Ubiquitination of TLR3 by TRIM3 signals its ESCRT-mediated trafficking to the endolysosomes for innate antiviral response

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Edited by George R. Stark, Cleveland Clinic Lerner College of Medicine, Cleveland, OH, and approved August 7, 2020 (received for review February 9, 2020)

Trafficking of toll-like receptor 3 (TLR3) from the endoplasmic reticulum (ER) to endolysosomes and its subsequent proteolytic cleavage are required for it to sense viral double-stranded RNA (dsRNA) and trigger antiviral response, yet the underlying mechanisms remain enigmatic. We show that the E3 ubiquitin ligase TRIM3 is mainly located in the Golgi apparatus and transported to the early endosomes upon stimulation with the dsRNA analog poly(I:C). TRIM3 mediates K63-linked polyubiquitination of TLR3 at K831, which is enhanced following poly(I:C) stimulation. The polyubiquitinated TLR3 is recognized and sorted by the ESCRT (endosomal sorting complex required for transport) complexes to endolysosomes. Deficiency of TRIM3 impairs TLR3 trafficking from the Golgi apparatus to endosomes and its subsequent activation. *Trim3*$^{-/-}$ cells and mice express lower levels of antiviral genes and show lower levels of inflammatory response following poly(I:C) but not lipopolysaccharide (LPS) stimulation. These findings suggest that TRIM3-mediated polyubiquitination of TLR3 represents a feedback-positive regulatory mechanism for TLR3-mediated innate immune and inflammatory responses.

TLR3 | TRIM3 | polyubiquitination | ESCRT | innate antiviral response

Toll-like receptors (TLRs) are a family of pattern-recognition receptors (PRRs) that recognize structurally conserved components of various pathogens and then mediate host innate immune and inflammatory responses (1). The membrane localization, recognition of ligands, and recruitment of adaptor molecules of TLRs are divergent (2–5). TLR3 is a member of the TLR family which mostly located in the endoplasmic reticulum (ER), and a small fraction of TLR3 continuously traffics to the Golgi apparatus and endosomes under physiological condition (6). After stimulation of double-stranded RNA (dsRNA) such as the synthetic dsRNA analog poly(I:C), a fraction of TLR3 traverses sequentially from the Golgi apparatus to early endosomes and then further to late endosomes. The late endosomes and lysosomes ultimately fuse into acidified endolysosomes, where TLR3 is proteolytically cleaved between residues 252 and 346 by cathepsins B and H to yield a mature and functional receptor for sensing viral dsRNA. The functional TLR3 recruits the adaptor protein TRIF to mediate downstream pathways that lead to activation of NF-κB and IRF3 as well as induction of innate immune and inflammatory genes (7, 8).

The mechanisms responsible for TLR3-mediated signaling pathways have been well investigated (9, 10). However, how TLR3 traffics from the ER to endolysosomes remains enigmatic. It has been demonstrated that nucleic-sensing TLRs are continually transported to endosomes even in the absence of infection (4). However, it is also evident that the trafficking of these TLRs to endosomes is regulated by ligand-specific stimulation, which provides the host a way to set up feedback mechanisms in response to nucleic acid ligands (11, 12).

The ESCRT (endosomal sorting complex required for transport) machinery performs a widespread and crucial role in various biological processes (13). In mammalian cells, it consists of four distinct heterooligomeric protein complexes, ESCRT-0, -I, -II, and -III, plus several accessory components, which are able to sequentially self-assemble and sort ubiquitinated cargoes in a highly ordered manner at the endosomal membrane (14). The ESCRT-0 is composed of two subunits: HRS and STAM1/2. The ubiquitin-interacting motif (UIM) of both HRS and STAM1/2 allows them to recognize and bind ubiquitinated cargoes, and the FYVE-type zinc finger domain of HRS facilitates its anchoring to the endosomal membrane by binding lipid phosphatidylinositol 3-phosphate (PtdIns3P). HRS is believed to serve as an initial and predominant regulator in the early endosomes. ESCRT-I has four subunits: TSG101, VPS28, VPS37, and MVB12. This complex is subsequently recruited by direct binding of the ubiquitin E2 variant (UEV) domain of TSG101 to P(S/T)AP-like motif in HRS. The UEV domain of TSG101 can also recognize ubiquitin. ESCRT-II consists of VPS22, VPS25, and VPS36 subunits, and VPS36 has two GRAM-like ubiquitin-binding in Eap45 (GLUE) domains. The C-terminal GLUE domain of VPS36 binds to the C-terminal domain of the ESCRT-I subunit VPS28, and the N-terminal GLUE domain of VPS36 not only recognizes ubiquitinated cargoes but also binds PtdIns3P at the endosomal membrane. ESCRT-III is composed of VPS2, VPS20, VPS24, and VPS32, which is recruited to ESCRT-II via a direct interaction between its subunit VPS20 and the

