Mint3 potentiates TLR3/4- and RIG-I–induced IFN-β expression and antiviral immune responses

Wanwan Huai[a,1], Hui Song[a,1], Zhongxia Yu[a], Wenwen Wang[a], Lihui Han[a], Takeharu Sakamoto[b], Motoharu Seiki[c], Lining Zhang[a], Qunye Zhang[d], and Wei Zhao[a,2]

[a]Department of Immunology and Key Laboratory of Infection and Immunity of Shandong Province, Shandong University School of Medicine, Jinan, Shandong 250012, China; [b]Division of Molecular Pathology, The Institute of Medical Science, The University of Tokyo, Minato-ku, Tokyo 108-8639, Japan; [c]Faculty of Medicine, Institute of Medical, Pharmaceutical, and Health Sciences, Kanazawa University, Takara-machi, Kanazawa 920-8640, Japan; and [d]Key Laboratory of Cardiovascular Remodeling and Function Research, Chinese Ministry of Education and Ministry of Public Health, Qilu Hospital, Shandong University, Jinan, Shandong 250012, China

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Type I IFNs (IFN-α/β) play crucial roles in the elimination of invading viruses. Multiple immune cells including macrophages recognize viral infection through a variety of pattern recognition receptors, such as Toll-like receptors (TLRs) and retinoic acid-inducible gene-I (RIG-I)–like receptors, and initiate type I IFN secretion and subsequent antiviral immune responses. However, the mechanisms by which host immune cells can produce adequate amounts of type I IFNs and then eliminate viruses effectively remain to be further elucidated. In the present study, we show that munc18-1–interacting protein 3 (Mint3) expression can be markedly induced during viral infection in macrophages. Mint3 enhances TLR3/4- and RIG-I–induced IRF3 activation and IFN-β production by promoting K63-linked polyubiquitination of TNF receptor-associated factor 3 (TRAF3). Consistently, Mint3 deficiency greatly attenuated antiviral immune responses and increased viral replication. Therefore, we have identified Mint3 as a physiological positive regulator of TLR3/4 and RIG-I–induced IFN-β production and have outlined a feedback mechanism for the control of antiviral immune responses.

interferon | viral infection | TLR | RIG-I | TRAF3

The production of type I IFNs (IFN-α/β) is a fundamental step for countering viral infections (1, 2). Pattern-recognition receptors, including Toll-like receptors (TLRs) and retinoic acid-inducible gene-I (RIG-I)–like receptors (RLRs), could signal viral infection and then activate immune cells to produce type I IFNs (3–6). Secreted type I IFNs bind to IFN-α/β receptor and trigger the production of numerous antiviral genes through the JAK/STAT pathway, which could suppress viral replication and promote viral clearance (7).

TLR3 and TLR4 initiate IFN-β signaling through the recruitment of the Toll/IL-1R domain-containing adaptor inducing IFN-β (TRIF) (3, 4). RLRs, including RIG-I and the melanoma differentiation-associated gene 5 (MDA5), recognize viral RNAs and poly(I:C) in the cytoplasm and subsequently recruit another antiviral signaling adaptor—mitochondrial antiviral signaling protein (MAVS; also called IPS-1, Cardif, or VISA)—to initiate IFN-β signaling (5, 6). Both TRIF and MAVS recruit TNF receptor-associated factor 3 (TRAF3), and the binding results in its K63-linked polyubiquitylation and the recruitment TANK-binding kinase 1 (TBK1). Activated TBK1 phosphorylates IRF3 and triggers its dimerization and nuclear translocation. IRF3 then forms active transcriptional complexes that bind to IFN stimulation response elements and activate type I IFN gene expression (3–6). Fine-tuning the expression of type I IFNs is vital for the elimination of invading viruses quickly and effectively. However, the underlying mechanisms of how immune cells secret adequate type I IFNs following viral infections is not fully understood.

Munc18-1–interacting protein 3 (Mint3), also known as amyloid-β A4 precursor protein-binding family A member 3 (APBA3), is a member of the Mint protein family (8). Unlike the other family members (Mint1 and Mint2, which are expressed primarily in neurons), Mint3 is ubiquitously expressed (8). Mint3 was originally identified as an Alzheimer disease β-amyloid precursor protein (APP) interactor (9). Mint3 associates with several membrane proteins such as APP and furin in Golgi and regulates the traffic of these proteins from the trans-Golgi network (10–12). Mint3 regulates ATP production in macrophages and augments LPS-induced septic shock by enhancing the activity of hypoxia-inducible factor-1 (13, 14). However, the potential role of Mint3 in antiviral immune responses remains unknown.

In the present study, we found that Mint3 expression was markedly increased during viral infection in macrophages, suggesting that Mint3 possessed potential roles in antiviral immune responses. Mint3 interacts with TRAF3 and promotes K63-linked polyubiquitination of TRAF3, resulting in subsequent enhancement of IRF3 activation and IFN-β secretion. Consistently with the reduced IFN-β expression, Mint3 deficiency greatly attenuated antiviral immune responses. Therefore, we have identified Mint3 as a positive regulator in TLR3/4 and RIG-I–induced antiviral signaling and have outlined a feedback mechanism for the control of antiviral immune responses.

