TRIF, MAVS, and STING, recruit the kinase called TBK1 to activate the transcription factor IRF3, which leads to the production of type I IFNs and the following antiviral immune responses. As an essential kinase for IRF3 activation and IFN-β production in the innate immune system, how TBK1 activation is regulated is not well defined.

Production of type I IFNs is required for viral clearance; however, aberrant production of type I IFNs can have a pathological role in autoimmune disorders. Thus, tight regulation of type I IFN signaling is critical for maintaining the homeostasis of both innate and adaptive immunity. Published works have demonstrated that virus-induced type I IFN signaling is tightly regulated by ubiquitination. Several ubiquitin ligase enzymes have been identified to regulate these processes (13). However, the function of deubiquitinating enzymes (DUBs) on the regulation of antiviral immune response is limited. DUBs are proteases that cleave ubiquitin or ubiquitin-like proteins conjugated with target proteins. About 100 putative DUBs are encoded by the human genome, and they belong to five different families, including ubiquitin-specific proteases, C-terminal hydrolases, otubain proteases, the Machado-Joseph disease protease, and the metalloproteases referred to as JAMM (14). A few DUBs have been shown to regulate virus-induced type I IFN signaling. For example, A20 was shown to negatively regulate the RIG-I–induced antiviral state (15). DUBA was identified to be...
required for efficient deubiquitination of TNFR-associated factor (TRAF3) and to function as a negative regulator of innate immune responses (16). CYLD, known as a tumor suppressor, was recently reported to negatively regulate the activation of TBK1/IK kinase–related kinase ε (17). Ubiquitin-specific protease (USP)17 was required in virus-triggered type I IFN signaling by deubiquitinating of RIG-I and MDA5 (18). We also demonstrated that USP4 positively regulated for RIG-I signaling pathway through deubiquitinating K48-linked ubiquitin chains and stabilizing RIG-I (19).

In this study, we provided evidence to show that USP2b negatively regulates IFN-β production and antiviral activity by deubiquitinating K63-linked polyubiquitin chains of TBK1. Therefore, we identified a new DUB for host cells to regulate virus-induced type I IFN signaling by targeting TBK1. At the same time, our results delineated a novel pathway to control excessive innate immune response after viral infection.

Materials and Methods
Reagents and Abs
LPS (Escherichia coli, 055:B5) was purchased from Sigma-Aldrich (St. Louis, MO). Polyinosinic-polycytidylic acid [Poly(I:C)] was purchased from Invivogen (San Diego, CA). DNA oligonucleotides corresponding to IFN stimulatory DNA (ISD) were obtained from Biosune and annealed in PBS. LPS and poly(I:C) were used at a final concentration of 100 ng/ml and 25 μg/ml, respectively. For transfection, poly(I:C) and ISD were used at a final concentration of 1 and 5 μg/ml, respectively. Sendai virus (SeV) was purchased from China Center for Type Culture Collection (Wuhan, China). The USP2 Ab was purchased from Abgent (San Diego, CA). The Abs specific for actin, hemagglutinin (HA) epitopes, and protein G agarose used for immunoprecipitation (IP) were from Santa Cruz Biotechnology (Santa Cruz, CA). The Abs specific to Myc and TBK1 were from Cell Signaling Technology (Beverly, MA). The Ab for Flag was from Sigma-Aldrich. Their respective HRP-conjugated secondary Abs were purchased from Santa Cruz Biotechnology.

Cell culture
HEK293 cell line and THP-1 cell line were obtained from American Type Culture Collection (Manassas, VA). The cells were cultured at 37°C under 5% CO2 in DMEM supplemented with 10% FCS (Invitrogen-Life Technologies). 100 U/ml penicillin, and 100 μg/ml streptomycin (Sigma-Aldrich).

