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The Ubiquitin Ligase TRIM56 Regulates Innate Immune Responses to Intracellular Double-Stranded DNA

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SUMMARY

The innate immune system detects pathogen- and host-derived double-stranded DNA exposed to the cytosol and induces type I interferon (IFN) and other cytokines. Here, we identified interferon-inducible tripartite-motif (TRIM) 56 as a regulator of double-stranded DNA-mediated type I interferon induction. TRIM56 overexpression enhanced IFN-β promoter activation after double-stranded DNA stimulation whereas TRIM56 knockdown abrogated it. TRIM56 interacted with STING and targeted it for lysine 63-linked ubiquitination. This modification induced STING dimerization, which was a prerequisite for recruitment of the antiviral kinase TBK1 and subsequent induction of IFN-β. Taken together, these results indicate that TRIM56 is an interferon-inducible E3 ubiquitin ligase that modulates STING to confer double-stranded DNA-mediated innate immune responses.

INTRODUCTION

The innate immune system has evolved as the first line of host defense against a variety of invading pathogens. The initiation of innate immune responses relies on the recognition of pathogen components by germline-encoded pattern-recognition receptors (PRRs), which include membrane-bound Toll-like receptors (TLRs) and cytosolic RIG-I-like receptors (RLRs), NOD-like receptors (NLRs; also known as nucleotide-binding domain and leucine-rich repeat containing molecules), and unidentified DNA sensors (Baccala et al., 2009; Medzhitov, 2007; Schroder and Tschopp, 2010; Takeuchi and Akira, 2010; Yoneyama and Fujita, 2009). Upon recognition, they initiate signal transduction pathways that lead to the induction of type I interferon (IFN) and proinflammatory cytokines, which are required for innate immune responses as well as for shaping subsequent adaptive immune responses.

DNA derived from DNA viruses and bacteria as well as damaged host cells or cells that have escaped from apoptosis are triggers for innate immune responses (Hornung and Latz, 2010; Ishii and Akira, 2006; Nagata et al., 2010; Yanai et al., 2009). Although TLR9 is known to recognize CpG DNA derived from viruses and bacteria in the endolysosome, there is evidence that double-stranded (ds) DNA delivered to the cytoplasm is recognized by one or more cytosolic sensors (Ishii et al., 2006; Stetson and Medzhitov, 2006; Yoshida et al., 2005). DNA-dependent activator of IFN-regulatory factors (DAI) (also known as ZBP1 or DLM-1) was identified as an intracellular dsDNA sensor that binds to dsDNA and increases type I IFN induction (Takaoka et al., 2007). However, a dispensable role of DAI in dsDNA-elicited innate and adaptive immune responses in vivo was revealed by a study on DAI-deficient mice (Ishii et al., 2008). It was also suggested that AT-rich dsDNA serves as a template for transcription to dsRNA by RNA polymerase III, which is recognized by the RLR member RIG-I, suggesting that RIG-I recognizes intermediates of dsDNA rather than dsDNA itself (Ablasser et al., 2009; Chiu et al., 2009). IFN-inducible absent in melanoma 2 (AIM2), which contains a pyrin and a HIN-200 DNA-binding domain, was identified to bind to dsDNA and form an ASC-containing inflammasome, which triggers caspase-1-dependent IL-1β production rather than type I IFN (Bürckstümmer et al., 2009; Fernandes-Alnemri et al., 2009; Hornung et al., 2009; Roberts et al., 2009). Collectively, these findings suggest the existence of unknown cytosolic PRRs that sense dsDNA and induce type I IFN production.

Stimulator of interferon genes (STING) (also known as MITA, ERIS, or MPYS) was identified as a molecule that activates the IFN-β promoter after overexpression and is a transmembrane protein that resides in the ER and/or mitochondria (Ishikawa and Barber, 2008; Jin et al., 2008; Sun et al., 2009; Zhong et al., 2008). Cells derived from STING-deficient mice fail to induce type I IFN production in response to dsDNA and infection with herpes simplex virus 1 (HSV-1) and *Listeria monocytogenes* (*L. monocytogenes*) that deliver DNA to the host cytosol. STING-deficient mice are also susceptible to HSV-1 infection (Ishikawa et al., 2009). In addition, STING-deficient mice show a remarkable reduction in cytotoxic T cell responses after plasmid DNA

vaccination (Ishikawa et al., 2009), indicating that STING is an essential component of dsDNA-mediated immune responses in vivo. Furthermore, STING-deficient cells display a considerable reduction in type I IFN production after infection with the negative single-stranded RNA virus vesicular stomatitis virus (VSV) that is recognized by RIG-I (Ishikawa and Barber, 2008). STING interacts with IPS-1 (also known as MAVS, CARDIF, or VISA), an essential adaptor for RLRs, at mitochondria (Ishikawa and Barber, 2008; Zhong et al., 2008). Thus, in addition to its pivotal role in the responses to cytosolic DNA, STING plays a pivotal role in RIG-I-mediated antiviral innate immune responses. In response to dsDNA stimulation, STING becomes relocalized to punctate structures, where the kinase TBK1 is recruited (Ishikawa and Barber, 2008; Saitoh et al., 2009; Zhong et al., 2008). It is likely that this recruitment induces TBK1 activation that results in phosphorylation of the transcription factor IRF3 and transcription of type I IFN genes during dsDNA-driven signaling. However, it remains to be defined how STING activation is controlled.

In the present study, we performed a functional screening and identified tripartite-motif (TRIM) 56. TRIM56 was induced by type I IFN and its overexpression enhanced dsDNA-mediated type I IFN induction. TRIM56 interacted with STING and promoted STING ubiquitination, which was a prerequisite for TBK1 recruitment and type I IFN induction.

RESULTS

Identification of TRIM56

To search for molecules involved in the innate immune responses to cytosolic DNA, we carried out an expression screening of a mouse carcinoma cDNA library. HEK293 cells were transiently transfected with a plasmid derived from the library along with an IFN-β promoter-luciferase reporter plasmid. The cells were then stimulated with dsDNA [poly (dA:dT)-poly (dT:dA)], herein referred to as poly (dA:dT), or dsRNA [poly (I:C)] by transfection, followed by measurement of the luciferase expression by a reporter assay. In a primary screening of around 1000 clones, we obtained one clone that displayed a marked increase in luciferase expression compared with the control plasmid-transfected cells (Figure 1A). This clone contained an open reading frame for TRIM56, a member of the TRIM family proteins (accession numbers BC045615, BG976873) (Figure 1B). Mouse TRIM56 contains an open reading frame of 2205 bases, encoding 734 amino acids. TRIM56 contains a RING domain, B-box domain, and coiled-coil domain in the N-terminal region (Figure 1B). The C-terminal region of TRIM56 did not share considerable homology with the C-terminal regions of other TRIMs. Expression of TRIM56 was upregulated after stimulation with poly (I:C) and poly (dA:dT) as evaluated by RT-PCR analysis (Figure 1C), consistent with a previous report showing that TRIM56 is IFN inducible (Carthagena et al., 2009).

We constructed an expression plasmid encoding FLAG-tagged TRIM56 to confirm its capability of increasing the IFN-β promoter activity. Overexpression of FLAG-TRIM56 alone into primary mouse embryonic fibroblast (MEF) cells resulted in 8-fold increases in the reporter gene expression (Figure 1D). Furthermore, FLAG-TRIM56 overexpression enhanced poly (dA:dT)- and poly (I:C)-stimulated IFN-β promoter activation (Figure 1D). Similarly, enhanced IFN-β promoter activation by TRIM56 was also observed in HEK293 cells (Figure S1A available online). Moreover, TRIM56 overexpression also increased the IFN-β promoter activation induced by 5,6-dimethylxanthenone-4-acetic acid (DMXAA), an antitumor agent that induces type I IFN via TBK1 and IRF3 (Figure S1B; Roberts et al., 2007).

Next, we transduced a retroviral vector encoding TRIM56 into MEF cells to investigate whether TRIM56 regulates innate immune responses to poly (dA:dT). We infected MEF cells derived from TBK1-deficient mice with retrovirus encoding TBK1 and found that TBK1 transduction restored poly (dA:dT) stimulation-induced production of IP-10, an IFN-inducible chemokine (Figure 1E). Notably, cotransduction of TBK1 together with TRIM56 markedly enhanced IP-10 production in response to poly (dA:dT) stimulation (Figure 1E). These findings suggested that TRIM56 positively regulates dsDNA-mediated IFN-β induction.