Results

Viral Infection and IFN-β Stimulation Induced Mint3 Expression. To determine the potential role of Mint3 in antiviral immune responses,

Significance

Optimal productions of type I IFNs are crucial for maintaining immune homeostasis and elimination of invading viruses. A couple of enhancers of host antiviral innate immunity are induced following vial infection and then potentiate type I IFN production. Here, we identified munc18-1–interacting protein 3 (Mint3) as a viral infection-induced physiological enhancer of antiviral immune responses. Viral infection and IFN-β secretion induced Mint3 expression in the virus-infected cells, and Mint3 then feedback-promoted Toll-like recptor- and retinoic acid-inducible gene-I–induced IFN-β expression. These results will be valuable for understanding the crosstalk between virus and host immune systems. Furthermore, as IFN-β has manifested in diverse pathogenic autoimmune diseases, control of IFN-β production via suppressing Mint3 will be a potential target for designing therapeutic strategies against autoimmune disorders.

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[1]W.H. and H.S. contributed equally to this work.

[2]To whom correspondence should be addressed. Email: wzhao@sdu.edu.cn.

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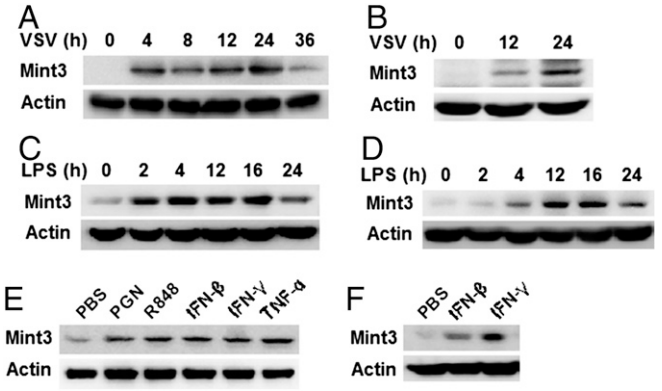

Fig. 1. Expression patterns of Mint3 during viral infection. (*A* and *B*) Western blot analysis of Mint3 expression in mouse peritoneal macrophages (*A*) or phorbol myristate acetate (PMA)-pretreated THP-1 cells (*B*) infected with VSV for the indicated time periods. (*C* and *D*) Western blot analysis of Mint3 expression in mouse peritoneal macrophages (*C*) or PMA-pretreated THP-1 cells (*D*) stimulated with LPS for the indicated time periods. (*E* and *F*) Western blot analysis of Mint3 expression in mouse peritoneal macrophages (*E*) or PMA-pretreated THP-1 cells (*F*) stimulated as indicated for 12 h. Data are representative of three independent experiments with similar results.

its expression during viral infection was examined. Vesicular stomatitis virus (VSV, a kind of single-stranded RNA virus recognized by RIG-I) infection markedly induced Mint3 expression in mouse primary peritoneal macrophages and THP-1 cells (Fig. 1 *A* and *B*). LPS stimulation also greatly induced Mint3 expression (Fig. *C* and *D*). Following viral infection, multiple immune cells including macrophages secreted amounts of type I IFNs and proinflammatory cytokines to initiate host antiviral responses. Then, we investigated Mint3 expression during the process. IFN-β and proinflammatory cytokines (such as IFN-γ and TNF-α) greatly induced Mint3 expression (Fig. 1 *E* and *F*). Collectively, these data indicate that Mint3 expression is greatly induced during viral infection, suggesting that Mint3 is involved in the regulation of antiviral responses.

Mint3 Facilitates IFN-β Expression. To further investigate whether Mint3 was involved in the regulation of TLR and RLR signaling, we silenced the endogenous expression of Mint3 in mouse peritoneal macrophages. Mint3 protein expression was greatly decreased with transfection of Mint3-specific silencing RNA duplexes (siRNA1 and siRNA2) (Fig. 2*A*). Mint3 knockdown significantly decreased LPS-induced IFN-β production in mouse peritoneal macrophages (Fig. 2*A*). Notably, Mint3 siRNA 1, which has a higher efficiency to knock down Mint3 expression, has a greater potential to decrease LPS-induced IFN-β production than Mint3 siRNA 2 (Fig. 2*A*). Therefore, Mint3 siRNA 1 was used in the following knockdown experiments. Mint3 knockdown significantly decreased LPS-, poly(I:C)-, and Sendai virus (SeV)-induced IFN-β production in mouse peritoneal macrophages (Fig. 2 *B* and *C*).

To further confirm the function of Mint3 in IFN-β production, the effects of Mint3 deficiency on IFN-β production in mouse peritoneal macrophages were observed. As shown in Fig. 2*D*, LPS-, poly(I:C)-, SeV-, and interferon-stimulatory DNA (ISD)-induced IFN-β production was significantly decreased in Mint3-deficient macrophages compared with that in wild-type macrophages. LPS- and SeV-induced IFN-β mRNA expression was also significantly decreased in Mint3-deficient macrophages (Fig. 2*E*). We intraperitoneally injected wild-type and Mint3-deficient mice with LPS and then assessed the secretion of IFN-β in serum. LPS-induced secretion of IFN-β was significantly inhibited in Mint3-deficient mice