Sequences, plasmid constructs
USP2b cDNA was amplified from HEK293 cell by RT-PCR and cloned into pFLAG-CMV-2 expression vector (Sigma-Aldrich) with the following primers: 5'-CCCAAGCTTATGGCCGACCTCTAAGCCGTCGTTAGCCATATGG-3' and 5'-CC-GGCCGGCCGTGATCCACGCGTTCG-3'. USP2b C67A mutant was constructed using the KOD-Plus-Mutagenesis kit (Toyobo, Osaka, Japan). All constructs were confirmed by DNA sequencing. USP2a and USP2c expression plasmids were provided by S. Grimm (Imperial College, London, U.K.). Expression plasmids for RIG-I, MDA5, TBK1, IRF3, IRF3 SD, and IκB-β, and IRF3 reporter plasmids were obtained, as previously described (20).

RT-PCR
Total RNA was extracted with TRIzol reagent, according to the manufacturer’s instructions (Invitrogen). The primer sequences were 5’-CACAAGGTGGTATGCCGACCTCTAATGGCCGAG-3’ for IFN-β; 5’-ATGCGGACCTCTGACCTACT-3’ and 5’-CC-GATCAATTCTTCCAATCCGAC-3’ for USP2b; and 5’-GGAAGAATGCTGGGAGTCG-3’ for actin.

Transfection and reporter assays
HEK293T cells were plated in 96-well plates and transfected using Lipofectamine 2000 (Invitrogen) with plasmid encoding IFN-β or IRF3 luciferase reporter (firefly luciferase; 50 ng) and pRL-TK (renilla luciferase plasmid; 10 ng) together with increasing concentrations (0, 50, 100, and 150 ng) of plasmid encoding Flag-USP2b or indicated plasmids. Empty pDNA3.1 vector was used to maintain equal amounts of DNA among wells. Twenty hours after transfection, cells were infected with SeV or left uninfected for 8 h. Cells were collected and luciferase activity was measured with a dual-luciferase assay (Promega), according to the manufacturer’s protocol. Reporter activity was determined by normalization of the firefly luciferase activity to renilla luciferase activity.

Small interfering RNA transfection
For transient silencing, duplexes of small interfering RNA (siRNA) were transfected into HEK293 cells or THP-1 cells with the Lipofectamine 2000 (Invitrogen), according to the standard protocol. Target sequences for transient silencing were 5’-CGGGGUUGGGAGAAUUCACTT-3’ (siRNA 1), 5’-GGCCGGCUUGGGCUUAAUTT-3’ (siRNA 2), and 5’-GGUGACACUGAAGCAUTT-3’ (siRNA 3) for USP2b; scrambled control sequence was 5’-UUCUGGAGCUUCGCUU-3’.

IP and Western blot analysis
For IP, HEK293 cells were collected 24 h after transfection, and whole-cell extracts were prepared by lysing in IP buffer containing 1% (v/v) Nonidet P40, 50 mM Tris-HCl (pH 7.4), 50 mM EDTA, 150 mM NaCl, and a protease inhibitor mixture (Merck). After centrifugation for 10 min at 14,000 × g, supernatants were collected and incubated with protein G Plus-agarose IP reagent (Santa Cruz Biotechnology) together with 1 μg anti-Flag mAb or 1 μg anti-Myc Ab. After 6 h of incubation, beads were washed five times with IP buffer. Immunoprecipitates were eluted by boiling with 1% (w/v) SDS sample buffer. For Western blot analysis, immunoprecipitates or whole-cell lysates were loaded and subjected to SDS-PAGE, transferred onto nitrocellulose membranes, and then blotted, as described previously (21).

Ubiquitination assays
For analysis of the ubiquitination of TBK1, HEK293 cells were transfected with Myc-TBK1, HA-UB (wild type [WT]), or HA-UB mutants and Flag-USP2b, C76A, or USP2 siRNA, and then whole-cell extracts were immunoprecipitated with anti-Myc and analyzed by immunoblot with anti-HA Ab.

TBK1 kinase assay
TBK1 kinase in total cell extracts was immunoprecipitated with anti-TBK1 Ab plus protein G beads by gentle rocking for 2 h at 4°C, followed by centrifugation for 5 min at 4°C. Pellets were washed three times with lysis buffer (20 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1 mM EDTA, 1% v/v Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM b-glycerophosphate, 1 mM Na3VO4, and 1 mM PMSF). After the final wash, the pellet was resuspended in kinase assay buffer for 30 min at 30°C (25 mM Tris-HCl [pH 7.5], 5 mM b-glycerophosphate, 2 mM DTT, 0.1 mM Na3VO4, and 10 mM MgCl2). TBK1 kinase activity was assayed with Adp-Glo and TBK1 Kinase Enzyme System, according to the manufacturer’s instructions (Promega).