TRIM56 Knockdown Abrogates the Responses to dsDNA and Poly (I:C)

To address the physiological function of TRIM56 in dsDNA-induced responses, we employed a knockdown strategy involving two distinct siRNAs targeting the human *TRIM56* gene (Tr56-1 and Tr56-2). HEK293 cells treated with these siRNAs displayed a considerable reduction in TRIM56 expression, as confirmed by RT-PCR (Figure 2A, top) and immunoblot (Figure 2A, bottom) analyses. These siRNAs also decreased TRIM56 expression in HeLa cells (data not shown). In TRIM56 knockdown cells, IFN-β and IFN-stimulation responsive element (ISRE) promoter activation was significantly reduced after poly (dA:dT) transfection (Figure 2B). IFN-β promoter activation induced by poly (I:C) was also reduced by TRIM56 knockdown (Figure 2C). Moreover, poly (dA:dT)-induced upregulation of *IFNB1* and *CXCL10* was reduced in TRIM56-knockdown HEK293 cells (Figure 2D). *CXCL10* mRNA induction after *Listeria monocytogenes* infection was also reduced in TRIM56-knockdown HeLa cells (Figure 2E). Furthermore, TRIM56 knockdown in HEK293 cells resulted in impaired IRF3 nuclear translocation by poly (dA:dT) (Figure 2F). These results strongly suggest that TRIM56 is involved in poly (dA:dT) and poly (I:C)-induced type I IFN production.

We next investigated whether TRIM56 is required for poly (dA:dT)-mediated responses in normal human cells. We nucleofected normal human lung fibroblasts with control siRNA or siRNA for TRIM56 (Tr56-1) and found that the amount of *TRIM56* mRNA was considerably reduced in Tr56-1-nucleofected cells compared with control siRNA-nucleofected cells (Figure 2G, top). TRIM56 knockdown abrogated *IFNB1* and *IL-6* mRNA induction by poly (dA:dT) (Figure 2G, top) and *CXCL10* and *CCL5* (RANTES) induction after infection with Newcastle disease virus (NDV) that stimulates the RIG-I pathway (Figure 2G, bottom). We then examined an involvement of TRIM56 in poly (dA:dT)- and poly (I:C)-induced IFN-β promoter activation by a reporter assay. In TRIM56 knocked down cells, poly (dA:dT) and poly (I:C) stimulation failed to enhance IFN-β promoter activity (Figure 2H). Thus, TRIM56 is required for poly (dA:dT)- and poly (I:C)-mediated type I IFN induction in human primary cells.

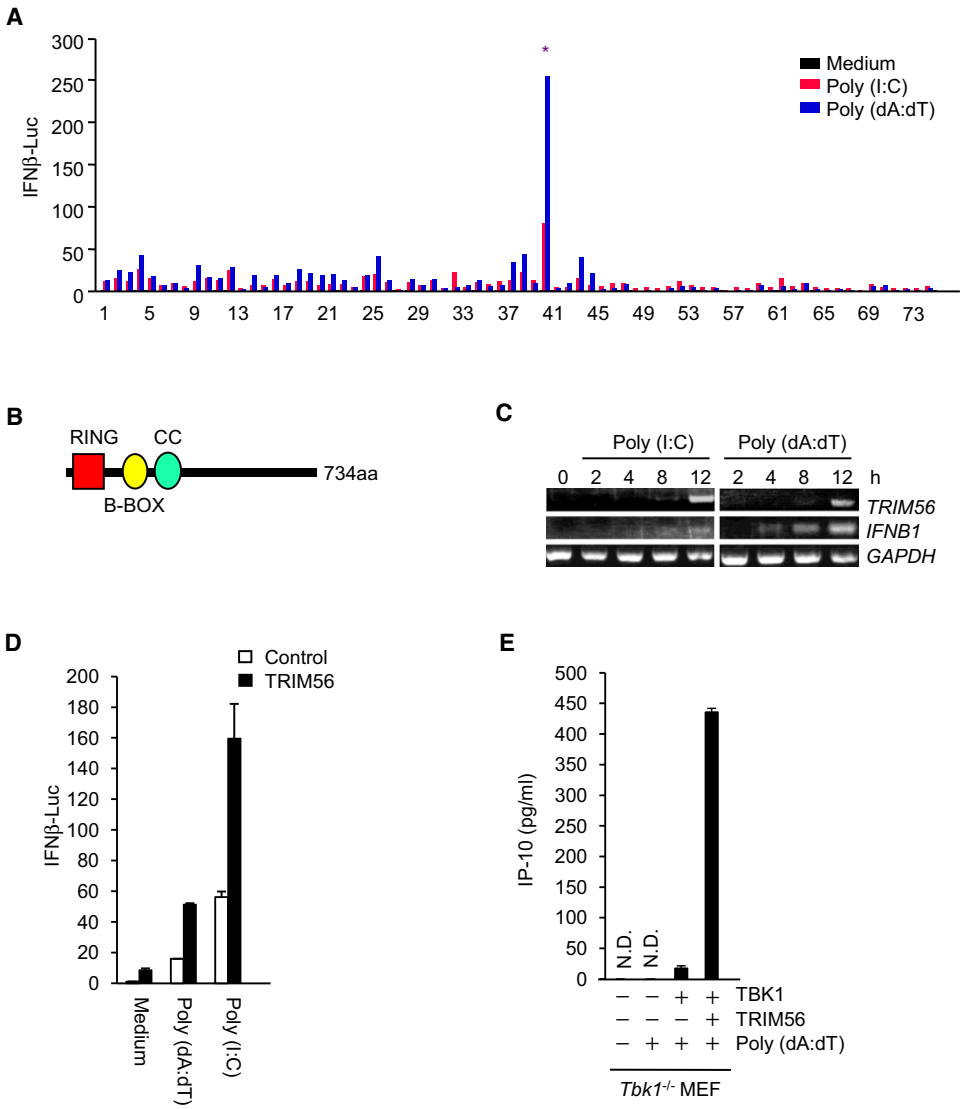

Figure 1. Identification of TRIM56

(A) HEK293 cells were transiently transfected with a plasmid derived from a mouse carcinoma cDNA library along with a reporter plasmid carrying the IFN-β promoter (IFN-β-Luc). After stimulation with poly (I:C) or poly (dA:dT) by transfection, the cells were analyzed for IFN-β promoter activity by a reporter gene assay. Asterisk: contained an open reading frame for TRIM56.

(B) Schematic representation of mouse TRIM56. CC; coiled-coil.

(C) Total RNA prepared from HEK293 cells stimulated with poly (I:C) or poly (dA:dT) for the indicated times was subjected to RT-PCR analysis for the expressions of *TRIM56, IFNB1*, and *GAPDH*.

(D) MEF cells transfected with control or TRIM56 expression plasmid along with the IFN-β-Luc reporter plasmid were left unstimulated or stimulated with poly (dA:dT) or poly (I:C) for 12 hr, and analyzed for IFN-β promoter activity by a reporter gene assay. Data represent means ± SD (n = 3).

(E) TBK1-deficient MEF cells infected with TBK1 and TRIM56 retrovirues were stimulated with poly (dA:dT) for 24 hr and analyzed for IP-10 production by ELISA. Data represent means ± SD (n = 3). N.D., not detected.

TRIM56 Is Localized to the Cytoplasm and Does Not Interact with Poly (dA:dT)

To determine the cellular localization of TRIM56, we expressed RFP-TRIM56 in HeLa cells via a retrovirus system. We found that RFP-TRIM56 was localized to the cytoplasm in the unstimulated condition (Figure 3A). In response to poly (dA:dT) and DMXAA stimulation, RFP-TRIM56 displayed a punctate pattern in the cytoplasm (Figure 3A). We found that TRIM56 was not localized at the ER, endosome, peroxisome, or mitochondria in unstimulated and poly (dA:dT)-stimulated cells (Figures S2A–S2D).