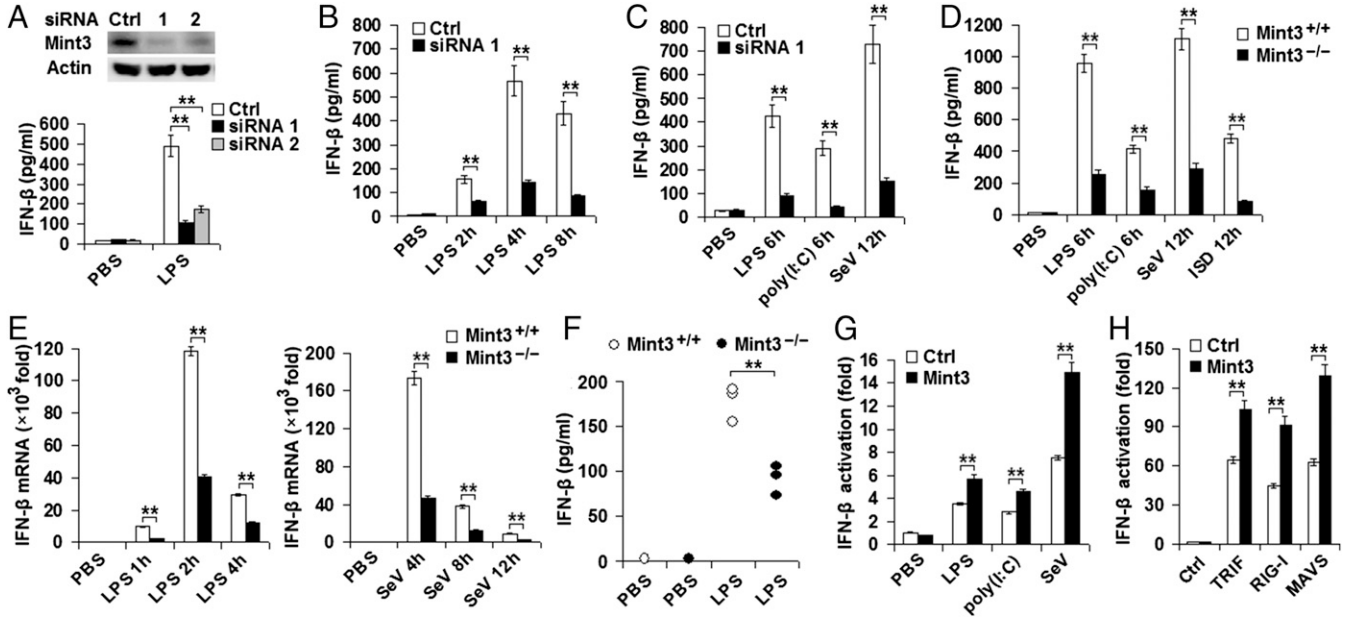

Fig. 2. Mint3 facilitates IFN-β expression. (*A*) Western blot analysis of Mint3 expression in mouse peritoneal macrophages transfected with scrambled control siRNA (Ctrl) and Mint3 siRNA 1(siRNA 1) or 2 (siRNA 2) for 36 h. ELISA analysis of IFN-β in the supernatants of peritoneal macrophages transfected with siRNA as indicated and stimulated with LPS for 6 h. (*B* and *C*) ELISA analysis of IFN-β in the supernatants of peritoneal macrophages transfected with control siRNA (Ctrl) or Mint3 siRNA 1 (siRNA 1) and then treated as indicated. (*D* and *E*) ELISA (*D*) or quantitative real-time RT-PCR (*E*) analysis of IFN-β expression in peritoneal macrophages from $Mint3^{+/+}$ or $Mint3^{-/-}$ mice after treatment as indicated. (*F*) ELISA of IFN-β in serum from $Mint3^{+/+}$ or $Mint3^{-/-}$ mice after i.p. administration with PBS or LPS for 1 h. (*G*) RAW264.7 cells were transiently transfected with IFN-β reporter plasmid together with Mint3 expression plasmid or control plasmid and analyzed for luciferase activity after stimulation with LPS, poly(I:C) for 6 h or infection with SeV for 12 h. (*H*) HEK293T cells were transiently transfected with IFN-β reporter plasmid and adaptor plasmids as indicated, together with the Mint3 expression plasmid or control plasmid, and analyzed for luciferase activity. $^{**}P < 0.01$. Data are from three independent experiments (*A*–*H*: mean ± SD, $n = 3$).

relative to that in wild-type mice (Fig. 2*F*). Taken together, these results indicate that Mint3 is a positive regulator of IFN-β production both in vitro and in vivo. Supporting this conclusion, Mint3 overexpression enhanced LPS-, poly(I:C)-, and SeV-induced IFN-β luciferase reporter gene expression in RAW264.7 cells (Fig. 2*G*). In addition, Mint3 overexpression also enhanced TRIF-, RIG-I–, and MAVS-induced IFN-β luciferase reporter gene expression in HEK293T cells (Fig. 2*H*).

Mint3 Promotes IRF3 Activation. IRF3 is the key transcription factor that is activated and mediates the expression of IFN-β in TLR3/4-RIG-I–, and ISD-mediated signal transduction (3–6). We observed the effect of Mint3 on IRF3 activation. Mint3 overexpression significantly enhanced poly (I:C) transfection- and SeV infection-induced IRF3 activation in HEK293T cells (Fig. 3*A*). TRIF-, RIG-I–, MAVS-, TBK1-, and IKK-ε–induced IRF3 promoter activation was also significantly promoted by Mint3 overexpression (Fig. 3*B*). However, Mint3 had no effects on NF-κB activation (Fig. 3*C*). To investigate the physiological function of Mint3 in IRF3 activation, peritoneal macrophages from wild-type or Mint3-deficient mice were stimulated with LPS. Mint3 deficiency substantially inhibited LPS-induced phosphorylation of IRF3 (Fig. 3*D*). However, Mint3 deficiency had no effects on LPS-induced phosphorylation of ERK, JNK, p38, and IκBα (Fig. 3*D*), indicating that Mint3 bears no regulatory effects on MAPK and NF-κB activation induced by TLR4 engagement. In addition, similar results were observed in Mint3 knockdown experiments (Fig. 3*E*). Consequently, Mint3 deficiency had no effects on LPS-induced secretion of TNF-α and IL-6 in macrophages (Fig. 3*F*). In addition, Mint3 deficiency had no effects on IL-1β secretion in LPS-primed macrophages triggered by multiple NLRP3 inflammasome activators, such as ATP and nigericin (Fig. 3*G*). Collectively, these data indicate that Mint3 enhances TLR4-induced IRF3 activation with no effects on NF-κB activation and subsequent secretion of proinflammatory cytokines.