Vesicular stomatitis virus plaque virus assay and detection of virus replication
Vesicular stomatitis virus (VSV) plaque assay was performed, as previously described (21). The HEK293 cells (2 × 105) were transfected with the indicated plasmids or USP2 siRNA for 24 h before VSV infection (multiplicity of infection of 0.1). At 1 h postinfection, cells were washed with PBS three times and then fresh medium was added. The culture supernatants were harvested 24 h after washing. The supernatants were diluted 1:106 and then used to infect confluent HEK293 cells cultured on 24-well plates. At 1 h postinfection, the supernatant was removed, and 3% methylene blue was overlaid. At 3 d postinfection, overlay was removed, and cells were fixed with 4% formaldehyde for 20 min and stained with 0.2% crystal violet. Plaques were counted, averaged, and multiplied by the dilution factor to determine viral titer as LOG10 (PFU/ml). Total HEK293 cellular DNA was extracted, and VSV RNA replicates were examined by quantitative RT-PCR, as previously described (21). Primers for VSV were as follows: 5’-AGCGCCTAATCCAGATG-3’ (sense) and 5’-CTCG-GTTCAAGATCCGTT-3’ (antisense).

Statistical analysis
All data are presented as means ± SD of one representative of three or four experiments. Analysis was performed using a Student t test. The p values < 0.05 were considered significant.

Results
USP2b negatively regulates virus-induced IFN-β expression
USP2, a deubiquitinating enzyme, is a member of the USP family. At least three USP2 isoforms have been described due to alternative splicing, as follows: USP2-69 (USP2a), USP2-45 (USP2b), and
USP2-41 (USP2c), which produce a 69-kDa protein, a 45-kDa protein, and a 41-kDa protein, respectively. All three isoforms share the same catalytic core with different N-terminal extensions (22).

Western blot analysis showed that isoforms 2a and 2b were expressed in HEK293 cells (Fig. 1A), whereas the main isoform expressed in THP-1 cells was USP2b (Fig. 1A). Isoform 2c was not detected in both cell types. Importantly, infection with SeV slightly decreased USP2a and USP2b expression (Fig. 1A), indicating USP2 may regulate antiviral immune responses.

To investigate whether USP2 plays a role in antiviral immunity, IFN-β-luciferase reporter and increasing amounts of USP2 expression plasmids were transfected into HEK293 cells, and then the cells were infected with SeV for 8 h and IFN-β-luciferase activity was measured. As shown in Fig. 1B, overexpression of USP2b inhibited SeV-induced IFN-β luciferase activity in a dose-dependent manner. Similarly, overexpression of USP2b inhibited SeV-induced IFN-β activation. In contrast, SeV-induced IFN-β activation was not inhibited by USP2a overexpression. Because USP2a could not inhibit SeV-induced IFN-β activation and USP2c was not detected in the cells that we studied, USP2b was chosen in the following studies. To further demonstrate the inhibitory effects of USP2b on the expression of IFN-β gene, USP2b expression plasmid was transfected into HEK293 cells, and then the cells were infected with SeV. As shown in Fig. 1D (upper), SeV infection could induce the expression of IFN-β (lane 3), whereas the induced IFN-β expression was markedly inhibited by USP2b overexpression (Fig. 1C, lane 4). Consistent with the reporter assays, SeV-induced IFN-β expression was also inhibited by USP2c overexpression (lane 6), but not by USP2a overexpression (lane 5) (Fig. 1C, bottom). IRF3 is the key transcription factor that is responsible for the expression of IFN-β after virus infection (23). Consistent with the data that USP2b inhibited virus-induced activation of the IFN-β promoter, SeV-induced IRF3 activation was also substantially inhibited by USP2b overexpression in a dose-dependent manner (Fig. 1D). To investigate whether the deubiquitinating enzymatic activity is involved in USP2b-mediated inhibition of IFN-β signaling, the conserved cysteine residue at position 67 in the enzymatic core was mutated to alanine. Compared with WT USP2b, this C67A mutant lost the ability to inhibit SeV-induced IFN-β promoter activation and expression of IFN-β mRNA (Fig. 1E, 1F). Collectively, these data suggested that USP2b negatively regulates SeV-induced production of type I IFN, which depends on its deubiquitinating enzymatic activity.