The findings that TRIM56 is an IFN-inducible gene and controls the poly (dA:dT)-mediated pathway suggest that TRIM56 may serve as an intracellular sensor for dsDNA. We therefore examined whether or not TRIM56 colocalizes with poly (dA:dT). We coexpressed RFP-TRIM56 along with FITC-labeled poly (dA:dT) into HeLa cells and examined their localization. There was no colocalization between RFP-TRIM56 and FITC-labeled

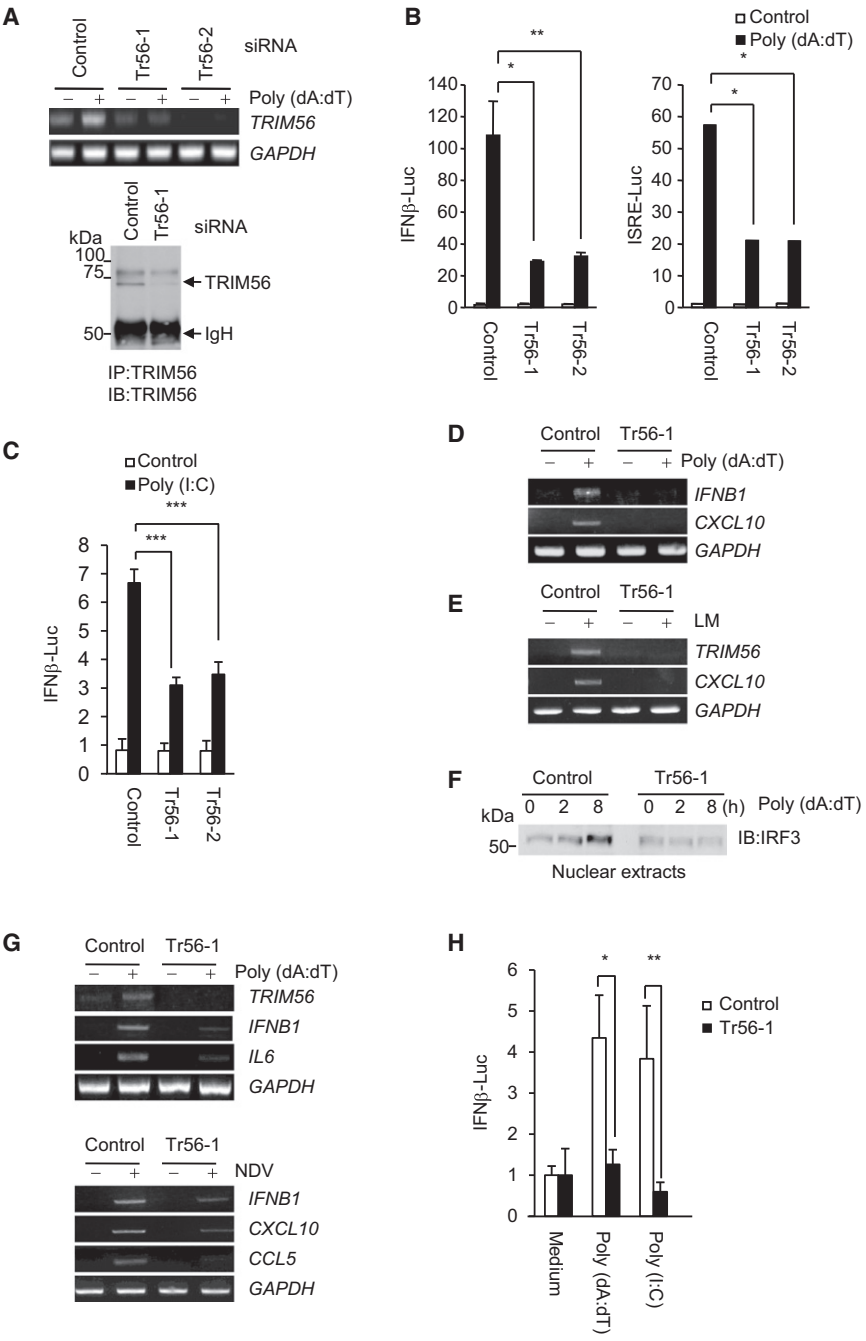

Figure 2. Requirement of TRIM56 for dsDNA and Poly (I:C)-Induced Responses

(A) HEK293 cells treated with a control siRNA or siRNAs targeting TRIM56 (Tr56-1 and Tr56-2) were stimulated with poly (dA:dT) for 12 hr. The cells were analyzed by RT-PCR for their expressions of *TRIM56* and *GAPDH* (top). Whole-cell lysates prepared from HEK293 cells treated with the indicated siRNAs were immunoprecipitated (IP) and immunoblotted (IB) with anti-TRIM56 (bottom).

(B and C) HEK293 cells treated with the indicated siRNAs were transfected with IFN-β-Luc or ISRE-Luc plasmids. Luciferase expression was analyzed after stimulation with poly (dA:dT) (B) or poly (I:C) (C). *$p < 0.001$, **$p < 0.002$, ***$p < 0.01$. Data represent means ± SD (n = 3).

(D) HEK293 cells treated with the indicated siRNAs were stimulated with poly (dA:dT) for 12 hr. Total RNA was prepared and analyzed for the expressions of *IFNB1, CXCL10*, and *GAPDH* by RT-PCR.

(E) HeLa cells treated with the indicated siRNAs were infected with *L. monocytogenes* (LM) at a MOI of 10 for 12 hr. Thereafter, total RNA was prepared and analyzed for the expressions of *TRIM56*, *CXCL10*, and *GAPDH* by RT-PCR.

(F) HEK293 cells treated with the indicated siRNAs were stimulated with poly (dA:dT) for the indicated periods. Nuclear extracts were prepared and analyzed by immunoblotting via anti-IRF3.

(G) Normal human lung fibroblasts nucleofected with control siRNA or siRNA targeting to TRIM56 (Tr56-1) were stimulated with poly (dA:dT) for 4 hr (top) or infected with NDV (moi = 5) for 8 hr (bottom). Total RNA was prepared and analyzed for the expressions of the indicated genes by RT-PCR.

(H) Normal human lung fibroblasts were nucleofected with control siRNA or siRNA targeting to TRIM56 (Tr56-1) along with IFN-β-Luc plasmid. Luciferase expression was analyzed after stimulation with poly (dA:dT) or poly (I:C). *$p < 0.001$, **$p < 0.02$. Data represent means ± SD (n = 3).

poly (dA:dT) in HeLa cells (Figure 3B). We next tested interaction between TRIM56 and poly (dA:dT). To this end, we precipitated cell lysates prepared from HEK293 cells overexpressing FLAG-human TRIM56, FLAG-mouse TRIM56, FLAG-p202, or FLAG-IPS-1 with biotin-conjugated poly (dA:dT) plus streptavidin agarose, and blotted with FLAG antibody. FLAG-p202 was used as a positive control because p202 (also known as IFI202b) was identified to bind to dsDNA (Roberts et al., 2009). We found that both human and mouse TRIM56 did not associate with poly (dA:dT) (Figure 3C). Collectively, these findings suggest that TRIM56 is unlikely to be a DNA sensor.

TRIM56 Interacts with STING and Accelerates IFN-β Induction

Next, we addressed the issue of whether TRIM56 is involved in STING- and IPS-1-mediated IFN-β induction. IFN-β promoter activation induced by STING and IPS-1 overexpression was significantly reduced in TRIM56 knockdown cells (Figure 4A). In contrast, IFN-β promoter activation induced by IRF3 overexpression was unimpaired in TRIM56 knockdown cells (Figure 4A), suggesting that TRIM56 is required for the function of STING and IPS-1. IFN-β promoter activation induced by STING overexpression was increased by coexpression of TRIM56 but not TRIM29 (Figure 4B). Furthermore, IPS-1-mediated IFN-β promoter activation was also increased by TRIM56 overexpression (Figure 4B). In contrast, TRIM56 did not enhance TRIF-mediated IFN-β promoter activation (Figure 4B).

We further evaluated the interaction between TRIM56 and STING by coimmunoprecipitation assays. FLAG-STING was

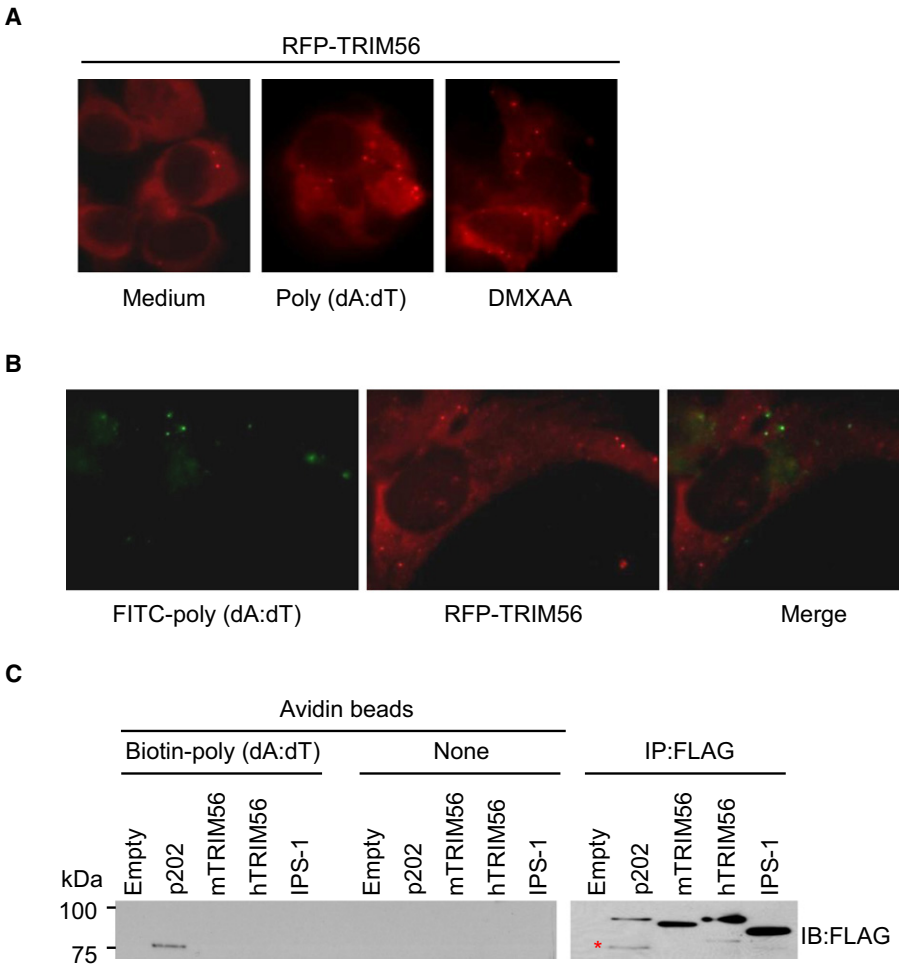

Figure 3. Cellular Localization of TRIM56

(A) HEK293 cells stably expressing RFP-TRIM56 were stimulated with poly (dA:dT) or DMXAA for 12 hr. The cells were fixed and observed by fluorescence microscopy.