Significance

Trafficking of TLR3 from the ER to endolysosomes and its subsequent proteolytic cleavage are necessary for it to sense viral dsRNA and trigger antiviral response. Understanding how TLR3 trafficking is regulated is important for deciphering the mechanism of TLR3-mediated innate immune and inflammatory responses. In this study, we report that TRIM3 mediates K63-linked polyubiquitination of TLR3 at K831. Subsequently, the polyubiquitinated TLR3 is recognized and sorted by the ESCRT complexes to endolysosomes to promote the activation of downstream signaling. This study provides insights into how the trafficking and activation of TLR3 are regulated for efficient innate immune response to extracellular and endosomal viral dsRNA.

Author contributions: W.-W. Li, Z.-S.X., and Y.-Y.W. designed research; W.-W. Li, Y.N., Y.Y., Y.R., W.-W. Luo, and M.-G.X. performed research; S.-Y.W. contributed new reagents/analytic tools; W.-W. Li, Z.-S.X., and Y.-Y.W. analyzed data; and W.-W. Li, Z.-S.X., and Y.-Y.W. wrote the paper.

The authors declare no competing interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at https://www.pnas.org/lookup/suppl/doi:10.1073/pnas.2002472117/-/DCSupplemental.

First published September 2, 2020.

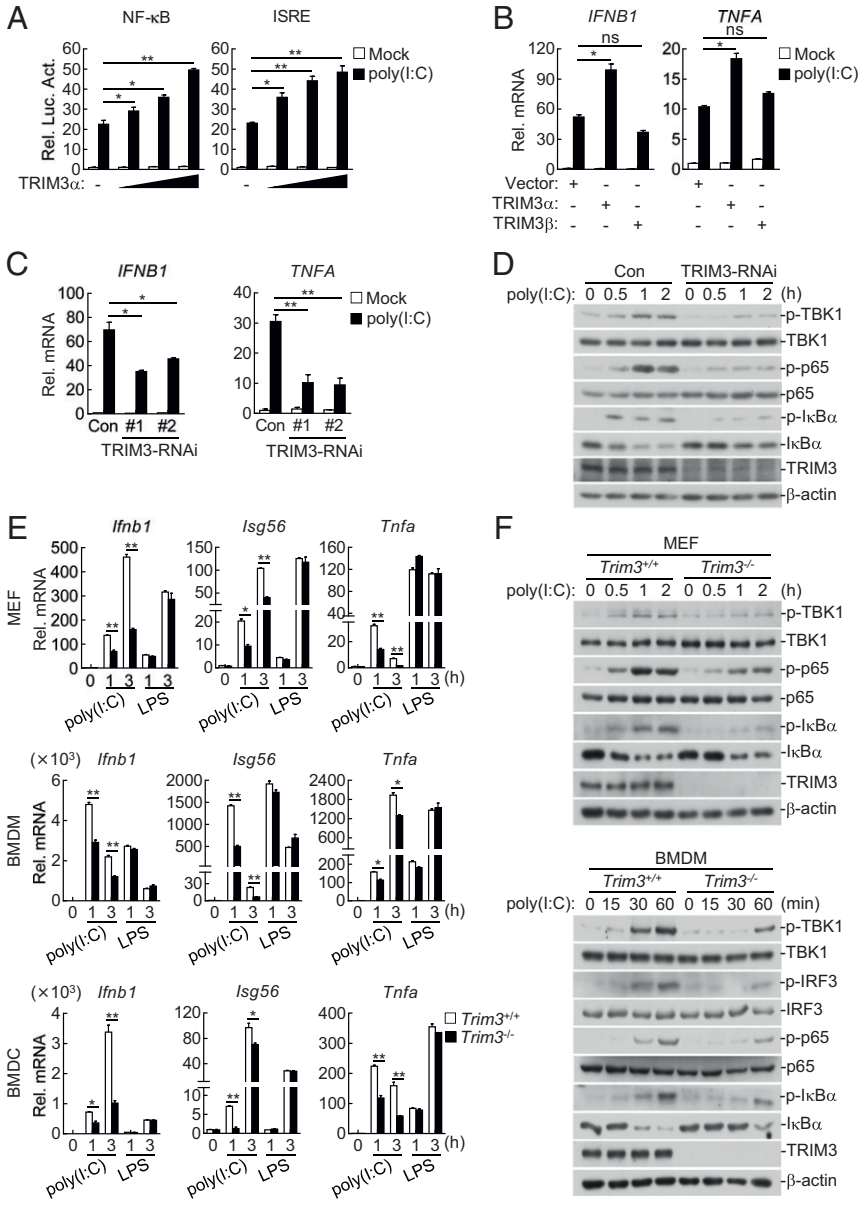

Fig. 1. TRIM3 positively regulates TLR3-mediated signaling. (*A*) Effects of TRIM3α on poly(I:C)-induced activation of NF-κB and ISRE. HEK293-TLR3 cells (1×10^5) were cotransfected with the indicated reporter (NF-κB [0.002 μg], ISRE [0.05 μg]), pRL-TK (*Renilla* luciferase) reporter (0.01 μg) and increased amounts of TRIM3α expression plasmids for 24 h. Cells were then left untreated or treated with poly(I:C) (50 μg/mL) for 10 h before luciferase assays. Rel. Luc. Act.: relative luciferase activity. (*B*) Effects of TRIM3α and TRIM3β on