We next examined the effects of Mint3 deficiency on IRF3 phosphorylation in other signal pathways. As shown in Fig. 3 *H–J*, Mint3 deficiency also greatly inhibited poly(I:C)-, SeV-, and ISD-induced phosphorylation of IRF3. Altogether, these data suggest that Mint3 facilitates TLR3/4-, RIG-I–, and cGAS-induced IRF3 activation.

Mint3 Promotes TRAF3 Ubiquitination. TLR3/4 and RIG-I induced IRF3 activation and IFN-β production through a complex cascade composed of various molecules including TRIF, MAVS, TBK1, and IRF3 (3–6). To determine the molecular targets of Mint3 in IFN-β signaling, the effects of Mint3 on IFN-β promoter activation mediated by various adaptors were examined. TRIF-, RIG-I–, MAVS-, TBK1-, and IKK-ε–induced IFN-β promoter activation was significantly enhanced by Mint3 overexpression (Figs. 2*H* and 4*A*). However, no difference was observed in IRF3-induced IFN-β promoter activation (Fig. 4*A*). Therefore, we conclude that Mint3 targets IRF3 or molecules upstream of IRF3 to promote the signal transduction. To clarify the Mint3 target, we examined the association between Mint3 and several adaptor factors. Myc-tagged TRAF3, TBK1, IKK-ε, IRF3 plasmids and V5-tagged Mint3 plasmid were cotransfected into HEK293T cells. Mint3 was coprecipitated with TRAF3, TBK1, and IKK-ε (Fig. 4*B*). However, Mint3 was not coprecipitated with IRF3 (Fig. 4*B*). Next, endogenous interaction was examined in macrophages stimulated with LPS. As shown in Fig. 4*C*, Mint3 interacted with TRAF3. However, no interaction was observed between Mint3 and TBK1 (Fig. 4*D*). Collectively, these results indicate that Mint3 binds specifically to TRAF3.