(B) HeLa cells stably expressing RFP-TRIM56 were transfected with FITC-labeled poly (dA:dT). At 8 hr after transfection, cells were fixed and observed by fluorescence microscope.

(C) HEK293 cells were transfected with control (lane 1), FLAG-p202 (lane 2), FLAG-mouse TRIM56 (lane 3), FLAG-human TRIM56 (lane 4), or FLAG-human IPS-1 (lane 5). Cell lysates were incubated with streptavidin beads with or without biotinylated poly (dA:dT) (left) or anti-FLAG beads (right) for 4 hr. Bounded protein was analyzed by immunoblot with anti-FLAG. Asterisk: p202 band.

coprecipitated with TRIM56 antibody but not with two control antibodies (Figure 4C), indicating that endogenous TRIM56 forms a complex with FLAG-STING. We then searched for the region of TRIM56 that was responsible for the interaction with STING by constructing several deletion mutants and found that the STING C-terminal region was coprecipitated with Myc-TRIM56 FL (encoding full-length TRIM56), Myc-TRIM56 ΔR (lacking the RING finger domain), and Myc-TRIM56 ΔN (lacking the RING-finger and B-box domains) but not with Myc-TRIM56 ΔC (encoding the N-terminal RING-finger and B-box domains) (Figure 4D), indicating that the C-terminal region of TRIM56 is necessary for the interaction with the C-terminal region of STING. Unlike STING, we were unable to detect an interaction between TRIM56 and IPS-1 (data not shown).

STING was previously shown to display punctate structures in response to poly (dA:dT) stimulation. This finding prompted us to investigate whether TRIM56 colocalizes with STING. To this end, we coexpressed GFP-STING and RFP-TRIM56 and determined their cellular localizations after poly (dA:dT) stimulation. GFP-STING displayed punctate structures, and several dots were merged with those of RFP-TRIM56 (Figure S3A). It was reported that STING becomes relocalized from the ER to perinuclear vesicles containing exocyst components (Ishikawa et al., 2009; Saitoh et al., 2009). However, colocalization between TRIM56 and exocyst complex component 2 (EXOC2) (also known as SEC5) was not observed (Figure S3B). These findings suggest that TRIM56 is localized with STING after poly (dA:dT) stimulation and rapidly removed before STING relocalizes to exocyst.

Induction of STING Ubiquitination by TRIM56

In HEK293 cells transiently transfected with FLAG-STING, immnoblotting with anti-FLAG showed two bands with sizes of

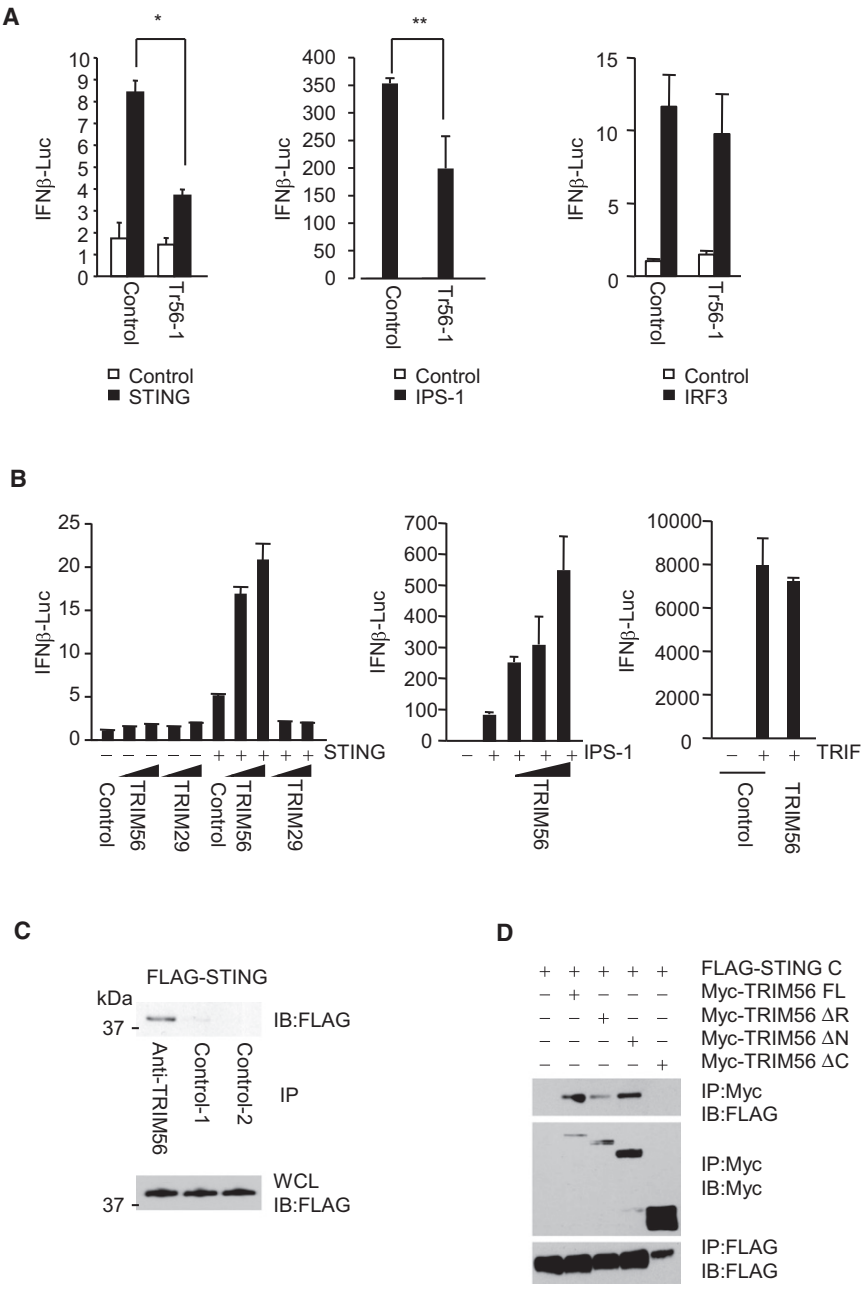

Figure 4. TRIM56 Interacts with STING and Enhances IFN-β Promoter Activation

(A) HEK293 cells treated with the indicated siRNAs were transfected with control, FLAG-STING, FLAG-IPS-1, or FLAG-IRF3 expression plasmids along with an IFN-β-Luc reporter plasmid, and analyzed for IFN-β promoter activity by a reporter gene assay. Data represent means ± SD (n = 3). *p < 0.01, **p < 0.002.

(B) HEK293 cells were cotransfected with the indicated combinations of expression plasmids encoding STING (100 ng), IPS-1 (1 ng), TRIF (1 ng), TRIM56 (100 or 250 ng), or TRIM29 (100 or 250 ng) along with an IFN-β-Luc reporter plasmid and subjected to a reporter assay. Data represent means ± SD (n = 3).

(C) Cell lysates prepared from HEK293 cells transfected with FLAG-STING were immunoprecipitated with the indicated antibodies and immunoblotted with anti-FLAG (top). Whole cell lysates (WCL) were immunoblotted with anti-FLAG (bottom).

(D) Cell lysates prepared from HEK293 cells cotransfected with the indicated combinations of expression plasmids were immunoprecipitated and immunoblotted with the indicated antibodies.

approximately 40 and 80 kDa. The 80 kDa band disappeared when anti-FLAG immunoprecipitates were treated with 8M urea, indicating that the 80 kDa band corresponds to a STING dimer, which was consistent with a previous report (Figure S4A; Sun et al., 2009). Notably, the 80 kDa band was increased by poly (dA:dT) stimulation and slightly increased by poly (I:C) stimulation (Figure 5A). Moreover, the anti-FLAG detected smeared bands over the 80 kDa band region in the poly (dA:dT)-stimulated cells (Figure 5A). These results suggest that poly (dA:dT) stimulation induces STING dimerization as well as modifications such as ubiquitination. Immunoblot analysis with ubiquitin antibody indicated that poly (dA:dT) stimulation increased the ubiquitination of anti-FLAG-STING immunoprecipitates, which was impaired by TRIM56 knockdown (Figure 5B, top). Furthermore, TRIM56 knockdown abrogated poly (dA:dT)-induced smeared bands over the 80 kDa band region as detected by anti-FLAG (Figure 5B, bottom). These results suggest that poly (dA:dT) stimulation triggered ubiquitination of STING or STING-associated proteins in a manner dependent on TRIM56.