**Knockdown of USP2b enhances IFN-β expression**

To further confirm the negative regulatory role of USP2 under physiological conditions, expression of endogenous USP2b was knocked down with three USP2-specific siRNA. RT-PCR analysis showed that the level of USP2b mRNA was greatly decreased by transient transfection of the three USP2-specific siRNAs (Fig. 2A). Similarly, overexpression of Flag-tagged USP2b protein was also markedly attenuated by transfection of USP2-specific siRNAs (Fig. 2B). Endogenous USP2b expression was markedly decreased by these siRNAs in both HEK293 cells and THP-1 cells (Fig. 2C). USP2a is the longest form of USP2, which covers the coding frame for USP2b. Consistently, USP2-specific siRNAs also attenuated both endogenous USP2a protein expression in HEK293 cells (Fig. 2C). These data indicated that USP2-specific siRNAs could efficiently knock down USP2b expression.

**FIGURE 1.** USP2b negatively regulates virus-induced IFN-β expression. (A) Western blot of USP2 protein expression in HEK293 cells and THP-1 cells infected with SeV for different times. (B) Luciferase activity in HEK293 cells transiently transfected with IFN-β-luc reporter plasmid together with increasing amount of USP2a, USP2b, and USP2c expression plasmid for 20 h, and then infected with SeV for 8 h. (C) RT-PCR analysis of IFN-β mRNA expression in HEK293 cells transfected with USP2a, USP2b, and USP2c expression plasmids or control plasmid for 20 h, and then infected or left uninfected with SeV for 8 h. (D) Luciferase activity in HEK293 cells transiently transfected with IRF3-luc reporter plasmid together with increasing amount of USP2b expression plasmid for 20 h, and then infected with SeV for 8 h. (E) Luciferase activity in HEK293 cells transiently transfected with IFN-β-luc reporter plasmid together with USP2b or USP2b C67A expression plasmid for 20 h, and then infected with SeV for 8 h. (F) RT-PCR analysis of IFN-β mRNA expression in HEK293 cells transfected with USP2b and USP2b C67A expression plasmids or control plasmid for 20 h, and then infected or left uninfected with SeV for 8 h. Data are shown as mean ± SD (n = 6) of one representative experiment in (B), (D), and (E). Similar results were obtained from three independent experiments in (A), (C), and (F).
Next, USP2b siRNA was transfected into HEK293 cells, and then SeV-induced activation of IFN-β reporter and transcription of IFN-β gene were measured. SeV infection could induce IFN-β luciferase reporter activation in HEK293 cells (Fig. 2D). However, transfection with USP2-specific siRNA resulted in increased IFN-β luciferase activation triggered by SeV infection in HEK293 cells, compared with control siRNA transfection (Fig. 2D). Consistent with these observations, knockdown of USP2b could significantly enhance expression of IFN-β gene in SeV-infected HEK293 cells (Fig. 2E). Similarly, knockdown of USP2b expression in THP-1 cells further increased SeV-induced expression of IFN-β mRNA (Fig. 2F). Taken together, these data demonstrated that knockdown of USP2b expression potentiates SeV-induced IFN-β expression.

**USP2b targets TBK1**

As USP2b inhibited Sev-induced IFN-β expression, we next sought to determine the molecular mechanisms by which USP2b inhibits type I IFN signaling. First, the effects of USP2b on the IFN-β expression mediated by various adaptors in the RLR pathway were examined using RT-PCR. As shown in Fig. 3A, expression plasmid of RIG-I, MDA5, MAVS, and TBK1 could induce IFN-β expression in HEK293 cells. The IRF-3 D mutant, in which residues at positions 396, 398, 402, 404, and 405 were replaced by the phosphomimetic aspartate amino acid, induced strong IFN-β expression (Fig. 3A). Cotransfection of USP2b attenuated RIG-I, MDA5, MAVS, and TBK1-induced IFN-β expression (Fig. 3A). In contrast, IRF3 5D-induced IFN-β expression was not impaired by USP2b overexpression (Fig. 3A).