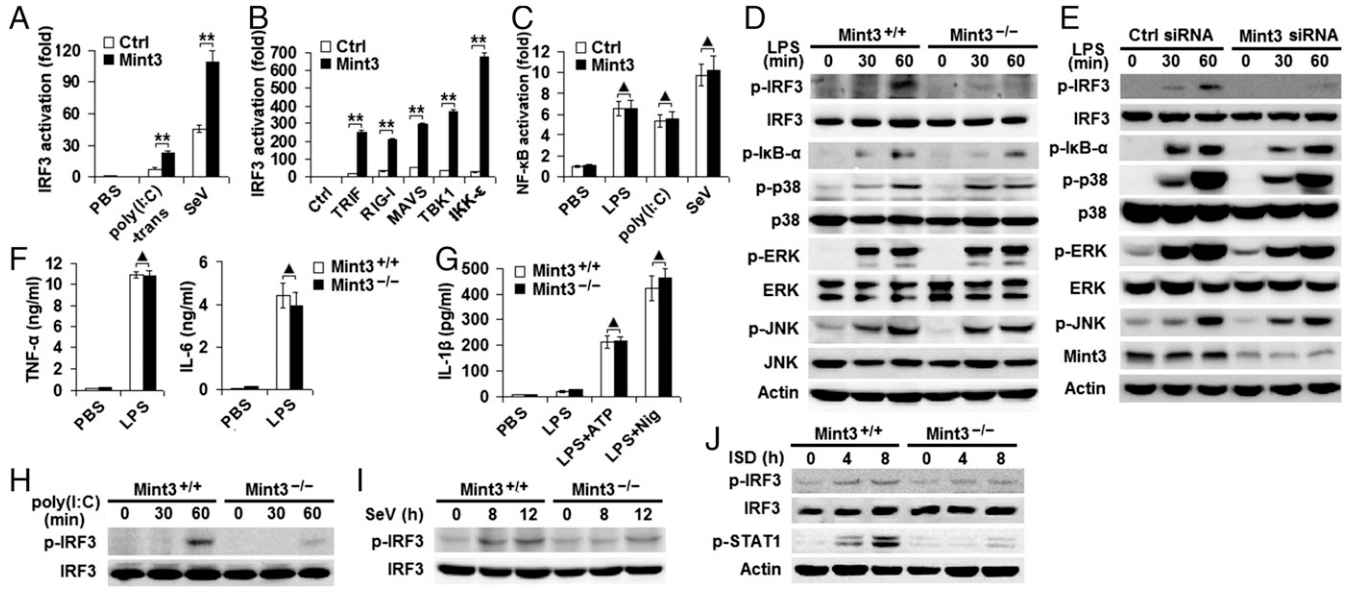

Fig. 3. Mint3 promotes IRF3 activation. (*A*) HEK293T cells were transiently transfected with IRF3 reporter plasmid together with Mint3 expression plasmid or control plasmid and analyzed for luciferase activity after transfection with poly(I:C) for 6 h or infection with SeV for 12 h. (*B*) HEK293T cells were transfected with IRF3 reporter plasmid and adaptor plasmids as indicated, together with Mint3 expression plasmid or control plasmid, and analyzed for luciferase activity. (*C*) RAW264.7 cells were transiently transfected with NF-κB reporter plasmid together with Mint3 expression plasmid or control plasmid and analyzed for luciferase activity after stimulation with LPS and poly(I:C) for 6 h or infection with SeV for 12 h. (*D*) Western blot analysis of indicated phosphorylated and total signaling proteins in peritoneal macrophages from Mint3$^{+/+}$ or Mint3$^{-/-}$ mice stimulated with LPS. (*E*) Western blot analysis of indicated phosphorylated and total signaling proteins in mouse peritoneal macrophages transfected with control siRNA or Mint3 siRNA stimulated with LPS. (*F*) ELISA analysis of TNF-α and IL-6 secretion in peritoneal macrophages from Mint3$^{+/+}$ or Mint3$^{-/-}$ mice after stimulation with LPS for 4 h. (*G*) ELISA analysis of IL-1β secretion in peritoneal macrophages from Mint3$^{+/+}$ or Mint3$^{-/-}$ mice, primed with LPS for 4 h followed by stimulation with ATP (5 mM) or Nigericin (Nig) (50 μM) for 30 min. (*H* and *I*) Western blot analysis of indicated phosphorylated-IRF3 in peritoneal macrophages from Mint3$^{+/+}$ or Mint3$^{-/-}$ mice stimulated with poly(I:C) (*H*) or infected with SeV (*I*). (*J*) Western blot analysis of indicated phosphorylated-IRF3 and phosphorylated-STAT1 in peritoneal macrophages from Mint3$^{+/+}$ or Mint3$^{-/-}$ mice stimulated with ISD. $^{**}P < 0.01$. ▲, $P > 0.05$. Data are representative of three independent experiments with similar results (*D*, *E*, and *H–J*) or are from three independent experiments (*A–C*, *F*, and *G*: mean ± SD, $n = 6$).

The formation of functional TBK1-containing complexes including TBK1, TRAF3, and IRF3 is critical for IFN-β expression. We therefore investigated whether Mint3 could modulate TRAF3–TBK1–IRF3 complex formation. However, Mint3 had no effects on the interaction between TRAF3 and TBK1, as well as TBK1 and IRF3 (Fig. 4*E*). Mint3 is known to enhance export of proteins through the trans-Golgi network. We therefore investigated the effects of Mint3 on TRAF3 localization. In wild-type mouse embryonic fibroblasts (MEFs), TRAF3 was distributed in a speckle pattern (Fig. 4*F*), which was vital for the downstream signal transduction (15). However, TRAF3 distribution was dramatically changed from a speckle pattern to a diffuse pattern in Mint3-deficient MEFs (Fig. 4*F*). These data indicate that Mint3 deficiency can impair TRAF3 localization and greatly inhibit subsequent signal transduction.

K63-linked polyubiquitination of TRAF3 is required for downstream signals leading to production of type I IFNs (16, 17). We therefore investigated the function of Mint3 in TRAF3 polyubiquitination. Marked enhancement of K63-linked polyubiquitination of TRAF3 was observed in Mint3 overexpression HEK293T cells (Fig. 4*G*). Under physiological conditions, the ubiquitination of endogenous TRAF3 was examined in wild-type and Mint3-deficient mouse peritoneal macrophages upon LPS stimulation. TRAF3 polyubiquitination was almost completely abolished in Mint3-deficient macrophage (Fig. 4*H*). Collectively, these data demonstrate that Mint3 interacts with TRAF3 and is required for the formation of TRAF3-containing speckles and for the promotion of subsequent K63-linked ubiquitination of TRAF3.

The C-terminal Region of Mint3 Is Crucial for the Enhancement of TRAF3 Ubiquitination. Mint3 contains a long isoform-specific N-terminal sequence, a central phosphotyrosine-binding (PTB) domain, and two C-terminal PSD-95/DLG-A/ZO-1 (PDZ) domains (11). To further explore the domains of Mint3 that are crucial for the enhancement of IFN-β and TRAF3 ubiquitination, a series of Mint3-truncated mutants were constructed (Fig. 5*A*). TRAF3 was coprecipitated with Mint3 wild-type, PTB domain deletion mutant (ΔPTB), PDZ domain deletion mutant (ΔPDZ) and CT (C-terminal, including two PDZ domains) region mutant, but not with PTB domain mutant (Fig. 5*B*), indicating that Mint3 interacted with TRAF3 via its C-terminal region. Mint3 WT and CT mutants greatly enhanced polyubiquitination of TRAF3 (Fig. 5*C*). However, PTB mutant had no effects on TRAF3 polyubiquitination (Fig. 5*C*). Consistently, Mint3 WT, CT mutant, and ΔPTB mutant (containing the C-terminal region) greatly enhanced SeV-induced IFN-β reporter gene activation (Fig. 5*D*). Taken together, these data indicate that the C-terminal region (including two PDZ domains and the last 15 amino acids) is crucial for the function of Mint3 in TRAF3 polyubiquitination and subsequent IFN-β expression.

Mint3 Promotes Antiviral Responses. IFN-β possesses critical roles in the immune responses against viral infection (1, 2). The fact that Mint3 facilitates IFN-β production prompted us to investigate the function of Mint3 in antiviral immunity. VSV RNA replicates were greatly increased in peritoneal macrophages from Mint3-deficient mice, compared with that from wild-type mice (Fig. 6*A*). We next