We overexpressed FLAG-STING along with or without Myc-TRIM56, and STING ubiquitination was examined by immunoblot analysis via ubiquitin antibody. Ubiquitination of FLAG-STING was induced in cells expressing TRIM56 (Figure 5C, top). Moreover, an amount of the 80 kDa band corresponding to the dimer of FLAG-STING in TRIM56-expressing cells was increased compared with that in control cells (Figure 5C, middle).

Immunoblot analysis via ubiquitin lysine (K) 63 antibody indicated that TRIM56 overexpression increased the STING ubiquitination linked to K63 (Figure 5D). By contrast, TRIM56 overexpression did not affect the K48-linked ubiquitination of STING as determined by immunoblot analysis via ubiquitin K48 antibody (data not shown). These results suggest that TRIM56 preferentially catalyzes K63-linked ubiquitination of STING.

We denatured anti-FLAG immunoprecipitates of cell lysates prepared from cells transfected with FLAG-STING and Myc-TRIM56 by boiling in a buffer containing 1% SDS, followed by re-immunoprecipitated with anti-FLAG and analyzed by immunoblotting with antiubiquitin. In this condition, ubiquitination was

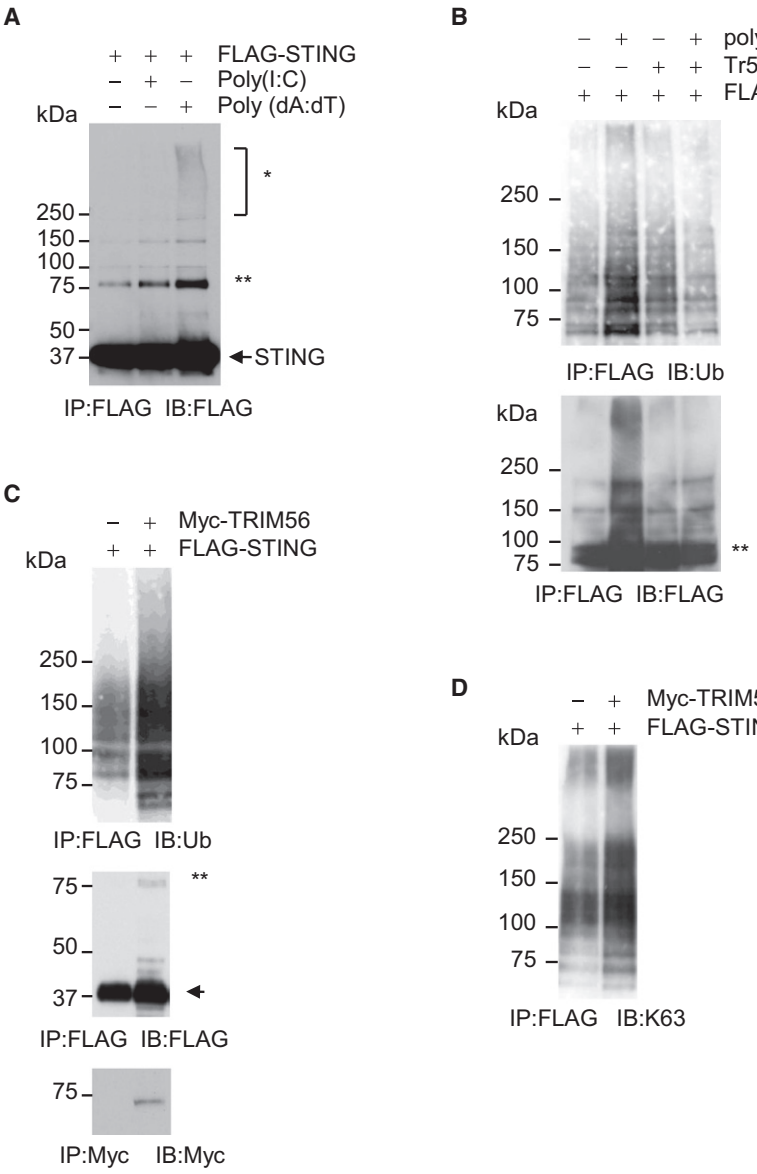

Figure 5. Ubiquitination of STING by TRIM56

(A) HEK293 cells transfected with FLAG-STING were stimulated with poly (I:C) or poly (dA:dT) for 12 hr. Cell lysates were immunoprecipitated and immunoblotted with anti-FLAG.

(B) HEK293 cells treated with siRNA for control (lane 1, 2) or TRIM56 (lane 3, 4) were stimulated with poly (dA:dT) for 12 hr. Cell lysates were immunoprecipitated with anti-FLAG and blotted with anti-ubiquitin (top) or -FLAG (bottom).

(C) HEK293 cells were transfected with FLAG-STING and Myc-TRIM56 as indicated. Cell lysates were immunoprecipitated with anti-FLAG or anti-Myc and immunoblotted with anti-ubiquitin, anti-FLAG, or anti-Myc as indicated.

(D) Cell lysates prepared in (C) were immunoprecipitated with anti-FLAG and blotted with anti-ubiquitin K63. Single asterisk: high molecular weight bands; double asterisk: 80 kDa band corresponding to the STING dimer.

still observed (Figure S4B), which strongly suggests that ubiquitin chains are covalently attached to STING. It was recently demonstrated that RIG-I binds to unanchored K63-linked polyubiquitin chains that are likely to be synthesized by TRIM25, activating downstream signaling (Zeng et al., 2010). This suggests the possibility that in addition to covalent attachment of ubiquitin, STING is also associated with unanchored ubiquitin chains. In this regard, we lysed cells transfected with FLAG-STING and Myc-TRIM56 in the regular lysis buffer that did not contain SDS, treated anti-FLAG immunoprecipitates with Isopeptidase T (Iso T; also known as USP5) that removes unanchored ubiquitin chains, and blotted with anti-ubiquitin. Iso T treatment decreased the amount of ubiquitination (Figure S4C), suggesting that STING is associated with unanchored ubiquitin chains or their associated proteins such as RIG-I under nondenatured condition.

Role of STING Lysine 150 in the Responses to dsDNA

To determine the lysine residue(s) in STING that are critical for TRIM56-mediated ubiquitination, we constructed a series of FLAG-STING mutants in which individual lysine residues were substituted with an arginine residue (K20R, K137R, and K150R). HA-ubiquitin and Myc-TRIM56 were coexpressed with FLAG-STING or STING mutants in HEK293 cells, and anti-FLAG immunoprecipitates were immunoblotted with HA antibody. The amount of ubiquitination was unaffected by mutation of lysine 20 or 137, but was severely abrogated by mutation of lysine 150 (Figure 6A). Notably, immunoblot analysis with anti-FLAG demonstrated that STING K150R failed to form a dimer (Figure 6A). Moreover, poly (dA:dT) stimulation resulted in increased dimer formation as well as ubiquitination of STING, and these increases were abrogated by mutation of lysine 150 (Figure 6B). We next performed coimmunoprecipitation assay to examine whether STING lysine 150 is required for the dimer formation. When FLAG-STING and Myc-STING were expressed together in HEK293 cells, FLAG-STING was coprecipitated with Myc antibody, indicating that FLAG-STING interacts with Myc-STING. In contrast, interaction between FLAG-STING K150R and Myc-STING K150R was reduced (Figure 6C). Furthermore, poly (dA:dT) stimulation failed to induce the interaction between FLAG-STING K150R and Myc-STING K150R (data not shown). These results strongly suggest that STING lysine 150 is the major acceptor site for the TRIM56-mediated K63-linked ubiquitin chains and that this lysine residue is required for the dimer formation.