Next, RIG-I, MDA5, MAVS, TBK1, and IRF3 5D-induced activation of IFN-β luciferase reporter was examined in the presence or absence of USP2b expression plasmid. As shown in Fig. 3B, RIG-I, MDA5, MAVS, and TBK1-induced IFN-β luciferase activity was greatly attenuated in the presence of USP2b overexpression, whereas IRF3 5D-induced IFN-β luciferase activity was not impaired by USP2b overexpression. WT IRF3-induced IFN-β luciferase activity was not impaired by USP2b overexpression (Fig. 3C). All together, these data indicated that USP2b negatively regulates virus-triggered IFN-β signaling at the TBK1 level.

**USP2b deubiquitates K63-linked polyubiquitination of TBK1**

It was reported that TBK1 activation is regulated by ubiquitination. USP2b has deubiquitinating enzymatic activity and functions at the TBK1 level. Therefore, it is possible that USP2b modulates TBK1 ubiquitination to regulate TBK1 activity. To confirm this possibility, the interaction between USP2b and TBK1 was first examined. Expression plasmid of Flag-USP2b was transfected into HEK293 cells together with expression plasmid for HA TBK1. IP and immunoblot analysis demonstrated USP2b could interact with TBK1 (Fig. 4A). Interaction between Flag-USP2b and endogenous TBK1 was also examined in HEK293 cells transfected with Flag-USP2b and stimulated with SeV for indicated time periods. As
shown in Fig. 4B, IP and immunoblot analysis demonstrated endogenous TBK1 interacted with Flag-USP2b in nonstimulated cells and SeV infection could increase this interaction. As a control, the interaction could not be detected with control IgG (Fig. 4B). Interaction between endogenous USP2b and TBK1 was also detected in SeV-infected THP-1 cells (Fig. 4C). Collectively, these data demonstrated that USP2b interacts with TBK1.

Next, the effect of USP2b on the status of TBK1 ubiquitination was examined. Myc-TBK1 and HA-ubiquitin were transfected into HEK293 cells with or without USP2b expression plasmid, and then IP and immunoblot analysis were performed to examine the ubiquitination status of TBK1. As shown in Fig. 4D, TBK1 ubiquitination could be easily detected (lane 2), whereas overexpression of USP2b greatly attenuated the ubiquitin of TBK1 (Fig. 4D, lane 3). As controls, polyubiquitination of RIG-I and MAVS, two molecules in the RLR pathway upstream of TBK1, was not affected by USP2b overexpression (Fig. 4D). To further determine the role of USP2b on TBK1 ubiquitination under physiological conditions, USP2b-specific siRNA was transfected into HEK293 cells to knock down endogenous USP2 expression. Compared with control siRNA transfection, USP2b-specific siRNA transfection greatly enhanced the ubiquitination of TBK1 (Fig. 4E, lane 4), indicating that USP2b mainly removes K63-linked polyubiquitination chains from TBK1. Consistent with the inability to inhibit IFN-β signaling, USP2 C67A lost the ability to cleave polyubiquitin chains from TBK1 (Fig. 4G, lane 4 compared with lane 3). Taken together, these data demonstrated that USP2b negatively regulates virus-mediated type I IFN signaling by deconjugating K63-linked polyubiquitination chain from TBK1.

