# TRAF-interacting protein (TRIP) negatively regulates IFN-β production and antiviral response by promoting proteasomal degradation of TANK-binding kinase 1

Meng Zhang,[1] Lijuan Wang,[1] Xueying Zhao,[1] Kai Zhao,[1] Hong Meng,[2] Wei Zhao,[1] and Chengjiang Gao[1]

[1]Key Laboratory for Experimental Teratology of the Ministry of Education and Department of Immunology, Shandong University School of Medicine, Shandong 250012, China
[2]Institute of Basic Medicine, Shandong Academy of Medical Sciences, Jinan, Shandong 250012, China

**TANK-binding kinase 1 (TBK1) plays an essential role in Toll-like receptor (TLR)– and retinoic acid–inducible gene I (RIG-I)–mediated induction of type I interferon (IFN; IFN-α/β) and host antiviral responses. How TBK1 activity is negatively regulated remains largely unknown. We report that TNF receptor-associated factor (TRAF)–interacting protein (TRIP) promotes proteasomal degradation of TBK1 and inhibits TLR3/4– and RIG-I–induced IFN-β signaling. TRIP knockdown resulted in augmented activation of IFN regulatory factor 3 (IRF3) and enhanced expression of IFN-β in TLR3/4– and RIG-I–activated primary peritoneal macrophages, whereas overexpression of TRIP had opposite effects. Consistently, TRIP impaired Sendai virus (SeV) infection–induced IRF3 activation and IFN-β production and promoted vesicular stomatitis virus (VSV) replication. As an E3 ubiquitin ligase, TRIP negatively regulated the cellular levels of TBK1 by directly binding to and promoting K48-linked polyubiquitination of TBK1. Therefore, we identified TRIP as a negative regulator in TLR3/4– and RIG-I–triggered antiviral responses and suggested TRIP as a potential target for the intervention of diseases with uncontrolled IFN-β production.**

CORRESPONDENCE
Wei Zhao: wzhao@sdu.edu.cn
OR Chengjiang Gao: cgao@sdu.edu.cn

Abbreviations used: IRF3, IFN regulatory factor 3; MAVS, mitochondrial antiviral signaling protein; MBP, myelin basic protein; MDA5, melanoma differentiation-associated gene 5; poly(I:C), polyinosinic:poly-cytidylic acid; RIG-I, retinoic acid–inducible gene I; SeV, Sendai virus; siRNA, small interference RNA; TBK1, TANK-binding kinase 1; TLR, Toll-like receptor; TRAF, TNF receptor-associated factor; TRIF, TIR domain-containing adaptor inducing IFN-β; TRIP, TRAF-interacting protein; VSV, vesicular stomatitis virus.

Host cells express various pattern recognition receptors (PRRs), including Toll-like receptors (TLRs) and retinoic acid–inducible gene I (RIG-I)–like helicases (RLRs), which sense invading pathogens (Akira et al., 2006; O'Neill, 2008). Once recognition of microbial components, such as LPS, polyinosinic:polycytidylic acid (poly(I:C)), and viral RNA, TLRs, and RLRs, are activated, they initiate a series of signaling events leading to production of type I IFN (IFN-α/β) and proinflammatory cytokines (Akira et al., 2006; O'Neill, 2008).

TLRs recruit a set of intracellular TIR domain–containing adaptors, including myeloid differentiation factor 88 (MyD88) and TIR domain–containing adapter inducing IFN-β (TRIF; Medzhitov, 2001; Beutler et al., 2006; Kawai and Akira, 2010). MyD88 is a universal adaptor that activates inflammatory pathways; it is shared by all TLRs with the exception of TLR3. Recruitment of MyD88 leads to the phosphorylation of IRAKs (IL-1R–associated

kinases) and subsequent activation of TNF receptor-associated factor (TRAF) 6, allowing for the activation of NF-κB and mitogen-activated protein kinase (MAPK) pathways and the induction of proinflammatory cytokines (Medzhitov, 2001; Beutler et al., 2006; Kawai and Akira, 2010). TRIF is recruited to TLR3 and TLR4 and activates an alternative pathway (TRIF-dependent pathway). TRIF associates with TANK-binding kinase 1 (TBK1) and activates downstream IFN regulatory factor 3 (IRF3), mediating TLR3- and TLR4-activated type I IFN production (Medzhitov, 2001; Beutler et al., 2006; Kawai and Akira, 2010). RLRs comprise three cytoplasmic DExD–H-box RNA helicases, RIG-I, melanoma differentiation-associated gene 5 (MDA5), and laboratory of genetics and

The Rockefeller University Press    $30.00
J. Exp. Med. 2012 Vol. 209 No. 10    1703-1711
www.jem.org/cgi/doi/10.1084/jem.20120024

1703
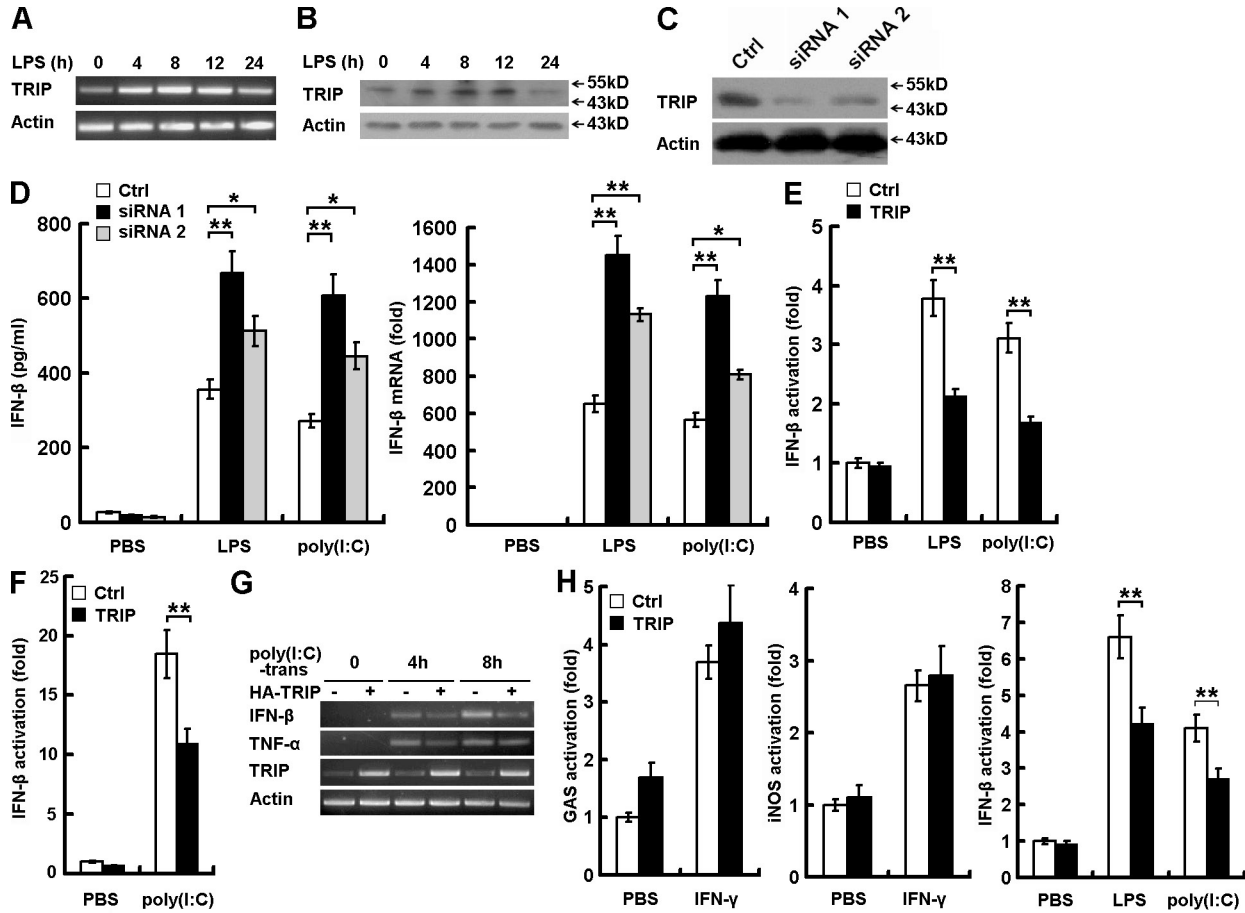
The Journal of Experimental Medicine