Next, we addressed whether STING ubiquitination is linked to IFN-β induction. Overexpression of STING K150R failed to

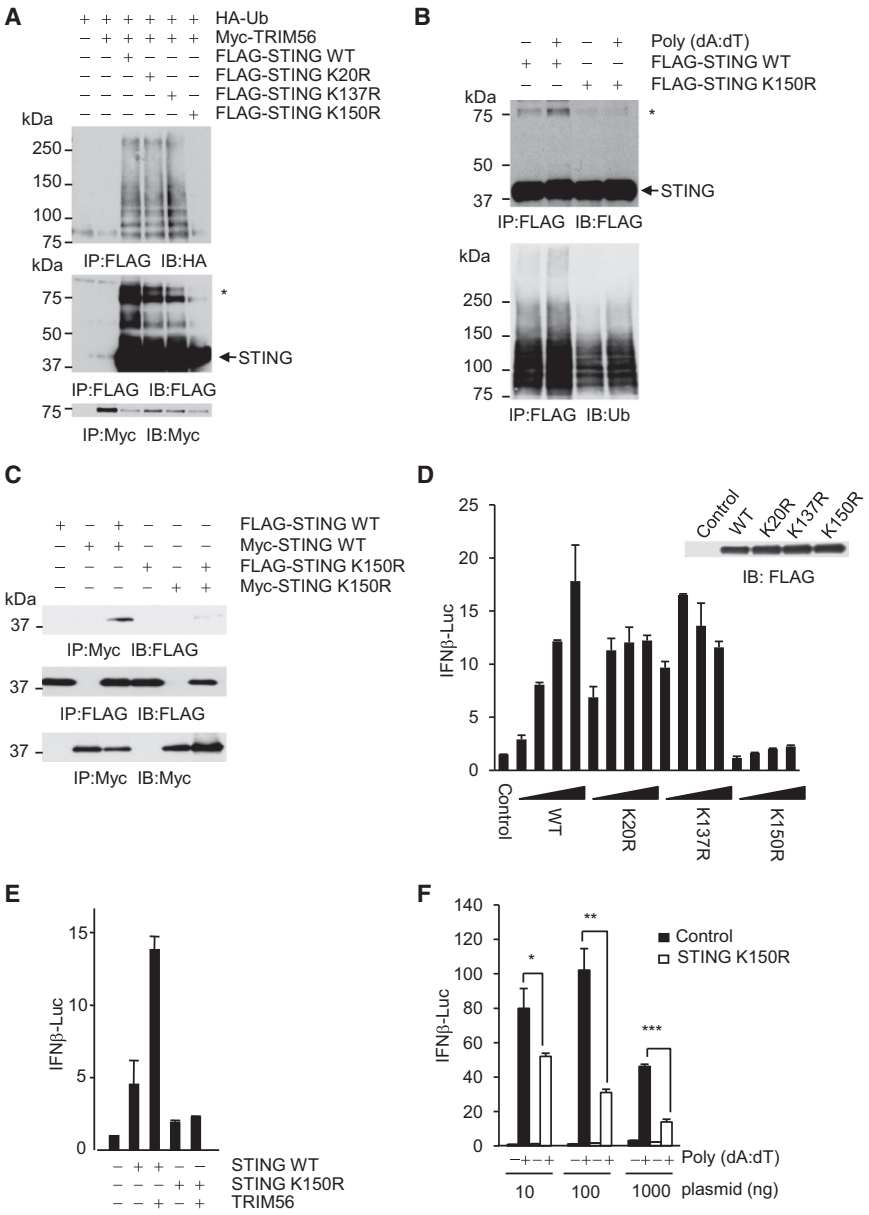

Figure 6. STING Lysine 150 Is Required for IFN-β Promoter Activation

(A) HEK293 cells were transfected with FLAG-STING WT, K20R, K137R, or K150R along with Myc-TRIM56 and HA-ubiquitin. Cell lysates were immunoprecipitated with anti-FLAG or anti-Myc and immunoblotted with anti-FLAG, anti-Myc, or anti-HA.

(B) HEK293 cells transfected with FLAG-STING WT or FLAG-STING K150R were stimulated with poly (dA:dT). Cell lysates were immunoprecipitated with FLAG antibody and immunoblotted with anti-FLAG (top) or anti-ubiquitin (bottom). Asterisk: 80 kDa band corresponding to the STING dimer (A, B).

(C) Cell lysates prepared from HEK293 cells cotransfected with the indicated combinations of expression plasmids were immunoprecipitated and immunoblotted with the indicated antibodies.

(D) HEK293 cells transfected with increasing amounts of control, STING WT, STING K20R, STING K137R, or STING K150R plasmids (1, 10, 100, or 250 ng) along with an IFN-β-Luc reporter plasmid were analyzed by a reporter assay (left). Cell lysates were immunoblotted with anti-FLAG (right).

(E) HEK293 cells transfected with the indicated combinations of plasmids for STING, STING K150R, and TRIM56 along with the IFN-β-Luc reporter plasmid were analyzed by a reporter gene assay.

(F) HEK293 cells transfected with the indicated amounts of control or FLAG-STING K150R plasmids along with the IFN-β-Luc reporter plasmid were stimulated with poly (dA:dT) and analyzed by a reporter gene assay. Data represent means ± SD (n = 3).

*p < 0.04, **p < 0.01, ***p < 0.001.

activate the IFN-β promoter whereas the activation induced by STING, STING K20R, and STING K137R were comparable (Figure 6D). Moreover, STING K150R remained unable to enhance IFN-β promoter activation even when coexpressed with TRIM56 (Figure 6E), and it acted as a dominant-negative form for suppression of the poly (dA:dT)-triggered IFN-β promoter activation (Figure 6F). Collectively, these findings indicate that TRIM56 catalyzes ubiquitination of STING lysine 150 during poly (dA:dT)-triggered signaling, which is important for the induction of IFN-β.

STING Ubiquitination Is Required for Recruitment of TBK1

It was shown that STING forms a complex with TBK1 and activates IRF3 (Ishikawa and Barber, 2008; Zhong et al., 2008). Therefore, we investigated whether TRIM56 influences the STING-TBK1 complex formation. We overexpressed FLAG-STING and Myc-TBK1 together with or without FLAG-TRIM56. FLAG-STING was coprecipitated with Myc-TBK1 in cells without overexpressing TRIM56 (Figure 7A). Moreover, FLAG-STING migrated slowly in the presence of Myc-TBK1. Phosphatase treatment abrogated the migration, suggesting that STING was phosphorylated in the presence of TBK1 (Figure S5A). When TRIM56 was coexpressed together, STING phosphorylation as well as ubiquitination was increased (Figure 7A). Moreover, interaction between STING and TBK1 was enhanced by TRIM56 overexpression (Figure 7A). These results suggest that TRIM56 facilitates interactions between STING and TBK1.

Overexpression of TRIM56 ΔN markedly inhibited IFN-β promoter activation induced by STING overexpression (Figure 7B), suggesting that TRIM56 ΔN limits STING function. Therefore, we examined whether this suppressive effect is due to inhibition of STING-TBK1 interaction. In this regard, we overexpressed FLAG-STING and Myc-TBK1 together with or without FLAG-TRIM56 ΔN and examined interactions between TBK1

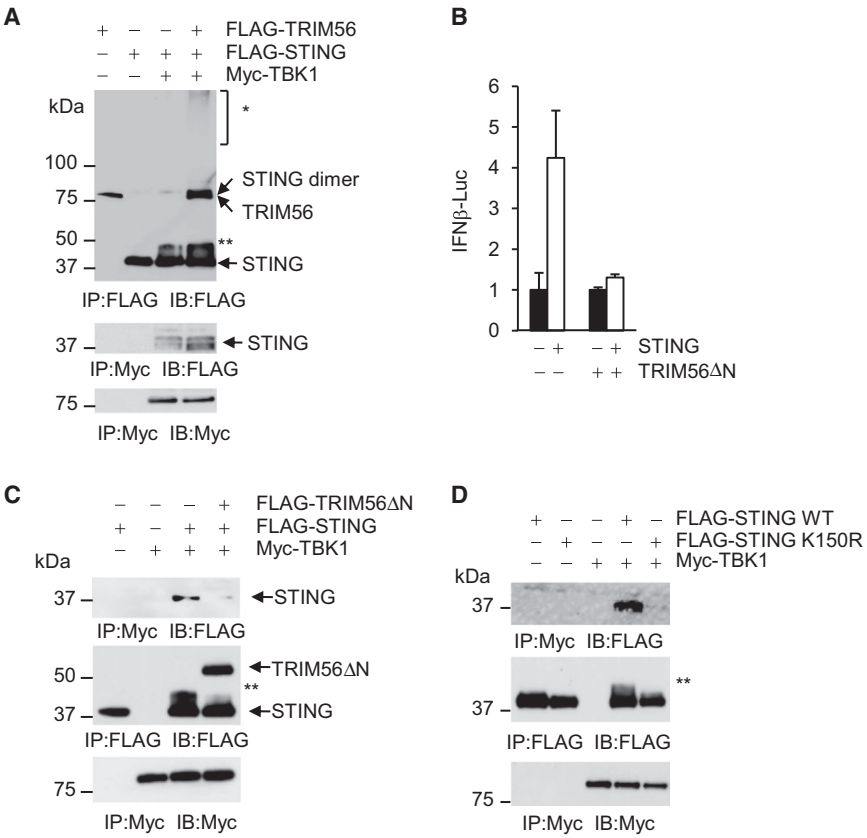

Figure 7. STING Ubiquitination Is Required for Recruitment of TBK1

(A) HEK293 cells were transfected with the indicated combinations of plasmids for FLAG-STING, FLAG-TRIM56, and Myc-TBK1. Cell lysates were immunoprecipitated and immunoblotted with the indicated antibodies.