**USP2b attenuates TBK1 kinase activity**

K63-linked polyubiquitination of TBK1 is required for TBK1 kinase activity. To investigate whether USP2b-mediated deubiquitination of TBK1 affects TBK1 activity, USP2b expression plasmids were transfected into HEK293 cells followed with SeV infection for 6 h. TBK1 kinase in total cell extracts was immunoprecipitated with anti-TBK1 Ab, and TBK1 kinase activity was measured. As shown in Fig. 5A, SeV infection increased TBK1 kinase activity, whereas USP2b overexpression substantially decreased TBK1 kinase activity in SeV-infected cells. To further confirm USP2b attenuates TBK1 kinase activity in physiological conditions, USP2b siRNA was transfected into HEK293 cells to knock down USP2b expression. SeV infection increased the TBK1 kinase activity in control siRNA-transfected cells. TBK1 kinase activity was further increased after SeV infection in USP2 siRNA-transfected cells, compared with that in control siRNA-transfected cells (Fig. 5B). All together, these data indicated that USP2b attenuates TBK1 kinase activity.

**USP2b negatively regulates cellular antiviral response**

Because USP2b negatively regulate virus-triggered IFN signaling, the roles of USP2b in cellular antiviral response were investigated.
using VSV, a kind of ssRNA virus recognized by RIG-I. Plaque assay of HEK293 cells infected with VSV showed that USP2b overexpression increased viral replication in the presence or absence of poly(I:C). Similarly, VSV RNA replicates were increased in USP2b-transfected cells compared with control vector-transfected cells (Fig. 6A). In contrast, USP2b C67A lost the ability to increase VSV replication, which is similar to control vector-transfected cells. To further confirm the function of USP2b on VSV replication under physiological conditions, USP2b expression was silenced by USP2-specific siRNA in HEK293 cells and then infected with VSV. USP2b knockdown decreased VSV viral replication in the presence or absence of poly(I:C). Accordingly, USP2b knockdown significantly decreased intracellular VSV RNA replicates (Fig. 6B). Similarly, siRNA knockdown of USP2b expression in THP-1 cells significantly inhibited VSV replication in THP-1 cells (Fig. 6C). Collectively, these data demonstrated that USP2b negatively regulates cellular antiviral response by inhibiting IFN-β expression through deubiquitination of TBK1.

**USP2b negatively regulates TLR3/4 and DNA-induced IFN-β signaling**

TBK1 is a key kinase for not just RNA sensing, but also for DNA sensing and for TLR3/4 signaling. TLR3 and TLR4 use TRIF as an adaptor to activate TBK1 and IRF3. Compared with control vector, overexpression of USP2b substantially attenuated TRIF-induced IFN-β promoter activation (Fig. 7A). Cyclic GMP-AMP synthase (cGAS) is a recently identified DNA sensor, which synthesizes 2′-3′-GAMP upon DNA binding. As a second messenger, cyclic GAMP binds to STING to initiate the DNA signaling. cGAS plus STING-induced IFN-β promoter activation was also attenuated by USP2b overexpression (Fig. 7A). In contrast, knockdown of USP2b could increase TRIF and cGAS plus STING-induced IFN-β luciferase activation (Fig. 7B). All together, these reporter assays suggested that USP2b also inhibits TLR3/4 and DNA-induced IFN-β signaling. To further confirm USP2b regulates TLR3/4 and DNA signaling, USP2b expression was knock down in THP-1 cells with transfection with siRNA, and then cells were stimulated with poly(I:C) (TLR3 ligand), LPS (TLR4 ligand), or ISD transfection (DNA molecule). RT-PCR analysis showed that the expression of IFN-β mRNA was increased after USP2b knockdown after stimulation with LPS and poly(I:C), or transfected with ISD (Fig. 7C). These data indicated that USP2b also regulates TLR3/4 and DNA-mediated IFN-β signaling.