poly(I:C)-induced transcription of downstream genes. HEK293-TLR3 cells (2×10^5) were transfected with an empty vector or the indicated form of TRIM3 expression plasmids (0.2 μg) for 24 h. Cells were then left untreated or treated with poly(I:C) (50 μg/mL) for 3 h before qPCR analysis. Rel. mRNA: relative transcription level of messenger RNA. (*C*) Effects of TRIM3 knockdown on poly(I:C)-induced transcription of downstream genes. HEK293-TLR3 cells were transduced with control (Con) or the indicated TRIM3-RNAi plasmids by retroviral-mediated gene transfer to establish the stable cell lines. The control and TRIM3-RNAi HEK293-TLR3 cells (2×10^5) were left untreated or treated with poly(I:C) (50 μg/mL) for 3 h before qPCR analysis. (*D*) Effects of TRIM3 knockdown on poly(I:C)-induced phosphorylation of TBK1, p65, and IκBα. The control and TRIM3-RNAi HEK293-TLR3 cells (2×10^5) (TRIM3-RNAi #1 plasmid was used) were left untreated or treated with poly(I:C) (50 μg/mL) for the indicated times before immunoblot analysis. (*E*) Effects of TRIM3 deficiency on poly(I:C)- or LPS-induced transcription of downstream genes in MEFs, BMDMs, and BMDCs. *Trim3*$^{+/+}$ and *Trim3*$^{-/-}$ cells (2×10^5) were left untreated or treated with poly(I:C) (50 μg/mL) or LPS (50 ng/mL) for the indicated times before qPCR analysis. (*F*) Effects of TRIM3 deficiency on poly(I:C)-induced phosphorylation of TBK1, IRF3, p65, and IκBα in MEFs and BMDMs. *Trim3*$^{+/+}$ and *Trim3*$^{-/-}$ cells (2×10^5) were left untreated or treated with poly(I:C) (50 μg/mL) for the indicated times before immunoblot analysis. Data in *A*–*C* were analyzed by one-way ANOVA, followed by the Dunnett post hoc test. Data in *E* were analyzed by unpaired, two-tailed Student's *t* test. Graphs show mean ± SD; $n = 3$. $^*P < 0.05$, $^{**}P < 0.01$, ns, not significant.