**Figure 1.   TRIP negatively regulates TLR3/4– and RIG–I–induced IFN–β production.** (A and B) RT-PCR and Western blot analysis of TRIP expression in mouse peritoneal macrophages stimulated with LPS for indicated time periods. (C) Western blot analysis of TRIP expression in mouse peritoneal macrophages transfected with control siRNA or TRIP siRNA 1 or 2 for 36 h. (D) Mouse peritoneal macrophages were treated as in C and then stimulated with LPS or poly(I:C) for 6 h. ELISA analysis of IFN–β in the supernatants and quantitative RT-PCR analysis of IFN–β mRNA in peritoneal macrophages. (E) Mouse peritoneal macrophages were transfected with IFN–β reporter plasmid together with TRIP expression plasmid or control plasmid, and luciferase activity was analyzed after treatment with LPS or poly(I:C) for 6 h. (F) HEK293-TLR3 cells were treated as in E. (G) HEK293 cells treated with the indicated plasmids were transfected with poly(I:C) for the indicated time periods. IFN–β, TNF, and TRIP mRNA expression were analyzed by RT-PCR. (H) RAW264.7 cells were transfected with iNOS, GAS, or IFN–β reporter plasmid together with TRIP expression plasmid or control plasmid, and luciferase activity was analyzed after treatment as indicated for 6 h. **, P < 0.01; *, P < 0.05. Data are representative of three experiments (mean and SD of six samples in D, E, F, and I). Similar results were obtained in at least three independent experiments in A, B, C, and G.

physiology 2 (LGP2; Takeuchi and Akira, 2009; Yoneyama and Fujita, 2009). The helicases RIG-I and MDA5 have been found to 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 (Yoneyama and Fujita, 2009; Takeuchi and Akira, 2009).

Although full activation of TLR and RIG-I signaling and secretion of type I IFNs are important for the elimination of invading microorganisms, inappropriate production of IFN–β and proinflammatory cytokines might promote the development of immunopathological conditions (Banchereau and Pascual, 2006; González-Navajas, et al., 2012). TBK1 is essential for both TLR and RLR signaling and is required for activation of IRF3 and subsequent induction of IFN–β (Fitzgerald et al., 2003; Takeuchi and Akira, 2009). The active state of

TBK1 is tightly regulated by posttranslational modifications such as phosphorylation and ubiquitination. GSK-3β promoted TBK1 dimerization and autophosphorylation in virus-triggered type I IFN induction and cellular antiviral response (Lei et al., 2010). Nrdp1 mediates K63-linked polyubiquitination and promotes TBK1 kinase activity (Wang et al., 2009). The mindbomb E3 ligases (MIB1 and MIB2) have been proposed to activate TBK1 by catalyzing its K63-linked polyubiquitylation (Li et al., 2011). During the revision of the manuscript, Cui et al. (2012) reported that NLRP4 could recruit the E3 ubiquitin ligase DTX4 to TBK1 for Lys48 (K48)–linked polyubiquitination, which led to degradation of TBK1. However, how TBK1 activity is negatively regulated remains largely unknown.

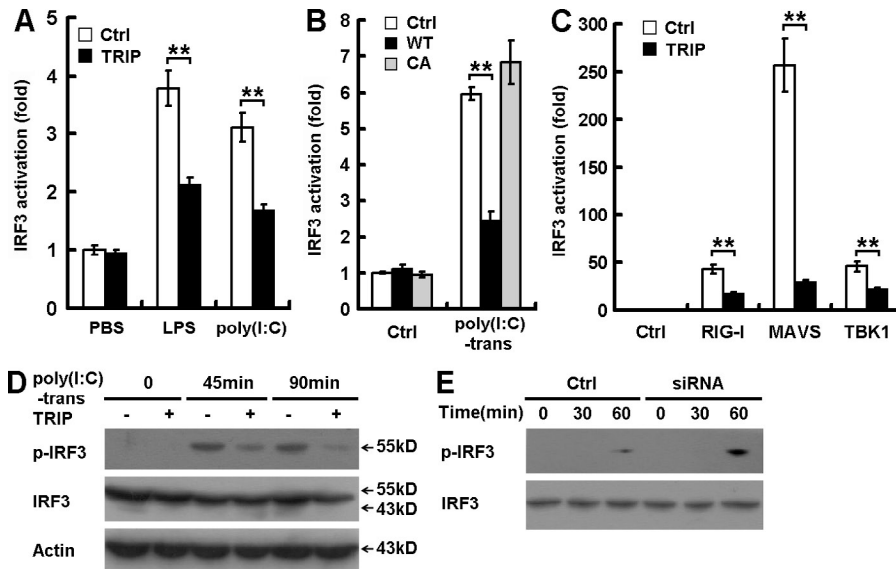TRAF-interacting protein (TRIP, or TRAIP) was initially identified as a TRAF1- and 2-interacting protein that

**Figure 2. TRIP inhibits IRF3 activation.** (A) Mouse peritoneal macrophages were transfected with IRF3 reporter plasmid together with TRIP expression plasmid or control plasmid, and luciferase activity was analyzed after treatment with LPS or poly(I:C) for 6 h. (B) HEK293 cells were transfected with IRF3 reporter plasmid together with control plasmid (Ctrl), TRIP WT plasmid, or TRIP point mutant plasmid (CA), and lucifer-ase activity was analyzed after transfection with poly(I:C) for 6 h. (C) HEK293 cells were transfected with RIG-I, MAVS, and TBK1, along with IRF3 reporter plasmid and TRIP plasmid, and luciferase activity was analyzed. (D) Western blot analysis of phosphorylated IRF3 and total IRF3 in HEK293 cells trans-fected with TRIP expression plasmid or control empty vector followed by transfected with poly(I:C). (E) Western blot analysis of phos-phorylated IRF3 and total IRF3 in mouse peri-toneal macrophages transfected with control siRNA (Ctrl) or TRIP siRNA (siRNA) and stimu-lated with poly(I:C). **, $P < 0.01$. Data are rep-resentative of three experiments (mean and SD of six samples in A, B, and C). Similar results were obtained in at least three independent experiments in D and E.

functions to inhibit NF-κB activation (Lee et al., 1997). TRIP contains an N-terminal RING finger and coiled-coil and leucine zipper regions that bind TRAF-family proteins (Besse et al., 2007) and a C-terminal region reported to interact with CYLD that facilitate the inhibition of TNF-mediated NF-κB activation (Regamey et al., 2003). TRIP interacts with the protein tyrosine kinase Syk and sensitizes cells to TNF-induced apoptosis (Zhou and Geahlen, 2009). TRIP plays important roles in the regulation of cell cycle progression and keratinocyte proliferation (Almeida et al., 2011). In ad-dition, TRIP-deficient mouse embryos died shortly after im-plantation as a result of proliferation defects and excessive cell death (Park et al., 2007). TRIP possesses RING-dependent Ub ligase activity (Besse et al., 2007), but its substrate and physiological functions remain largely unclear.

In the present study, we identified TRIP as a new regula-tor in TLR3/4- and RIG-I–mediated IFN-β signaling and antiviral responses by mediating ubiquitination and degrada-tion of TBK1 through the N-terminal RING domain. The identification of a physiological suppressor of TBK1 provides an explanation on the inability of the kinase to activate the IFN pathways under physiological conditions.