**Discussion**

Virus-triggered induction of type I IFNs is crucial for the early innate antiviral response as well as late-stage adaptive immunity. This process is delicately regulated in a spatiotemporal manner by various molecules and distinct mechanisms. TBK1 mediates the activation of IRF3 and IRF7, leading to the induction of type I IFN (IFN-αβ) in response to viral infection (26, 27). As a critical kinase in IFN-β production, TBK1 must be tightly regulated to maintain immune homeostasis. TBK1 activity can be regulated through different mechanisms, such as phosphorylation, ubiquitination, and modulation of its kinase activity. It has shown that the kinase GSK3β interacts with TBK1 in a viral infection-dependent manner. GSK3β enhances TBK1 self-association and autophosphorylation at Ser172, which is critical for virus-induced IRF3 activation and IFN-β induction (28). In addition to being regulated by phosphorylation, TBK1 can also be activated by K63-linked polyubiquitination by the ubiquitin ligase Nrdp1 after stimulation with LPS or by the E3 ligase Mib postinfection with a RNA virus (25, 29). TBK1 activity also can be regulated through K48-linked polyubiquitination. For example, NLR protein NLRP4 recruits the E3 ligase DTX4 to mediated K48-linked
infection could increase the interaction between TBK1 and
strated that USP2b interacted with TBK1. Importantly, SeV-triggered activation of IFN-
mRNA expression. Conversely, knockdown of USP2b potentiated
and TBK1-, but not IRF3-mediated IFN-
(TBK1) activation. Given the fact that TBK1 plays an
role downstream of virus-sensing pathways mediated by
by receptors including TLR3/4, RLRs, and DNA sensors, USP2b
may represent a general regulator in virus-triggered IFN production.

In conclusion, our findings demonstrated that USP2b deubi-
quitates K63-linked polyubiquitin chains from TBK1 to termi-
activate TBK1. Therefore, similar to USP2a, USP2b also has deubiquitination activity toward both K48- and K63-linked polyubiquitin chains. For example, USP2a has been reported to inhibit TLR/IL-1 and virus-triggered NF-κB activation through deubiquitination of K63-linked ubiquitin chain from TRAF6 (34). USP2b has also been demonstrated to remove K63-linked ubiquitin chains from receptor interacting protein 1 and TRAF2 to regulate TNF-induced cell death (35). USP2a also selectively deubiquitinates and stabilizes an E3 ligase Mdm2 to regulate p53 pathway (36). Similarly, limited publications reported that USP2b can cleave K48-linked polyubiquitin chains from target proteins, consequently leading to the stabiliza-
tion and prevention of the target proteins from undergoing degradation (37, 38). We demonstrated in this study that USP2a and USP2c attenuated SeV-induced IFN-β signaling, whereas knockdown of USP2b increased poly-
ubiquitination, whereas knockdown of USP2b increased poly-
ubiquitination of TBK1. Finally, our study demonstrated that USP2b trimmed K63-linked polyubiquitin chains from TBK1 using ubiquitin mutants for transfection. Consistent with the central role of TBK1 in TLR3/4 and DNA-mediated IFN-β signaling, TLR3/4 and DNA-induced activation of IFN-β promoter and expression of IFN-β mRNA were also attenuated by USP2b.

USP2b has at least three isoforms, as follows: USP2a, USP2b, and USP2c, due to alternative splicing of 5′ exons. USP2a is the longest isoform with 605 aa. Isoform 2b is composed of 396 aa, which is missing two exons from the 5′-end and contains an alternative 5′ terminal exon. Isoform 2c is composed of 362 aa, which lacks an internal exon in the 5′-end. Although these three isoforms have different N-terminal extensions, they share the same C-terminal catalytic core, which is encoded by exons 3–13. The C-terminal catalytic core contains the conserved Cys and His residues required for its catalytic activity.

Compared with USP2b and c, the function of USP2a has been studied extensively. USP2a has deubiquitination activity toward both K48- and K63-linked polyubiquitin chains. For example, USP2a has been reported to inhibit TLR/IL-1 and virus-triggered NF-κB activation through deubiquitination of K63-linked ubiquitin chain from TRAF6 (34). USP2b has also been demonstrated to remove K63-linked ubiquitin chains from receptor interacting protein 1 and TRAF2 to regulate TNF-induced cell death (35). USP2a also selectively deubiquitinates and stabilizes an E3 ligase Mdm2 to regulate p53 pathway (36). Similarly, limited publications reported that USP2b can cleave K48-linked polyubiquitin chains from target proteins, consequently leading to the stabilization and prevention of the target proteins from undergoing degradation (37, 38). We demonstrated in this study that USP2a and USP2c attenuated SeV-induced IFN-β signaling, whereas knockdown of USP2b increased polyubiquitination, whereas knockdown of USP2b increased poly-
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