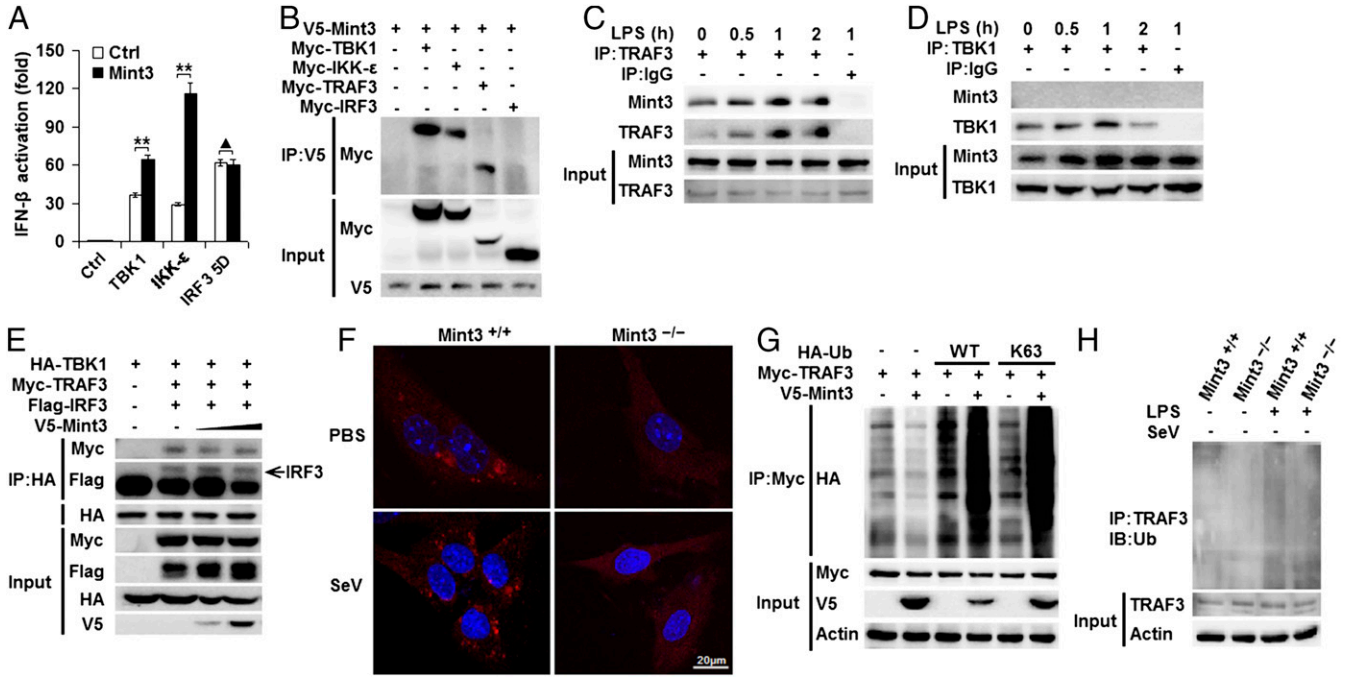

Fig. 4. Mint3 promotes TRAF3 ubiquitination. (*A*) HEK293T cells were transiently transfected with IFN-β reporter plasmid and adaptor plasmids as indicated, together with Mint3 expression plasmid or control plasmid, and analyzed for luciferase activity. (*B*) Lysates from HEK293T cells transiently transfected with V5-Mint3 and Myc-TBK1, IKK-ε, TRAF3, or IRF3 were subjected to immunoprecipitation with anti-V5 antibody followed by Western blot analysis with anti-Myc antibody. (*C* and *D*) Lysates from mouse peritoneal macrophages stimulated with LPS for indicated time periods were subjected to immunoprecipitation with anti-TRAF3 or anti-TBK1 antibody followed by Western blot analysis with anti-Mint3 antibody. Proteins in whole-cell lysate were used as positive control (Input). (*E*) Lysates from HEK293T cells transiently transfected with HA-TBK1, Myc-TRAF3, Flag-IRF3, and an increasing amount of V5-Mint3 were subjected to immunoprecipitation with anti-HA antibody followed by Western blot analysis with anti-Myc or anti-Flag antibody. (*F*) Mint3$^{+/+}$ or Mint3$^{-/-}$ MEFs transfected with ORF-TRAF3 were infected with SeV for 4 h and then fixed. Expression pattern of TRAF3 in cytoplasm was examined by confocal microscopy. (*G*) Lysates from HEK293 cells transiently cotransfected with Myc-TRAF3, V5-Mint3, or vector control and HA-Ub (WT) or HA-Ub (K63) plasmids were subjected to immunoprecipitation with anti-Myc antibody followed by Western blot analysis with anti-HA antibody. (*H*) Immunoblot analysis of lysates from LPS-stimulated Mint3$^{+/+}$ or Mint3$^{-/-}$ macrophages, followed by immunoprecipitation with anti-TRAF3, and probes with anti-Ub. $^{**}P < 0.01$. ▲, $P > 0.05$. Data are representative of three independent experiments with similar results (*B*–*H*) or are from three independent experiments (*A*: mean ± SD, $n = 6$).