## RESULTS AND DISCUSSION
### TRIP expression is induced by TLR stimulation in macrophages
To determine the potential role of TRIP in TLR signaling, we analyzed its expression upon stimulation with TLR ago-nists. TRIP mRNA (Fig. 1 A) and protein (Fig. 1 B) expression were both significantly increased in mouse peritoneal macro-phages stimulated with LPS (TLR4 ligand). TRIP expression was up-regulated after 4 h of stimulation and reached the peak level of expression at 8 h. After 24 h, TRIP expression declined to the basal level as in untreated cells. TRIP expres-sion was also up-regulated in mouse peritoneal macrophages stimulated with poly(I:C) (TLR3 ligand) or infected with Sendai virus (SeV; unpublished data). Collectively, these data

indicate that TRIP was up-regulated after TLR activa-tion and SeV infec-tion in macrophages, raising a possibility that TRIP may be involved in this signaling transduction.

A previous study identified the localization of over-expressed TRIP as predominantly perinuclear in COS-7 cells (Regamey et al., 2003). Another study identified that endog-enous TRIP and exogenously overexpressed TRIP predom-inantly localize in the nucleus in MCF7 cells and a small portion of TRIP localizes in punctate cytoplasmic structures (Zhou and Geahlen, 2009). To investigate the subcellular locali-zation of TRIP, GFP-TRIP was transfected into HeLa cells, followed by immunofluorescence staining and microscopic analysis. We found that TRIP was presented by small speck-led structures diffused throughout the cell and that its ex-pression pattern remains unchanged after TLR4 activation (unpublished data). Collectively, these data indicate that TRIP diffused in the whole cytoplasm and nucleus.

### TRIP negatively regulates TLR3/4– and RIG–I–induced IFN–β production
To investigate whether TRIP plays a role in TLR signaling, we initially examined the effects of TRIP on LPS– and poly(I:C)–induced IFN-β production in macrophages. Two synthesized interfering RNAs targeting mouse TRIP were used to suppress endogenous TRIP expression. TRIP expression was signifi-cantly decreased with transfection of TRIP specific small interference RNAs (siRNAs; Fig. 1 C). TRIP knockdown sub-stantially increased LPS– and poly(I:C)–induced IFN-β pro-duction in mouse peritoneal macrophages at both mRNA and protein levels (Fig. 1 D). TRIP siRNA 1, which has a higher efficiency to knockdown TRIP expression (Fig.1C), has a greater potential to increase the TLR3/4-induced IFN-β
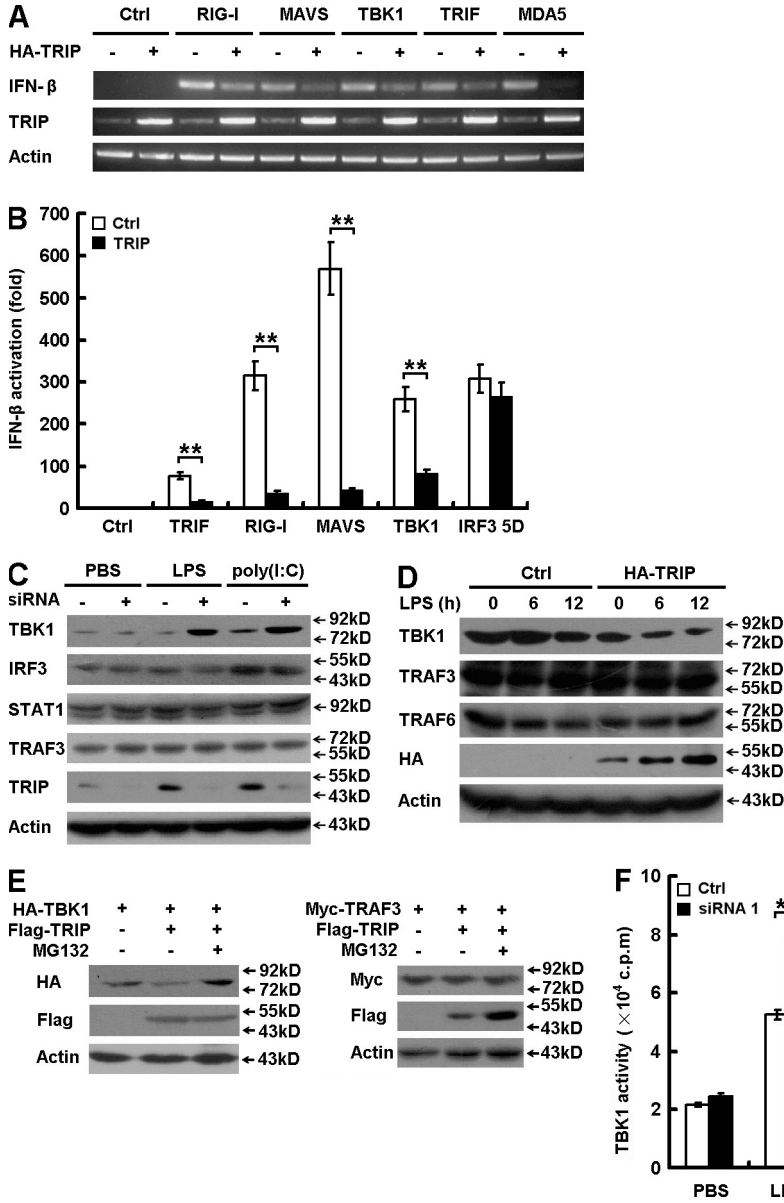
**Figure 3. TRIP Targets TBK1 for degradation.**
(A) HEK293 cells were transfected with RIG-I, MAVS, TBK1, TRIF, and MDA5 along with TRIP plasmid, and IFN-β mRNA was analyzed by RT-PCR. (B) HEK293 cells were transfected with TRIF, RIG-I, MAVS, TBK1, and IRF3 5D along with IFN-β reporter plasmid and TRIP plasmid, and luciferase activity was analyzed. (C) Western blot analysis of the expression of TBK1, IRF3, STAT1, and TRAF3 in peritoneal macrophages transfected with control siRNA or TRIP siRNA and then stimulated with LPS or poly (I:C) for 6 h. (D) Western blot analysis of the expression of TBK1, TRAF3, and TRAF6 in RAW264.7 cells stably transfected with control or HA-TRIP plasmid and then stimulated with LPS for indicated time periods. (E) Western blot analysis of TBK1 or TRAF3 expression in HEK293 cells cotransfected with HA-TBK1 or Myc-TRAF3 and Flag-TRIP or vector control and then treated with proteasome inhibitor MG-132 for 4 h. (F) In vitro kinase assays of TBK1 in the lysates of peritoneal macrophages transfected with control siRNA or TRIP siRNA 1 and then stimulated with LPS for 30 min, assessed with the substrates MBP (for TBK1). **, P < 0.01. Data are representative of three experiments (mean and SD of six samples in B and F). Similar results were obtained in at least three independent experiments in A, C, D, and E.

transfected with poly(I:C) to activate RIG-I signaling. RIG-I–induced up-regulation of IFN-β and TNF was attenuated in TRIP-overexpression HEK293 cells (Fig. 1 G).

To investigate whether TRIP specifically inhibited TLR and RLR pathways, we observed the effect of TRIP on IFN-γ–induced activation of GAS and iNOS promoter reporter in RAW264.7 cells. TRIP could not inhibit IFN-γ–induced activation of GAS reporter and iNOS reporter (Fig. 1 H). As a parallel control, TRIP overexpression was significantly decreased LPS- and poly(I:C)-induced IFN-β reporter activation in RAW264.7 cells (Fig. 1 H). Collectively, these data indicate that TRIP specifically repress TLR3/4- and RIG-I–induced IFN-β signaling.