ESCRT-II subunit VPS25. ESCRT-III catalyzes the scission of membrane necks to support the formation of enlarged multivesicular bodies (MVBs)/late endosomes. The MVBs containing ubiquitinated cargoes fuse with lysosomes to form the large endolysosomes (15–18). Previous studies have shown that the ESCRT complexes mainly determine whether endocytic plasma membrane proteins or receptors are recycled back to cell surface or are further sorted into the MVBs for their eventual degradation by the canonical ESCRT pathway (19, 20). However, recent reports indicated that some endosomal receptors are activated but not degraded by the ESCRT pathway (21).

Tripartite motif-containing protein 3 (TRIM3) is a member of the tripartite motif (TRIM) protein superfamily, which represents

the largest class of RING-finger E3 ligases in mammals. The TRIM proteins have been shown to be involved in a variety of biological processes including proliferation, apoptosis, and transcriptional regulation (22, 23). It has been shown that TRIM3 is associated with the CART (cytoskeleton-associated recycling or transport) complex, which is necessary for efficient recycling of actin-dependent and constitutive plasma membrane receptors (24–26). In addition, TRIM3 regulates motility of microtubule-dependent motor protein KIF21B and also controls hippocampal plasticity and learning by mediating polyubiquitination of synaptic γ-actin (27, 28). In this study, we found that TRIM3 mediated K63-linked polyubiquitination of TLR3 at K831. The polyubiquitinated TLR3 was recognized and sorted by the ESCRT complexes to endolysosomes where TLR3 was activated. Deficiency of TRIM3 impaired TLR3 trafficking to the endolysosomes and TLR3-mediated downstream signaling. Our findings suggest that TRIM3-mediated polyubiquitination of TLR3 represents a feedback-positive regulatory mechanism for TLR3-mediated innate immune and inflammatory responses.

Results

TRIM3 Positively Regulates TLR3-Mediated Signaling. Previously, it has been demonstrated that membrane protein cargoes are principally marked by ubiquitins (most typically K63-linked polyubiquitin chains) for their inclusion into MVBs and subsequent transportation to endolysosomes (17). To determine whether TLR3 is transported to endolysosomes by similar mechanisms, we attempted to identify ubiquitin-related enzymes that could regulate TLR3-mediated signaling. To do this, we screened 352 ubiquitin-related human complementary DNA expression plasmids by reporter assays for their effects on poly(I:C)-induced activation of IFN-β promoter in HEK293 cells stably expressing TLR3 (HEK293-TLR3) (10, 29). These efforts led to the identification of TRIM3 as a candidate protein. TRIM3 has two isoforms, the full-length isoform 1 (designated as TRIM3α) and the shorter isoform 2 (designated as TRIM3β). Endogenous TRIM3 mainly existed as TRIM3α (*SI Appendix*, Fig. S1*A*). Overexpression of TRIM3α potentiated poly(I:C)-induced activation of NF-κB and ISRE promoters in a dose-dependent manner, whereas TRIM3β showed little effects (Fig. 1*A* and *SI Appendix*, Fig. S1*B*). In addition, overexpression of either TRIM3α or TRIM3β had no marked effects on LPS-induced activation of NF-κB and ISRE in HEK293 cells stably expressing TLR4 (HEK293-TLR4) (*SI Appendix*, Fig. S1*C*). Consistently, overexpression of TRIM3α but not TRIM3β enhanced poly(I:C)-induced transcription of *IFNB1* and *TNFA* genes (Fig. 1*B*). However, none of the two TRIM3 isoforms had marked effects on SeV-triggered transcription of *IFNB1* and *TNFA* genes (*SI Appendix*, Fig. S1*D*). To investigate whether endogenous TRIM3 is involved in poly(I:C)-induced signaling, we constructed two RNA interference (RNAi) plasmids which targeted both isoforms of human TRIM3 and dramatically down-regulated the expression of TRIM3. Knockdown of TRIM3 markedly inhibited