(B) HEK293 cells transfected with the indicated combinations of plasmids for STING, and TRIM56 ΔN along with the IFN-β-Luc reporter plasmid were analyzed by a reporter gene assay. Data represent means ± SD (n = 3).

(C) HEK293 cells were transfected with the indicated combinations of plasmids for FLAG-STING WT, FLAG-TRIM56 ΔN, and Myc-TBK1. Cell lysates were immunoprecipitated and immunoblotted with the indicated antibodies.

(D) HEK293 cells were transfected with the indicated combinations of plasmids for FLAG-STING WT, FLAG-STING K150R, and Myc-TBK1. Cell lysates were immunoprecipitated and immunoblotted with the indicated antibodies.

Single asterisk: high molecular weight bands (A); double asterisk: phosphorylated STING (A, C, D).

and STING. Coprecipitation of FLAG-STING with Myc-TBK1 was disrupted by TRIM56 ΔN overexpression (Figure 7C). Moreover, TBK1-mediated STING phosphorylation was not observed in the presence of TRIM56 ΔN (Figure 7C). Thus, TRIM56 ΔN disrupts STING and TBK1 interaction probably through inhibiting STING ubiquitination.

Next, we investigated whether STING ubiquitination is required for TBK1 activation. We overexpressed Myc-TBK1 together with FLAG-STING or FLAG-STING K150R and examined their interactions. FLAG-STING was coprecipitated with TBK1 whereas FLAG-STING K150R was not (Figure 7D). Moreover, FLAG-STING, but not FLAG-STING K150R, migrated slowly in the presence of Myc-TBK1 (Figure 7D). These findings suggest that STING ubiquitination is a prerequisite for TBK1 recruitment and activation.

Previously, it was demonstrated that STING relocalizes to intracellular punctate structures along with TBK1 after poly (dA:dT) stimulation (Ishikawa et al., 2009; Saitoh et al., 2009). We therefore examined whether STING ubiquitination is prerequisite of STING translocation. We reconstituted GFP-TBK1 into TBK null MEFs by retrovirus infection. We further transduced RFP-STING WT or K150R into these cells and determined their localizations. STING WT was translocated to perinuclear vesicles from the cytoplasm in response to poly (dA:dT), where TBK1 was colocalized (Figure S5B). Similarly, STING K150R was also translocated to the perinuclear vesicles and merged with TBK1 (Figure S5B). These findings suggest that STING translocation does not require ubiquitination.

Taken together, these findings suggest that poly (dA:dT) stimulation induces TRIM56 expression, which promotes STING ubiquitination. Ubiquitinated STING in turn forms a dimer or oligomer to recruit TBK1, which is important for its kinase activation as well as subsequent type I IFN induction.

DISCUSSION

More than 60 TRIM proteins have been identified in humans and mice (Carthagena et al., 2009; Nisole et al., 2005; Ozato et al., 2008). They contain an N-terminal RBCC motif composed of a RING domain, one or two B-boxes, and a coiled-coil domain and have C-terminal regions with different structures. The members of this family are largely classified to 10 subfamilies based on their C-terminal domains (Ozato et al., 2008; Short and Cox, 2006). Notably, some of them are induced by stimulation with type I and II IFNs and are involved in antiviral innate immune responses through ubiquitination. TRIM25 promotes K63-linked ubiquitination of RIG-I to activate antiviral innate immune responses (Gack et al., 2007). TRIM21 (also known as Ro52 and SSA1), which is found in sera from patients with systemic lupus erythematosus and Sjögren's syndrome, influences activation of IRF family proteins via K48- or K63-linked ubiquitination (Higgs et al., 2008; Kong et al., 2007; Yang et al., 2009). In contrast, TRIM30α binds TAK1 protein kinase and induces degradation of the TAK1-binding proteins TAB2 and TAB3 to suppress TLR-mediated NF-κB activation (Shi et al., 2008). Therefore, TRIM family proteins may represent new components that regulate innate immune responses via ubiquitination. In the present study, we showed that TRIM56 regulates dsDNA-driven innate immune responses. TRIM56 interacted

with STING and targeted it for ubiquitination. It is likely that K63-linked polyubiquitin chains are covalently attached to STING, and this modification is critical for STING dimerization or oligomerization as well as IFN-β induction. In addition, it is also likely that STING is noncovalently associated with unanchored polyubiquitin chains that are removed by Iso T. It was recently reported that unanchored K63-polyubiquitin chains associate with RIG-I and activate downstream signaling (Zeng et al., 2010). Moreover, STING deficiency or STING knockdown abrogates the antiviral responses to certain RNA viruses that are recognized by RIG-I (Ishikawa et al., 2009), indicating that STING is involved in RIG-I signaling. Therefore, unanchored polyubiquitin chains may function as a platform that assembles a signaling complex involving RIG-I and STING. Collectively, it is speculated that STING is regulated by two different types of K63-linked ubiquitination. STING is initially attached with ubiquitin by TRIM56 to form a dimer or oligomer, which is then recruited to unanchored polyubiquitin chains along with RIG-I or an unknown DNA sensor for induction of antiviral innate immune responses.

We found that TRIM56 overexpression enhanced poly (I:C)- and IPS-1-induced IFN-β promoter activation and that TRIM56 knockdown abrogated this activation as well as NDV-induced cytokine gene expression, suggesting that TRIM56 is involved in RLR signaling pathways. However, we were unable to detect an interaction between TRIM56 and IPS-1 by coimmunoprecipitation assays (data not shown), and the TRIM56 signals were not merged with the signals for a mitochondrial marker where IPS-1 is localized. In addition, modifications of IPS-1 such as ubiquitination or phosphorylation were not induced when TRIM56 was overexpressed (data not shown). Therefore, the ability of TRIM56 to enhance IPS-1 function may be based on TRIM56-mediated modulation of the endogenous STING function. Recently, the phosphatase Eyes absent 4 (EYA4) was identified to enhance innate immune responses to dsDNA and RNA viruses (Okabe et al., 2009). EYA4 was shown to bind to both IPS-1 and STING. Therefore it is possible that EYA4 may be involved in TRIM56-mediated IPS-1 activation.

TBK1 is an essential protein kinase that phosphorylates IRF3 and induces type I IFN in dsDNA-stimulated cells (Ishii et al., 2008). TBK1 is localized in the cytoplasm in a different manner to STING but translocates to the punctate structures, where STING is colocalized, in response to dsDNA stimulation. This translocation is abrogated by STING deficiency (Ishikawa et al., 2009), indicating that translocation of TBK1 to STING-containing vesicles is required for IRF3 activation. These findings, together with our finding that STING K150R did not interact with TBK1, suggest that TBK1 is recruited to the punctate structures when STING is ubiquitinated by TRIM56. However, it is noteworthy that similar to STING WT, STING K150R also translocates to the perinuclear vesicles in response to poly (dA:dT) stimulation, where it colocalizes with TBK1. This observation suggests that TRIM56-mediated STING ubiquitination is dispensable for dsDNA-mediated translocation of STING to the punctate structures; rather this modification is required for physical interaction with TBK1 within the structure, which is important in IRF3 activation. We recently demonstrated that the autophagy-related protein Atg9a protects against aberrant dsDNA-triggered responses by preventing the dynamic translocation of STING to these punctate structures (Saitoh et al., 2009). Therefore, it is possible that dsDNA-driven STING translocation may be regulated by Atg9a or its related proteins but not TRIM56. It was demonstrated that exocyst components are involved in these punctate structures along with STING and TBK1 and that the exocyst components play a role in TBK1 activation (Ishikawa et al., 2009). However, colocalization between TRIM56 and EXOC2 was not observed in the present study. This finding suggests that TRIM56 is removed from the STING-containing vesicles after it causes STING ubiquitination. Alternatively, TRIM56 may be rapidly degraded within EXOC2-containing vesicles.

RNF5 interacts with STING and mediates K48-linked ubiquitination to induce degradation, thereby suppressing antiviral innate immune responses (Zhong et al., 2009). RNF5 is localized to the ER and mitochondria, and interactions between RNF5 and STING occur at the mitochondria during RNA virus infection. Thus, RNF5 may negatively regulate RLR signaling by targeting STING that is localized to mitochondria for degradation. These authors identified STING lysine 150 as an essential residue for K48-linked ubiquitination. In contrast, we found that TRIM56 preferentially promoted K63-linked ubiquitination of the same lysine residue of STING that was important for the dimer formation and TBK1 activation. Furthermore, mitochondrial localization of TRIM56 was not observed. Taken together, it is likely that STING is negatively and positively regulated by different types of ubiquitination. Specifically, TRIM56 induces STING dimerization during dsDNA-triggered signaling to potentiate antiviral responses while RNF5 may induce degradation of mitochondrial STING to suppress RLR-induced antiviral responses.