## TRIP inhibits IRF3 activation

IRF3 is the key transcription factor that mediates the expression of IFN-β in TLR3/4- and RIG-I–mediated signal transduction (Doyle et al., 2002; Yoneyama and Fujita, 2009). We then observed the effect of TRIP on IRF3 activation. TRIP significantly inhibited LPS- and poly(I:C)-induced IRF3 reporter activation in mouse peritoneal macrophages (Fig. 2 A). To address the function of TRIP in RIG-I–induced IRF3 activation, HEK293 cells were transfected with poly(I:C) to activated RIG-I signaling. TRIP significantly attenuated RIG-I–induced IRF3 reporter activation (Fig. 2 B). TRIP also significantly inhibited RIG-I–, MAVS-, and TBK1-dependent IRF3 activation (Fig. 2 C). Furthermore, TRIP overexpression inhibited

production (Fig. 1 D). Therefore, TRIP siRNA 1 was used in the following experiments. To further confirm the negatively regulatory role of TRIP in TLR3/4-mediated IFN-β production, the effect of TRIP on LPS- and poly(I:C)-induced IFN-β luciferase reporter gene expression was analyzed by dual-luciferase reporter assay. TRIP overexpression significantly decreased LPS- and poly(I:C)-induced IFN-β reporter activation in mouse peritoneal macrophages (Fig. 1 E). Consistently, TRIP overexpression also significantly decreased poly(I:C)-induced IFN-β reporter activation in HEK293-TLR3 cells (Fig. 1 F).

RIG-I is a key PRR for the detection of RNA virus (such as SeV and vesicular stomatitis virus [VSV]) and poly(I:C) (Yoneyama and Fujita, 2009; Takeuchi and Akira, 2009). To address the physiological function of TRIP in RIG-I–induced IFN-β production, HEK293 cells were
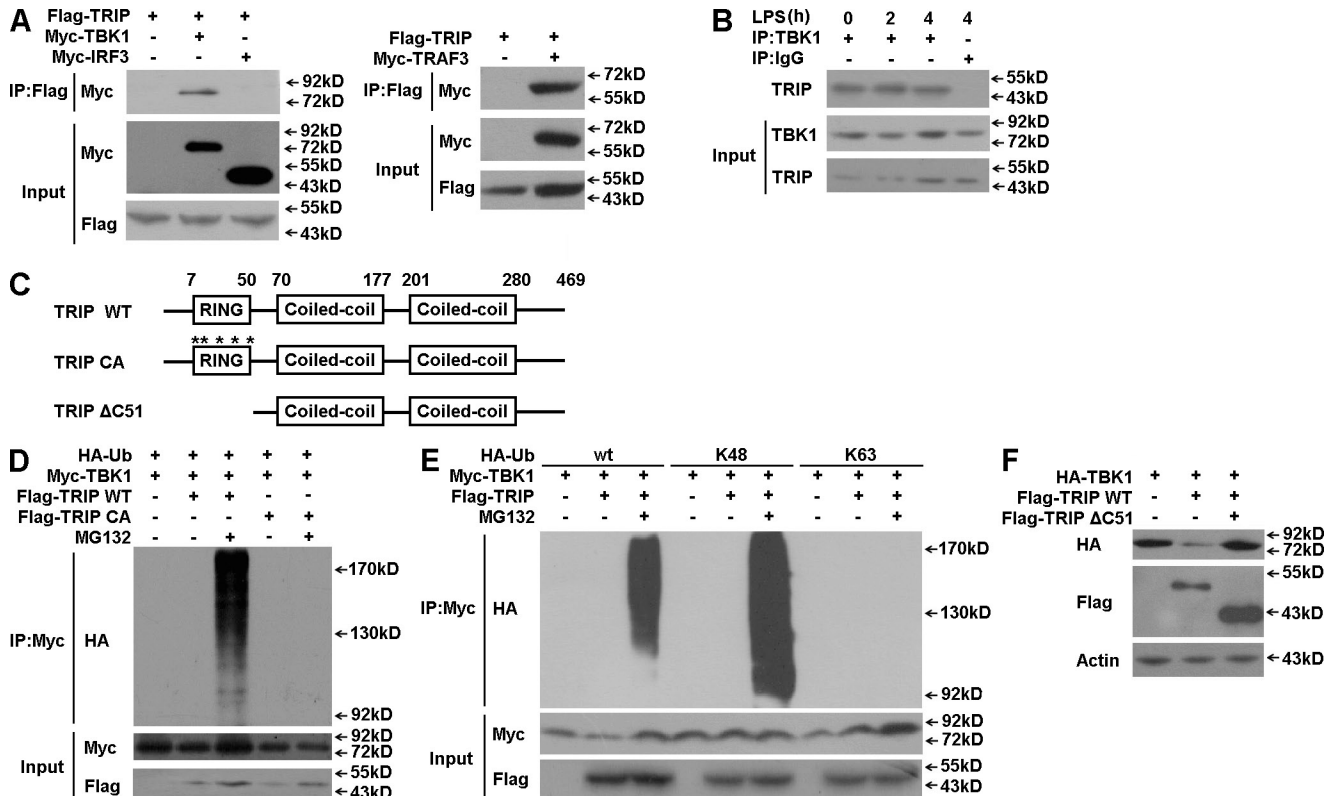
**Figure 4.  TRIP promotes K48–linked ubiquitination and proteasomal degradation of TBK1.** (A) Lysates from HEK293 cells transiently cotransfected with Flag-TRIP and Myc-TBK1, Myc-IRF3, or Myc-TRAF3 expression plasmids were subjected to immunoprecipitation with anti-Flag antibody followed by Western blot analysis with anti-Myc antibody. (B) Lysates from mouse peritoneal macrophages stimulated with LPS for indicated time periods were subjected to immuno-precipitation with anti-TBK1 or control IgG followed by Western blot analysis with anti-TRIP antibody. Proteins in whole-cell lysate were used as positive control (Input). (C) Schematic diagram of TRIP WT and mutant constructs. TRIP WT contains a RING domain, two coiled-coil domains, and a C-terminal CYLD interacting domain. In mutant CA, the cysteine residues at positions 7, 10, 25, 33, and 46 within the RING domain were substituted with alanine. In mutant ΔC51, the RING domain was simply deleted. (D) Lysates from HEK293 cells transiently cotransfected with Myc-TBK1, Flag-TRIP WT, or TRIP CA, and HA-Ub plasmids were subjected to immunoprecipitation with anti-Myc antibody followed by Western blot analysis with anti-HA antibody. (E) Lysates from HEK293 cells transiently cotransfected with Myc-TBK1, Flag-TRIP, or vector control, and HA-Ub (WT), HA-Ub (K48), or HA-Ub (K63) plasmids were subjected to immunoprecipitation with anti-Myc antibody followed by Western blot analysis with anti-HA antibody. (F) Western blot analysis of TBK1 expression in HEK293 cells cotransfected with HA-TBK1 and Flag-TRIP, Flag-TRIP ΔC51, or vector control. Similar results were obtained in three independent experiments in A, B, D, E, and F.

RIG-I–induced phosphorylation of IRF3 in HEK293 cells (Fig. 2 D). To investigate the physiological function of TRIP in IRF3 activation, mouse peritoneal macrophages were transfected with TRIP siRNA 1. TRIP knockdown substantially enhanced poly(I:C)–induced phosphorylation of IRF3 (Fig. 2 E). All together, these data suggest that TRIP negatively regulates TLR- and RIG-I–induced IRF3 activation.

## TRIP targets TBK1 for degradation

TLR3/4 and RIG-I recognize the microbial patterns and recruit adaptors TRIF and MAVS, respectively (Yoneyama and Fujita, 2009; Kawai and Akira, 2010). TRIF and MAVS mediate IRF3 activation and IFN-β production through a complex cascade composed of various molecules including TRIF, MAVS, TBK1, and IRF3 (Yoneyama and Fujita, 2009; Kawai and Akira, 2010). To determine the molecular targets of TRIP in TLR- and RIG-I–induced IFN-β signaling, the effects of TRIP on the IFN-β expression mediated by various adaptors

were examined. TRIP inhibited RIG-I–, MAVS–, TBK1–, TRIF–, and MDA5–induced IFN-β mRNA expression (Fig. 3 A). Consistently, TRIF–, RIG-I–, MAVS–, and TBK1–induced IFN-β promoter activation were also significantly inhibited by TRIP overexpression (Fig. 3 B). Previous investigations showed that WT IRF-3 induces marginal levels of type I IFN (Lin et al., 1999). In contrast, the IRF-3 5D mutant, in which residues at positions 396, 398, 402, 404, and 405 were replaced by the phosphomimetic aspartate amino acid, induces strong activation of the IFN-β promoter (Lin et al., 1999). We examined the effects of TRIP on the IFN-β promoter activation mediated by IRF3 using IRF3 5D mutation. IRF3–induced IFN-β promoter activation remains unchanged by TRIP overexpression (Fig. 3 B). Therefore, we conclude that TRIP targets molecules upstream of IRF3.