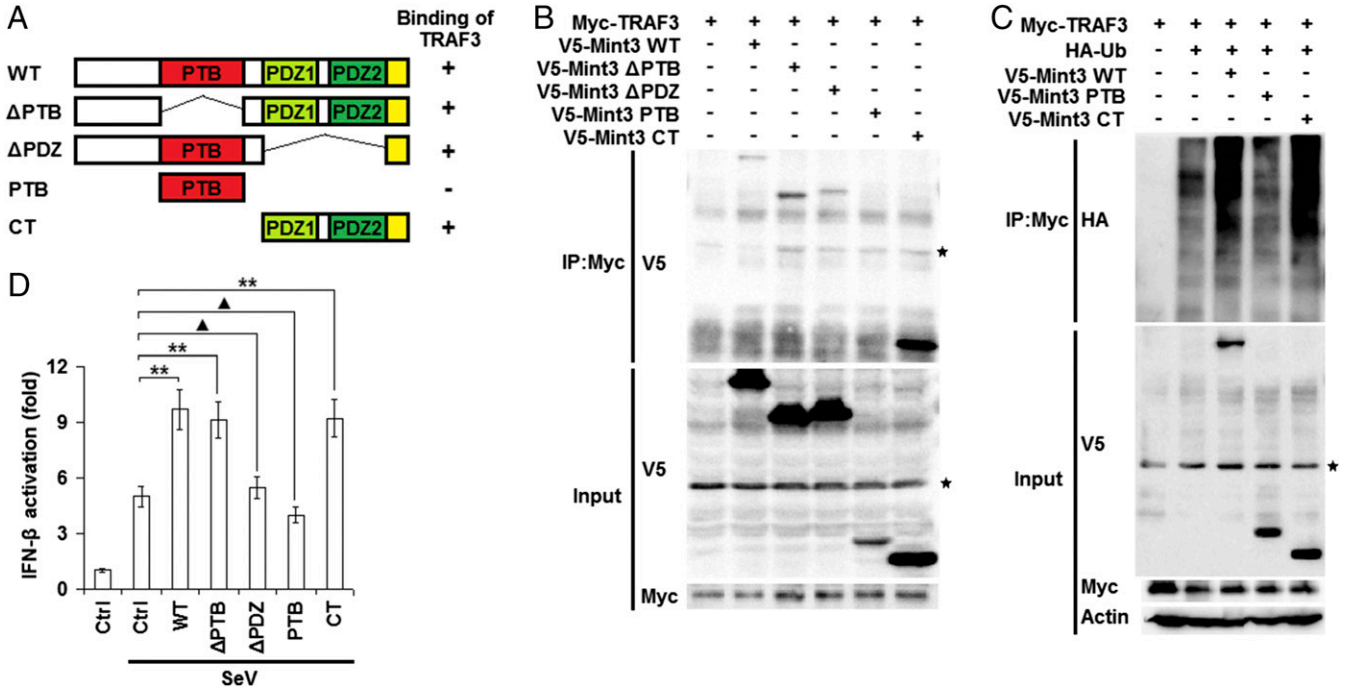

Fig. 5. The C-terminal region of Mint3 is crucial for the enhancement of TRAF3 ubiquitination. (*A*) Schematic diagram of Mint3 and its truncation mutants. (*B*) V5-tagged Mint3 or its mutants and Myc-TRAF3 were individually transfected into HEK293T cells. The cell lysates were immunoprecipitated with an anti-Myc antibody and then immunoblotted with the indicated antibodies. ★, nonspecific band. (*C*) Lysates from HEK293 cells transiently cotransfected with Myc-TRAF3, V5-Mint3, or its truncation mutants and HA-Ub (WT) plasmids were subjected to immunoprecipitation with anti-Myc antibody followed by Western blot analysis with anti-HA antibody. (*D*) HEK293T cells were transiently transfected with IFN-β reporter plasmid together with Mint3 wild type plasmid or its truncation mutants and analyzed for luciferase activity after infection with SeV for 12 h. $^{**}P < 0.01$. ▲, $P > 0.05$. Data are representative of three independent experiments with similar results (*B* and *C*) or are from three independent experiments (*D*: mean ± SD, $n = 6$).

investigated the physiological and pathological relevance of the regulatory effects of Mint3 in the context of VSV infection in vivo. IFN-β secretion induced by VSV infection was much less in sera of Mint3-deficient mice than in that of WT mice (Fig. 6*B*). In accordance with reduced IFN-β secretion, VSV replication in the lung and liver of Mint3-deficient mice was higher than WT controls (Fig. 6*C*). Severe injury was observed in the lungs of Mint3-deficient mice, compared with that of WT mice after VSV infection (Fig. 6*D*). Moreover, Mint3-deficient mice were more susceptible to VSV infection than WT mice (Fig. 6*E*). Collectively, these data indicate that Mint3 is an important positive regulator of IFN-β production and antiviral immune responses.

Discussion

Optimal productions of type I IFNs are crucial for maintaining immune homeostasis and elimination of invading viruses (18, 19). Thus, type I IFN secretion must be tightly controlled. Viruses have developed a variety of tactics to inhibit type I IFN expression and then attenuate antiviral immune responses, such as the induction of multiple inhibitory molecules targeting TLR/RLR signals. As for