The findings that TRIM56 failed to interact with dsDNA and that there was no colocalization between TRIM56 and dsDNA within cells suggest that TRIM56 is unlikely to be a dsDNA sensor, and instead facilitates the STING function by ubiquitination. Although a cytosolic dsDNA sensor responsible for type I IFN induction still remains to be identified, this study on TRIM56 characterization and future analyses of TRIM56-deficient animal models will help toward understanding the roles of the dsDNA sensor-driven signaling pathway in antiviral and antibacterial immune responses as well as the pathogenesis of autoimmune and inflammatory diseases that are caused by pathogen or host DNA.

EXPERIMENTAL PROCEDURES

Functional Screening

HEK293 cells seeded on 24-well plates (1×10^5 cells/well) were transiently transfected with IFN-β promoter-luciferase reporter plasmids (50 ng) and a plasmid DNA (100 ng) prepared from mouse or human cDNA library cloned into pCMV-SPORTS6 (Open Biosystems) via Lipofectamine 2000 (Invitrogen). After 12 hr, the cells were left unstimulated or stimulated with poly (dA:dT) (100 ng/ml) or poly (I:C) (1 μg/ml) by transfection. After another 24 hr, the cells were lysed with Passive Lysis Buffer (Promega) and measured for their luciferase activities via a Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions. As an internal control, 50 ng of a *Renilla* luciferase reporter gene was used simultaneously.

Cells and Reagents

HEK293, HeLa, and MEF cells were cultured in DMEM (Sigma) supplemented with 10% FBS (GIBCO) in a 5% CO_2 incubator. TBK1-deficient MEF cells were described previously (Hemmi et al., 2004). Normal human lung fibroblasts were obtained from Lonza. Human lung fibroblasts were maintained in Fibroblast

Basal Medium (Lonza; CC-3132) containing hFGF-B, insulin, FBS, and gentamicin/amphotericin-B (Lonza; CC-4126) according to the manufacturer's instruction. Poly (dA:dT) and poly (I:C) were purchased from InvivoGen. Biotin-conjugated poly (dA:dT), FITC-labeled poly (dA:dT) was purchased from Invitrogen. DMXAA was obtained from Sigma. *L. monocytogenes* and NDV were described previously (Kawai et al., 2005; Uematsu et al., 2007). Streptavidin agarose resin was purchased from Thermo Scientific. TRIM56 polyclonal, TRIM56 monoclonal, STING polyclonal, and ubiquitin (FK2) monoclonal antibodies were purchased from Novus Biologicals, Abcam, Imgenex, and Assay Designs, respectively. IRF3 (FL-425), c-*myc* (9E10), and HA (3F10) antibodies were purchased from Santa Cruz Biotechnology Inc. Anti-FLAG M2 Affinity Agarose Gel and anti-FLAG M2-Peroxidase antibody were purchased from Sigma. Anti-mouse IgG-Alexa Fluor 568 was purchased from Molecular Probes. Ub K63 monoclonal antibody was purchased from Millipore.

Plasmids

Mouse TRIM56, TRIM56 ΔR (amino acids 64–734), TRIM56 ΔN (amino acids 209–734), and TRIM56 ΔC (amino acids 1–208) were amplified by PCR and ligated into pFLAG-CMV6 or pEF-BOS to generate Flag- and Myc-tagged expression constructs, respectively. Human TRIM56, TRIM29, STING, STING-C (amino acids 174–379), and mouse p202 were amplified by RT-PCR and also ligated into pFLAG-CMV6 or pEF-BOS. TRIF, IPS-1, IRF3, TBK1 expression plasmids, and reporter plasmids were described previously (Kawai et al., 2005). HA-tagged ubiquitin was amplified by RT-PCR and ligated into pEF-BOS. STING K20R, STING K137R, and STING K150R were generated via a QuikChange II Site-directed Mutagenesis Kit (Stratagene). The expression plasmids pMRX-GFP-ires-puro and pMRX-RFP-ires-puro were described previously (Saitoh et al., 2009). The cDNA fragments encoding TRIM56 and TBK1 were amplified by RT-PCR and inserted into pMRX-RFP-ires-puro and pMRX-GFP-ires-puro, respectively. Packaging cell line Platinum E cells (Cell Biolabs Inc.) plated on 60 mm dishes (1×10^6 cells/dish) were transfected with 4 μg of retroviral expression plasmid together with 1 μg of pVSV-G plasmid via Lipofectamine 2000. At 48 hr after transfection, the viruses were harvested, mixed with Polybrene (Sigma), and infected into HeLa or MEF cells. The infected cells were selected in the presence of 3 μg/ml puromycin for 5–7 days.

Reporter Assay

HEK293 or MEF cells plated on 24-well plates (1×10^5 cells/well) were transiently transfected with 50 ng of luciferase reporter plasmid together with a total of 1.0 μg of expression plasmid or empty control plasmid with Lipofectamine 2000 or FuGENE 6 (Roche), respectively. Human lung fibroblasts plated on 24-well plates (5×10^4 cells/well) were electroporated with 100 ng of luciferase reporter plasmid with Amaxa Nucleofector (Amaxa Biosystems) according to the manufacturer's instructions. At 24 hr after the transfection, the cells were stimulated and luciferase activities in the total cell lysates were measured with a Dual-Luciferase Reporter Assay System (Promega). A *Renilla* luciferase reporter plasmid (50 ng) was used as an internal control.

RT-PCR

Total RNA was isolated with Trizol reagent (Invitrogen) and reverse transcribed with ReverTra Ace (Toyobo) according to the manufacturer's instructions. PCR was performed with the following primers: *IFNB1*, 5′-CAGCAATTTTCAGTGTCAGAAGCT-3′ and 5′-TCATCCTGTCCTTGAGGCAGTAT-3′; *CXCL10*, 5′-ATGAATCAAACTGCGATTCCTGATTTGCTGC-3′ and 5′-TTAAGGAGATCTTTTAGCCATTTCCTTGC-3′; *IL-6,* 5′-GTAGCCGCCCCACACAGACAGCC-3′ and 5′-GCCATCTTTGGAAGGTTC-3′; *CCL5,* 5′-ACCAGTGGCAAGTGCTCCA-3′ and 5′-TCCGAACCCATTTCTTCTCT3-′; and *GAPDH*, 5′-ACCACCATGGAGAAGGCTGG-3′ and 5′-CTGAGTGTAGCCCAGGATGC-3′.

Immunoprecipitation and Immunoblot Analysis

HEK293 cells seeded on 100 mm dishes were transiently transfected with a total of 10 μg of various plasmids. At 24 hr after the transfection, the cells were lysed in lysis buffer (1% Nonidet P-40, 50 mM Tris-HCl [pH 7.4], 150 mM NaCl) supplemented with a complete protease inhibitor cocktail tablet (Roche). For immunoblot with ubiquitin antibody, RIPA buffer (1% Nonidet P-40, 25 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1% SDS) was used. Extraction of nuclear proteins, immunoprecipitation, and immunoblot were carried out as described previously (Kawai et al., 2005).

RNA Interference

Double-stranded RNA duplexes corresponding to human *TRIM56* mRNA were purchased from Invitrogen. The targeting sequences were as follows: TRIM56-1 sense, 5′-GAGCAGCGACUUCCUGGCCUGUAAA-3′; TRIM56-2 sense, 5′-CCACGUGGAGGUGUACAAUAUGGAA-3′. Negative control oligo was purchased from Invitrogen. HEK293 and HeLa cells were transfected with 50 nM siRNA via Lipofectamine RNAi MAX (Invitrogen) according to the manufacturer's instructions. Human lung fibroblasts were electroporated with 100 nM siRNA via Amaxa Nucleofector (Amaxa Biosystems) according to the manufacturer's instructions. At 48 hr after transfection, the cells were used for further experiments. Knockdown of *TRIM56* mRNA was verified by RT-PCR with the primers 5′-GTGGAGGCTGCCGAAGAAGC-3′ and 5′-GATTACCACACTATTCTGCTG-3′.

Fluorescence Microscopy

Cells cultured on coverslips were fixed with 3% paraformaldehyde in phosphate-buffered saline for 12 min, mounted with Prolong Gold antifade reagent (Invitrogen), and examined with an Olympus IX81 microscope.

Statistical Analysis

Differences were analyzed for statistical significance with Student's t test. A p value of less than 0.05 was considered significant.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and five figures and can be found with this article online at doi:10.1016/j.immuni.2010.10.013.

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