To clarify TRIP target, the function of TRIP on the degradation of the molecules involved in TLR3/4 and RIG-I signaling was investigated. TRIP knockdown greatly increased TBK1

protein level in peritoneal macrophages (Fig. 3 C). IRF3, STAT1, and TRAF3 protein levels were not impaired. Consistently, TBK1 protein level in TRIP stable transfection RAW264.7 cells was greatly decreased (Fig. 3 D). TRAF3 and TRAF6 protein levels were not changed. Therefore, we speculate that TBK1 may be a TRIP target. To confirm, TRIP targets TBK1, HA-TBK1, and Flag-TRIP were cotransfected into HEK293 cells. TRIP promoted degradation of TBK1 (Fig. 3 E). As a control, TRIP overexpression had no effects on TRAF3 expression. To determine whether TRIP inhibits TBK1 activity, in vitro kinase assay of TBK1 was assessed with myelin basic protein (MBP) as substrate. As expected, LPS stimulation could increase TBK1 kinase activity; remarkably, TBK1 kinase activity was further increased in TRIP siRNA-transfected macrophages after LPS stimulation (Fig. 3 F). All together, these data indicate that TRIP targets TBK1 for degradation to inhibit TLR- and RIG-I–induced expression of IFN-β.

## TRIP promotes K48–linked ubiquitination and proteasomal degradation of TBK1

Protein ubiquitination is a crucial posttranscriptional modification to provide specificity and regulate the intensity of TLR signaling (Liu et al., 2005; Jiang and Chen, 2012). K48-linked protein ubiquitination leads to the degradation of the corresponding protein by 26S proteasome (Liu et al., 2005; Jiang and Chen, 2012). Ubiquitination requires the sequential actions of three enzymes: Ub–activating enzyme (E1), Ub–conjugating enzyme (E2), and Ub ligase (E3), and the E3 ligase dictates which target protein gets ubiquitinated (Liu et al., 2005; Jiang and Chen, 2012). Several proteins possessing E3 ubiquitin ligase activity have been implicated in regulation of TLR and RLR pathways, such as Nrdp1 (Wang et al., 2009), RAUL (Yu and Hayward, 2010), CHIP (Yang et al., 2011), and others. TRIP is a RING-dependent E3 ubiquitin ligase (Besse et al., 2007). Therefore, we speculated that TRIP promoted TBK1 degradation through the ubiquitin–proteasome pathway.

Physical binding to target proteins is a necessary step for E3 ligase to initiate ubiquitination. First, we investigated whether TRIP interacted with TBK1. Myc-TBK1, Myc-IRF3, or Myc-TRAF3 and Flag-TRIP were cotransfected into HEK293 cells, and immunoprecipitation experiments were performed with Flag antibody. TBK1 was coprecipitated with TRIP (Fig. 4 A). As a negative control, IRF3 was not coprecipitated with TRIP. As a positive control, TRAF3 was coprecipitated with TRIP, consistent with a previous study (Besse et al., 2007). Endogenous interaction was examined in mouse peritoneal macrophages stimulated with LPS for indicated time periods, followed by immunoprecipitation with TBK1 antibody. TBK1 interacted with TRIP in both LPS-stimulated and nonstimulated cells (Fig. 4 B). The interaction could not be detected with control IgG. Consistently, immunostaining experiments also demonstrated the colocalization between TBK1 and TRIP before and after LPS stimulation or SeV infection (unpublished data). Collectively, these results show that TRIP binds directly to TBK1.

To test the role of TRIP in TBK1 ubiquitination and degradation, we constructed various TRIP mutants (Fig. 4 C). In mutant CA, the cysteine residues at positions 7, 10, 25, 33, and 46 within the RING domain were substituted with alanine. ΔC51 lacks the RING domain. TRIP markedly increased polyubiquitination of TBK1 (Fig. 4 D, lane 3). In contrast, the TRIP CA lost the ability to promote polyubiquitination of TBK1 (Fig. 4 D, lane 5), indicating that the RING-finger domain is required for the TRIP-mediated ubiquitination of TBK1.

To investigate the forms of TRIP-mediated polyubiquitination of TBK1, ubiquitin mutant vectors K48 and K63, which contain arginine substitutions of all of its lysine residues except the one at position 48 and 63, respectively, were used in the transfection assays. TRIP-mediated polyubiquitination of TBK1 was significantly increased in the presence of both WT and K48 mutant ubiquitin plasmids (Fig. 4 E, lane 3 and 6), but not in the presence of K63 mutant ubiquitin plasmid, indicating that TRIP mediates K48-linked ubiquitination of TBK1. K48-linked polyubiquitination leads to the degradation of target proteins through 26S proteasome. Consistently, TRIP-induced degradation of TBK1 could be reversed by proteasome inhibitor MG-132 (Fig. 3 E). Furthermore, TRIP mutant ΔC51 lost the ability to promote degradation of TBK1 (Fig. 4 F). All together, these data demonstrate that TRIP interacts with TBK1 and mediates K48-linked ubiquitination and degradation through the E3 ligase activity.

## TRIP negatively regulates cellular antiviral response

Type I IFNs (IFN-α/β) play critical roles in the immune responses against viral infection. We further investigated the potential role of TRIP in cellular antiviral responses. TRIP inhibited IFN-β and RNATES expression in HEK293 cells infected with SeV (Fig. 5 A). Consistently, TRIP inhibited SeV-induced IFN-β and IRF3 promoter activation and TRIP CA mutant lost the inhibitory effects (Fig. 5 B). Importantly, SeV-induced IRF3 dimerization was significantly attenuated by transfection of TRIP expression plasmid in HEK293 cells (Fig. 5 C). Furthermore, SeV-induced IFN-β and RNATES expression was significantly enhanced by TRIP knockdown in peritoneal macrophages (Fig. 5 D). TBK1 protein level was greatly decreased in SeV-infected HEK293 cells with TRIP overexpression (Fig. 5 E). In contrast, IRF3, TRAF3, and STAT1 levels remain unchanged. Consistent with the observation of TRIP-mediated decrease of TBK1 expression, TRIP knockdown greatly increased TBK1 kinase activity after SeV infection (Fig. 5 F).