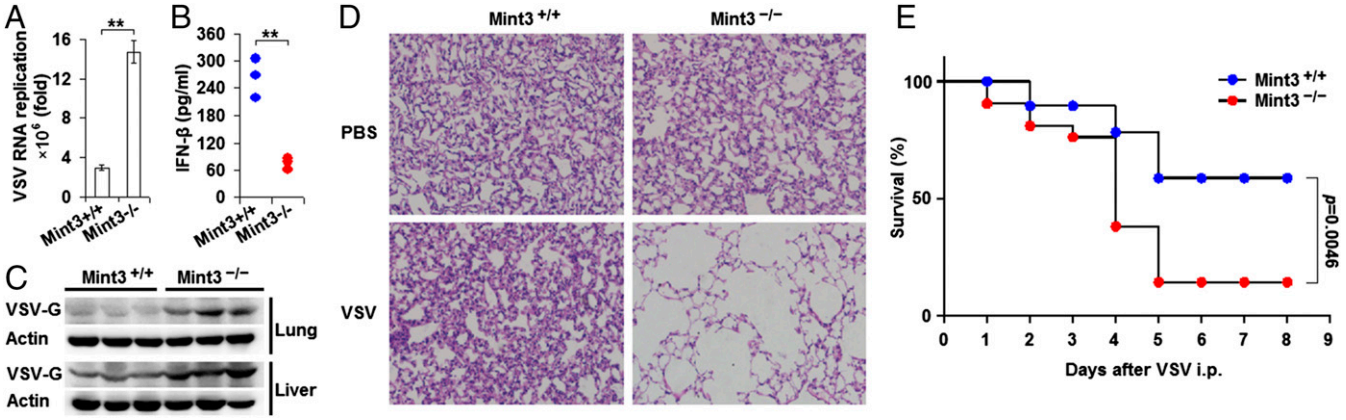

Fig. 6. Mint3 promotes antiviral responses. (*A*) Peritoneal macrophages from Mint3$^{+/+}$ or Mint3$^{-/-}$ mice were infected with VSV for 12 h. Intracellular VSV RNA replicates were measured by RT-PCR. $^{**}P < 0.01$. Data are representative of three experiments (mean and SD of six samples). (*B–D*) Mint3$^{+/+}$ or Mint3$^{-/-}$ mice ($n = 3$) were infected with VSV (4×10^7 pfu per mouse) for 48 h. Secretion of IFN-β in serum was examined by ELISA (*B*). Expression of VSV G protein in lung and liver was analyzed by Western blot with VSV-G antibody (*C*). H&E staining of lung tissue sections (*D*). (Magnification: 200×.) (*E*) Survival of Mint3$^{+/+}$ or Mint3$^{-/-}$ mice ($n = 16$) infected with VSV (4×10^7 pfu per mouse).

host, a couple of enhancers of innate immunity could be induced following virus infection and then potentiate type I IFN production. For example, ELF4 expression could be induced following viral infection and then promote type I IFN expression and subsequent antiviral immune responses (20). Induction of deubiquitinase ubiquitin-specific protease 25 (USP25) by SeV or VSV infection could promote type I IFN expression by mediating the stabilization of TRAF3 and TRAF6 (21). By these means, host immune systems could eliminate invading viruses quickly and effectively. In the present study, we found that both viral infection and IFN-β can greatly induce Mint3 expression in macrophages. Mint3 directly bound to TRAF3 and promoted K63-linked ubiquitination of TRAF3, resulting in enhancement of IFN-β production. Mint3-deficient mice showed much less IRF3 activation and IFN-β production. As a consequence, Mint3-deficient mice possessed impaired antiviral responses and increased virus replication.

TRAF3, as a key adaptor involved in the TLR- and RLR-induced antiviral innate immune responses, can activate TBK1 and initiate subsequent IFN-β expression (16, 17). In uninfected cells, TRAF3 resides in the ER-to-Golgi intermediate compartment and the cis-Golgi apparatus (15). Following viral infection, the Golgi apparatus fragmented into cytoplasmic punctated structures containing TRAF3, allowing its colocalization and interaction with other adaptors, such as MAVS (15). Thus, retention of TRAF3 at the ER-to-Golgi vesicular transport system blunted the ability of TRAF3 to interact with MAVS upon viral infection and consequently decreased type I IFN secretion (15). Mint3 associates with several membrane proteins in Golgi and regulate their traffic from the trans-Golgi network (10–12). We found that Mint3 deficiency dramatically changed the TRAF3 distribution from a speckle pattern to a diffuse pattern. Furthermore, Mint3 deficiency also markedly inhibited ubiquitination of TRAF3, which was critical for the activation of TRAF3 and downstream signal transduction. Thus, our results revealed an unpredicted role of Mint3 in the optimal activation of TRAF3 and the antiviral responses.

In conclusion, we identified Mint3 as a critical positive regulator of TLR3/4- and RIG-I–induced IFN-β production. Viral infection and IFN-β secretion induced Mint3 expression in the virus-infected cells, and Mint3 then feedback-facilitated host antiviral innate immune responses. The identification of a viral infection-induced physiological enhancer of antiviral immune responses will be valuable for understanding the crosstalk between virus and host immune systems. Furthermore, excessive IFN-β production has manifested in diverse pathogenic autoimmune diseases (22). Therefore, control of TRAF3 function via suppressing Mint3 will be a target for designing therapeutic strategies against autoimmune disorders.

Materials and Methods

Mice. Mint3$^{-/-}$ mice were established with the RIKEN Laboratory for Animal Resources and Genetic Engineering as described (14). C57BL/6 mice were obtained from the Vital River Laboratory Animal Technology Co. All animal experiments were undertaken in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals with the approval of the Scientific Investigation Board of Shandong University School of Medicine, Jinan, Shandong Province, China.

Cells. Mouse primary peritoneal macrophages were prepared as described (23, 24). Wild-type (Mint3$^{+/+}$) and Mint3-deficient (Mint3$^{-/-}$) MEFs were established and cultured as described previously (25). Mouse macrophage cell line RAW264.7 and HEK293T and human THP-1 cells were obtained from the American Type Culture Collection. The cells were cultured in endotoxin-free DMEM with 10% (vol/vol) FBS (Invitrogen-Gibco).

Viral Pathogenesis in Mice. C57BL/6J mice (female, 8 wk old) were intravenously infected with VSV (5×10^7 pfu per mouse) as described (24). Lungs from control or virus-infected mice were dissected, fixed in 10% (vol/vol) phosphate-buffered formalin, embedded into paraffin, sectioned, stained with H&E solution, and examined by light microscopy for histologic changes.

Statistical Analysis. Analysis was performed using a Student's *t* test or analysis of variance for paired samples. Animal survival was analyzed using the Kaplan–Meir analysis, and the survival rates were analyzed by Wilcoxon's test. Statistical significance was determined as $P < 0.05$.

Other Materials and Methods. The information for reagents, transfection, ELISA, RNA quantitation, immunoprecipitation, Western blot, confocal analysis, and luciferase assay used in this study are described in *SI Materials and Methods*.

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