To directly investigate the effects of TRIP on antiviral responses, VSV, a kind of single-stranded RNA virus recognized by RIG-I, was used to infect HEK293 cells and macrophages. Plaque assay of HEK293 cells infected with VSV showed that TRIP overexpression substantially increased viral replication in the presence of poly(I:C) (Fig. 5 G). In sharp contrast, TRIP mutant CA could not increase viral replication (Fig. 5 G). Similarly, VSV RNA replicates were greatly increased in TRIP-transfected cells compared with control vector- or TRIP CA-transfected cells (Fig. 5 G). To further
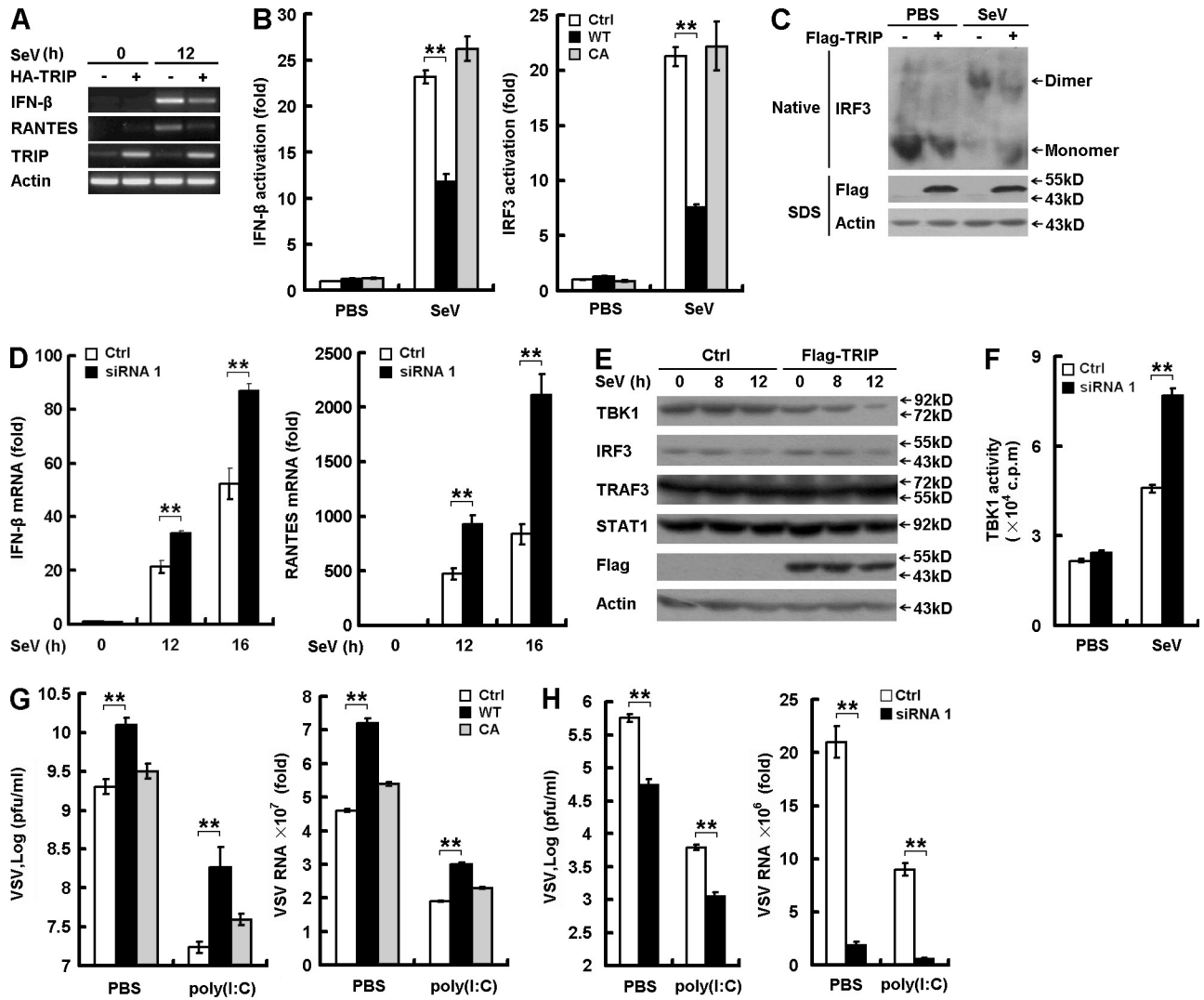
**Figure 5. TRIP negatively regulates cellular antiviral response.** (A) HEK293 cells transfected with the indicated plasmids were infected with SeV for 12 h. IFN-β, RANTES, and TRIP mRNA expressions were analyzed by RT-PCR. (B) HEK293 cells were transfected with IFN-β or IRF3 reporter together with TRIP or control plasmid, and luciferase activity was analyzed after infection with SeV for 12 h. (C) HEK293 cells were transfected with the indicated plasmids. 24 h after transfection, cells were infected with SeV or left uninfected for 6 h. Cell lysates were separated by native (top) or SDS (bottom two) PAGE and analyzed by immunoblotting with the indicated antibodies. (D) Mouse peritoneal macrophages were transfected with control siRNA or TRIP siRNA for 36 h. Quantitative RT-PCR analysis of IFN-β and RANTES mRNA in peritoneal macrophages infected with SeV for indicated time periods. (E) Western blot analysis of the expression of TBK1, IRF3, TRAF3, and STAT1 in HEK293 cells transfected with control or Flag-TRIP plasmid and then infected with SeV for indicated time periods. (F) In vitro kinase assays of TBK1 in the lysates of peritoneal macrophages transfected with control siRNA or TRIP siRNA 1 and then infected with SeV for 2 h, assessed with the substrates MBP (for TBK1). (G) HEK293 cells were transfected with the indicated plasmids. 24 h later, cells were further transfected with 0.1 µg poly(I:C). 18 h later, cells were infected with VSV (MOI, 0.1), and the supernatants were harvested at 12 h after infection. Supernatants were analyzed for VSV titers. Intracellular VSV RNA replicates were measured by quantitative RT-PCR. (H) Mouse peritoneal macrophages were transfected with control siRNA (Ctrl) or TRIP siRNA (siRNA). VSV titers and intracellular VSV RNA replicates were measured as in G. **, P < 0.01. Data are representative of three experiments (mean and SD of triplicate samples in B, D, F, G, and H). Similar results were obtained in three independent experiments in A, C, and E.

confirm the function of TRIP on VSV replication under physiological condition, TRIP expression was silenced by TRIP siRNA in mouse peritoneal macrophages, and then the macrophages were infected with VSV. TRIP knockdown greatly decreased VSV viral replication in macrophages in the presence or absence of poly(I:C) (Fig. 5 H). Accordingly, TRIP knockdown significantly decreased intracellular VSV

RNA replicates (Fig. 5 H). Collectively, these data demonstrate that TRIP negatively regulates cellular antiviral response by inhibiting IFN-β expression.

Given the important roles of IFN-β in host antiviral immunity, virus may escape immune surveillance by modulating TRIP expression in the infected cells, and then feedback may inhibit the innate immune response against itself. In contrast,

excessive IFN-β production has manifested in diverse pathogenic autoimmune diseases (Banchereau and Pascual, 2006; González-Navajas, et al., 2012). Our results demonstrate TBK1 as a substrate of TRIP, provide a strategy to downregulate IFN-β production, and they suggest that TRIP may have therapeutic potential for the intervention of diseases with uncontrolled IFN-β production.

## MATERIALS AND METHODS

**Mice, cells, and reagents.** C57BL/6J mice were obtained from Joint Ventures Sipper BK Experimental Animal. 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 Medical School of Shandong University. Mouse macrophage cell line RAW264.7, human HEK293 cells, and HeLa cells were obtained from American Type Culture Collection. HEK293-TLR3 cell lines were obtained from InvivoGen. Mouse primary peritoneal macrophages were prepared as previously described (Zhao et al., 2012). The cells were cultured at 37°C under 5% $CO_2$ in DME supplemented with 10% FCS (Invitrogen), 100 U/ml penicillin, and 100 µg/ml streptomycin. MG132 and LPS (*Escherichia coli*, 055:B5) were purchased from Sigma-Aldrich. Poly (I:C) was purchased from InvivoGen. IFN-γ was from PeproTech. LPS, poly(I:C), and IFN-γ were used at a final concentration of 100 ng/ml, 10 µg/ml, and 100 U/ml, respectively. For transfection, poly(I:C) was used at a final concentration of 1 µg/ml. The antibodies specific for IRF3, TRAF3, TRAF6, β-actin, HA, and protein G agarose used for immunoprecipitation were from Santa Cruz Biotechnology, Inc. The antibodies specific to Myc, TBK1, STAT1, and phospho-IRF3 were from Cell Signaling Technology. The antibody for Flag was from Sigma-Aldrich. The antibody for TRIP was from Abcam. Their respective horseradish peroxidase–conjugated secondary antibodies were purchased from Santa Cruz Biotechnology, Inc.

**Sequences, plasmid constructs, and transfection.** TRIP cDNA was amplified from RAW264.7 cells by PCR and cloned in pCMV6-Flag, pCMV-HA, and pEGFP plasmids (Promega). The primers used were 5′-CCAA-GCTTATGCCTATCCGCGCTCTGTG-3′ and 5′-GGTCTAGAT-TACTGACATAAGAAGGTAT-3′. The TRIP CA, TRIP ΔC51, and IRF3 5D mutations were generated using the KOD-Plus-Mutagenesis kit (Toyobo). In mutant CA, the cysteine residues at positions 7, 10, 25, 33, and 46 within the RING domain were substituted with alanine. ΔC51 lacks the RING domain. All constructs were confirmed by DNA sequencing. IFN-β and IRF3 reporter plasmids and TBK1 and TRIF plasmids were gifts from X. Cao (Second Military Medical University, Shanghai, China). The IRF3 reporter plasmids are composed of two plasmids, p-55UASGLuc and p-EFGAL4/IRF3, as previously described (Zhao et al., 2011). Expression vectors for HA-Ub WT and mutant K48, K63 were from H. Xiao (Institute Pasteur of Shanghai, Chinese Academy of Sciences, China). Expression plasmids for RIG-I, MDA5, and MAVS were provided by Z. Jiang (Peking University, Beijing, China). TRAF3 expression plasmid was a gift from M. Karin (University of California, San Diego, San Diego, CA). GAS and iNOS reporter plasmids were purchased from Stratagene. For transient transfection of plasmids into RAW264.7 cells, jetPEI reagents were used (Polyplus-transfection). For transient silencing, duplexes of siRNA were transfected into peritoneal macrophages with the GenePORTER 2 Transfection Reagent (Genlantis) according to the standard protocol. Target sequences for transient silencing were 5′-GCGGCAGGAUGAGACCAAA-3′ (siRNA 1) and 5′-GGCC-AAGAGUAAACAGAAA-3′ (siRNA 2) for TRIP, and scrambled control sequences were 5′-UUCUCCGAACGUGUCACGU-3′. For stable selection of cell lines overexpressing TRIP, transfected RAW264.7 macrophages were selected with 800 µg/ml G418 and were pooled for further experiments.

**ELISA and RT-PCR.** The concentrations of IFN-β in culture supernatants were measured by ELISA kits (R&D Systems). Total RNA was extracted with TRIzol reagent according to the manufacturer's instructions (Invitrogen). A LightCycler (ABI PRISM 7000; Applied Biosciences) and a SYBR RT-PCR kit (Takara Bio, Inc.) were used for quantitative RT-PCR analysis. Specific primers used for RT-PCR assays were 5′-CAA-CAAGTGTCTCCTCCAAAT-3′ and 5′-TCTCCTCAGGGATGTCA-AAG-3′ for h-IFN-β, 5′-TGAAGAGGACCTGGGAGTAGATGA-3′ and 5′-CAGGCAGTCAGATCATCTTCTCGA-3′ for h-TNF, 5′-ATCT-GCCTCCCCATATTCCTCGGA-3′ and 5′-TTCGGGTGACAAAGAC-GACTGCTG-3′ for h-RANTES, 5′-AGTGCCGAATCCAGGTTG-3′ and 5′-GCGTATCCCGCAGAGTGT-3′ for h-TRIP, 5′-GGAAATC-GTGCGTGACATTAA-3′ and 5′-AGGAAGGAAGGCTGGAAGAG-3′ for h-actin, 5′-ATGAGTGGTGGTTGCAGGC-3′ and 5′-TGACCT-TTCAAATGCAGTAGATTCA-3′ for m-IFN-β, 5′-CCTGCTGCTTT-GCCTACCTCT-3′ and 5′-GTATTCTTGAACCCACTTCTTCTCTG-3′ for m-RANTES, and 5′-TGTTACCAACTGGGACGACA-3′ and 5′-CTG-GGTCATCTTTTCACGGT-3′ for m-actin.

**Immunoprecipitation and Western blot analysis.** For immunoprecipitation, whole-cell extracts were collected 36 h after transfection and were lysed in immunoprecipitation buffer containing 1.0% (vol/vol) Nonidet P 40, 50 mM Tris-HCl, pH 7.4, 50 mM EDTA, 150 mM NaCl, and a protease inhibitor cocktail (Merck). After centrifugation for 10 min at 14,000 *g*, supernatants were collected and incubated with protein G Plus–Agarose Immunoprecipitation reagent (Santa Cruz Biotechnology, Inc.) together with 1 µg monoclonal anti-Flag or 1 µg anti-Myc. After 6 h of incubation, beads were washed five times with immunoprecipitation buffer. Immunoprecipitates were eluted by boiling with 1% (wt/vol) 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 (Zhao et al., 2012).

**Native PAGE.** The IRF3 dimerization assay was performed as described previously with a modification (Mori et al., 2004). In brief, HEK293 cells were harvested with 30 ml of ice-cold lysis buffer (50 mM Tris/HCl, pH 7.5, 150 mM NaCl, and 0.5% NP-40 containing protease inhibitor cocktail). After centrifugation at 13,000 *g* for 10 min, supernatants were quantified using a BCA assay (Thermo Fisher Scientific) and diluted with 2× native PAGE sample buffer (125 mM Tris/HCl, pH 6.8, 30% glycerol, and 0.1% Bromophenol blue), and then 20 µg of total protein was applied to a prerun 7.5% native gel for separation. After electrophoresis, the proteins were transferred onto a nitrocellulose membrane for immunoblotting.

**Assay of luciferase activity.** Luciferase activity was measured with the Dual-Luciferase Reporter Assay system according to the manufacturer's instructions (Promega) as described previously (Zhao et al., 2011). Data were normalized for transfection efficiency by division of firefly luciferase activity with that of Renilla luciferase.

**In vitro kinase assay.** Protein in total cell extracts was immunoprecipitated with anti-TBK1 antibody 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 with lysis buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% vol/vol Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM b-glycerophosphate, 1 mM $Na_3VO_4$, and 1 mM phenylmethylsulfonyl fluoride). Each washed pellet was resuspended for 30 min at 30°C in kinase assay buffer (25 mM Tris-HCl, pH 7.5, 5 mM b-glycerophosphate, 2 mM dithiothreitol, 0.1 mM $Na_3VO_4$, and 10 mM $MgCl_2$). TBK1 kinase activity was assayed with ADP-Glo and TBK1 Kinase Enzyme System according to the manufacturer's instructions (Promega).

**Ubiquitination assays.** For analysis of the ubiquitination of TBK1, HEK293 cells were transfected with Myc-TBK1, HA-Ub (WT), or HA-Ub mutants and Flag-TRIP WT or Flag-TRIP CA, and then whole-cell extracts were immunoprecipitated with anti-Myc and analyzed by immunoblot with anti-HA antibody.

**VSV plaque assay and detection of virus replication.** VSV plaque assay was performed as previously described (Zhao et al., 2012). The HEK293 cells ($2 \times 10^5$) were transfected with the indicated plasmids for 36 h before VSV infection (MOI of 0.1). At 1 h after infection, cells were washed with PBS three times and then medium was added. The supernatants were harvested 24 h after washing. The supernatants were diluted $1:10^6$ and then used to infect confluent HEK293 cells cultured on 24-well plates. At 1 h after infection, the supernatant was removed, and 3% methylcellulose was overlaid. At 3 d after infection, 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 RNA was extracted and VSV RNA replicates were examined by quantitative RT-PCR as previously described (Zhao et al., 2012). Primers for VSV were as follows: 5′-ACGGCGTACTTC-CAGATGG-3′ (sense) and 5′-CTCGGTTCAAGATCCAGGT-3′ (antisense).

**Statistical analysis.** All data are presented as mean ± SD of three or four experiments. Statistical significance was determined with the two-tailed Student's *t* test, with a P value of <0.05 considered statistically significant.

## REFERENCES

Akira, S., S. Uematsu, and O. Takeuchi. 2006. Pathogen recognition and innate immunity. *Cell.* 124:783–801. http://dx.doi.org/10.1016/j.cell.2006.02.015

Almeida, S., S. Ryser, M. Obarzanek-Fojt, D. Hohl, and M. Huber. 2011. The TRAF-interacting protein (TRIP) is a regulator of keratinocyte proliferation. *J. Invest. Dermatol.* 131:349–357. http://dx.doi.org/10.1038/jid.2010.329

Banchereau, J., and V. Pascual. 2006. Type I interferon in systemic lupus erythematosus and other autoimmune diseases. *Immunity.* 25:383–392. http://dx.doi.org/10.1016/j.immuni.2006.08.010

Besse, A., A.D. Campos, W.K. Webster, and B.G. Darnay. 2007. TRAF-interacting protein (TRIP) is a RING-dependent ubiquitin ligase. *Biochem. Biophys. Res. Commun.* 359:660–664. http://dx.doi.org/10.1016/j.bbrc.2007.05.149

Beutler, B., Z. Jiang, P. Georgel, K. Crozat, B. Croker, S. Rutschmann, X. Du, and K. Hoebe. 2006. Genetic analysis of host resistance: Toll-like receptor signaling and immunity at large. *Annu. Rev. Immunol.* 24:353–389. http://dx.doi.org/10.1146/annurev.immunol.24.021605.090552

Cui, J., Y. Li, L. Zhu, D. Liu, Z. Songyang, H.Y. Wang, and R.F. Wang. 2012. NLRP4 negatively regulates type I interferon signaling by targeting the kinase TBK1 for degradation via the ubiquitin ligase DTX4. *Nat. Immunol.* 13:387–395. http://dx.doi.org/10.1038/ni.2239

Doyle, S., S. Vaidya, R. O'Connell, H. Dadgostar, P. Dempsey, T. Wu, G. Rao, R. Sun, M. Haberland, R. Modlin, and G. Cheng. 2002. IRF3 mediates a TLR3/TLR4-specific antiviral gene program. *Immunity.* 17:251–263. http://dx.doi.org/10.1016/S1074-7613(02)00390-4

Fitzgerald, K.A., S.M. McWhirter, K.L. Faia, D.C. Rowe, E. Latz, D.T. Golenbock, A.J. Coyle, S.M. Liao, and T. Maniatis. 2003. IKKepsilon and TBK1 are essential components of the IRF3 signaling pathway. *Nat. Immunol.* 4:491–496. http://dx.doi.org/10.1038/ni921

González-Navajas, J.M., J. Lee, M. David, and E. Raz. 2012. Immunomodulatory functions of type I interferons. *Nat. Rev. Immunol.* 12:125–135.

Jiang, X., and Z.J. Chen. 2012. The role of ubiquitylation in immune defense and pathogen evasion. *Nat. Rev. Immunol.* 12:35–48.

Kawai, T., and S. Akira. 2010. The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. *Nat. Immunol.* 11:373–384. http://dx.doi.org/10.1038/ni.1863

Lee, S.Y., S.Y. Lee, and Y. Choi. 1997. TRAF-interacting protein (TRIP): a novel component of the tumor necrosis factor receptor (TNFR)- and CD30-TRAF signaling complexes that inhibits TRAF2-mediated NF-κB activation. *J. Exp. Med.* 185:1275–1285. http://dx.doi.org/10.1084/jem.185.7.1275

Lei, C.Q., B. Zhong, Y. Zhang, J. Zhang, S. Wang, and H.B. Shu. 2010. Glycogen synthase kinase 3β regulates IRF3 transcription factor-mediated antiviral response via activation of the kinase TBK1. *Immunity.* 33:878–889. http://dx.doi.org/10.1016/j.immuni.2010.11.021

Li, S., L. Wang, M. Berman, Y.Y. Kong, and M.E. Dorf. 2011. Mapping a dynamic innate immunity protein interaction network regulating type I interferon production. *Immunity.* 35:426–440. http://dx.doi.org/10.1016/j.immuni.2011.06.014

Lin, R., Y. Mamane, and J. Hiscott. 1999. Structural and functional analysis of interferon regulatory factor 3: localization of the transactivation and autoinhibitory domains. *Mol. Cell. Biol.* 19:2465–2474.

Liu, Y.C., J. Penninger, and M. Karin. 2005. Immunity by ubiquitylation: a reversible process of modification. *Nat. Rev. Immunol.* 5:941–952. http://dx.doi.org/10.1038/nri1731

Medzhitov, R. 2001. Toll-like receptors and innate immunity. *Nat. Rev. Immunol.* 1:135–145. http://dx.doi.org/10.1038/35100529

Mori, M., M. Yoneyama, T. Ito, K. Takahashi, F. Inagaki, and T. Fujita. 2004. Identification of Ser-386 of interferon regulatory factor 3 as critical target for inducible phosphorylation that determines activation. *J. Biol. Chem.* 279:9698–9702. http://dx.doi.org/10.1074/jbc.M310616200

O'Neill, L.A. 2008. The interleukin-1 receptor/Toll-like receptor superfamily: 10 years of progress. *Immunol. Rev.* 226:10–18. http://dx.doi.org/10.1111/j.1600-065X.2008.00701.x

Park, E.S., S. Choi, J.M. Kim, Y. Jeong, J. Choe, C.S. Park, Y. Choi, and J. Rho. 2007. Early embryonic lethality caused by targeted disruption of the TRAF-interacting protein (TRIP) gene. *Biochem. Biophys. Res. Commun.* 363:971–977. http://dx.doi.org/10.1016/j.bbrc.2007.09.103

Regamey, A., D. Hohl, J.W. Liu, T. Roger, P. Kogerman, R. Toftgard, and M. Huber. 2003. The tumor suppressor CYLD interacts with TRIP and regulates negatively nuclear factor κB activation by tumor necrosis factor. *J. Exp. Med.* 198:1959–1964. http://dx.doi.org/10.1084/jem.20031187

Takeuchi, O., and S. Akira. 2009. Innate immunity to virus infection. *Immunol. Rev.* 227:75–86. http://dx.doi.org/10.1111/j.1600-065X.2008.00737.x

Wang, C., T. Chen, J. Zhang, M. Yang, N. Li, X. Xu, and X. Cao. 2009. The E3 ubiquitin ligase Nrdp1 'preferentially' promotes TLR-mediated production of type I interferon. *Nat. Immunol.* 10:744–752. http://dx.doi.org/10.1038/ni.1742

Yang, M., C. Wang, X. Zhu, S. Tang, L. Shi, X. Cao, and T. Chen. 2011. E3 ubiquitin ligase CHIP facilitates Toll-like receptor signaling by recruiting and polyubiquitinating Src and atypical PKCζ. *J. Exp. Med.* 208:2099–2112. http://dx.doi.org/10.1084/jem.20102667

Yoneyama, M., and T. Fujita. 2009. RNA recognition and signal transduction by RIG-I-like receptors. *Immunol. Rev.* 227:54–65. http://dx.doi.org/10.1111/j.1600-065X.2008.00727.x

Yu, Y., and G.S. Hayward. 2010. The ubiquitin E3 ligase RAUL negatively regulates type i interferon through ubiquitination of the transcription factors IRF7 and IRF3. *Immunity.* 33:863–877. http://dx.doi.org/10.1016/j.immuni.2010.11.027

Zhao, W., L. Wang, M. Zhang, P. Wang, L. Zhang, C. Yuan, J. Qi, Y. Qiao, P.C. Kuo, and C. Gao. 2011. Peroxisome proliferator-activated receptor gamma negatively regulates IFN-β production in Toll-like receptor (TLR) 3- and TLR4-stimulated macrophages by preventing interferon regulatory factor 3 binding to the IFN-β promoter. *J. Biol. Chem.* 286:5519–5528. http://dx.doi.org/10.1074/jbc.M110.149823

Zhao, W., L. Wang, M. Zhang, P. Wang, C. Yuan, J. Qi, H. Meng, and C. Gao. 2012. Tripartite motif-containing protein 38 negatively regulates TLR3/4- and RIG-I-mediated IFN-β production and antiviral response by targeting NAP1. *J. Immunol.* 188:5311–5318. http://dx.doi.org/10.4049/jimmunol.1103506

Zhou, Q., and R.L. Geahlen. 2009. The protein-tyrosine kinase Syk interacts with TRAF-interacting protein TRIP in breast epithelial cells. *Oncogene.* 28:1348–1356. http://dx.doi.org/10.1038/onc.2008.493