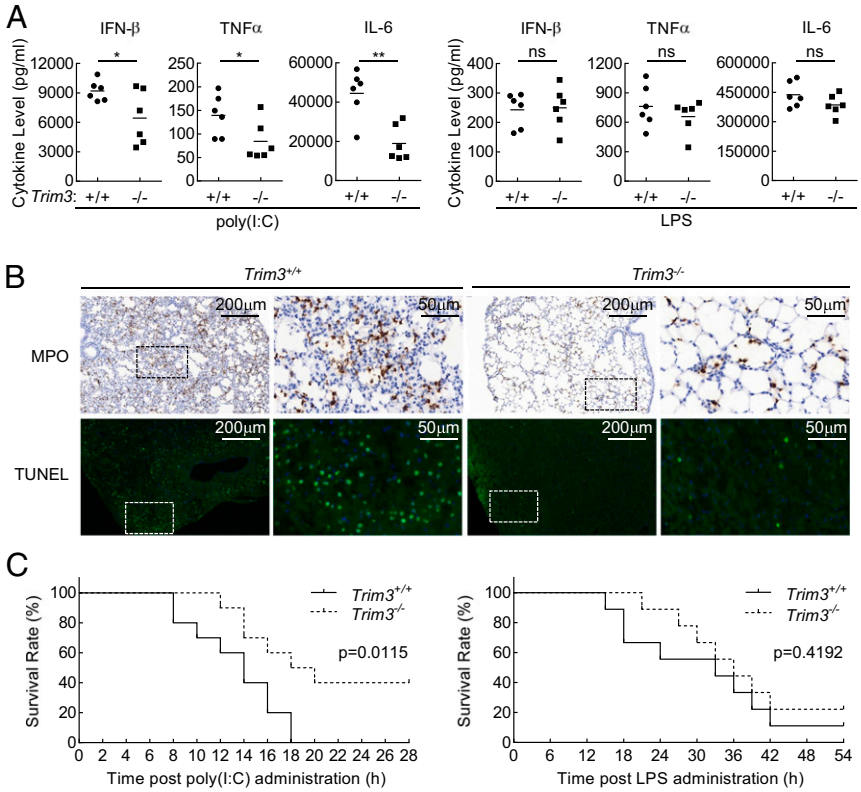

Fig. 2. TRIM3 deficiency attenuates poly(I:C)-triggered but not LPS-triggered innate immune and inflammatory responses in mice. (*A*) Effects of TRIM3 deficiency on serum levels of IFN-β, TNFα, and IL-6 induced by poly(I:C) or LPS. Sex- and age-matched *Trim3*$^{+/+}$ and *Trim3*$^{-/-}$ mice ($n = 6$) were injected i.p. with poly(I:C) (5 μg/g) or LPS (10 μg/g) for 2 h before measurement of the indicated serum cytokines by ELISA. Each symbol represents an individual mouse. *Trim3*$^{+/+}$: wild-type, *Trim3*$^{-/-}$: Trim3 deficiency. Data were analyzed by unpaired, two-tailed Student's *t* test. (*B*) Effects of TRIM3 deficiency on poly(I:C)-induced inflammation and apoptosis. Sex- and age-matched *Trim3*$^{+/+}$ and *Trim3*$^{-/-}$ mice were injected i.p. with poly(I:C) (0.6 μg/g) plus D-galactosamine (0.5 mg/g) for 4 h, then lung sections were used for immunohistochemical analysis (MPO staining, *Upper*), and liver sections were used for TUNEL analysis (fluorescein labeling for FITC-dUTP and DAPI, *Lower*). The areas in the dashed boxes on the *Left* are enlarged on the *Right*. (Scale bars, 200 μm.) (*C*) Effects of TRIM3 deficiency on poly(I:C)- or LPS-induced death in mice. Sex- and age-matched *Trim3*$^{+/+}$ and *Trim3*$^{-/-}$ mice were injected i.p. with poly(I:C) (0.6 μg/g) plus D-galactosamine (0.5 mg/g) ($n = 10$) or LPS (10 μg/g) ($n = 9$), and the survival rates of mice were recorded every 1 h in the following 28 or 54 h, respectively. The log-rank test was applied to analyze the statistical significance. Graphs show mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$, ns, not significant.

poly(I:C)-induced transcription of *IFNB1* and *TNFA* genes (Fig. 1*C*) and phosphorylation of TBK1, p65, and IκBα (Fig. 1*D*). These results suggest that TRIM3 positively regulates TLR3-mediated signaling.

To further confirm the physiological functions of TRIM3 in TLR3-mediated innate immune response, TRIM3-deficient mice were generated using the CRISPR/Cas9 method (*SI Appendix*, Fig. S2*A*). The successful deletion of *Trim3* in the knockout mice

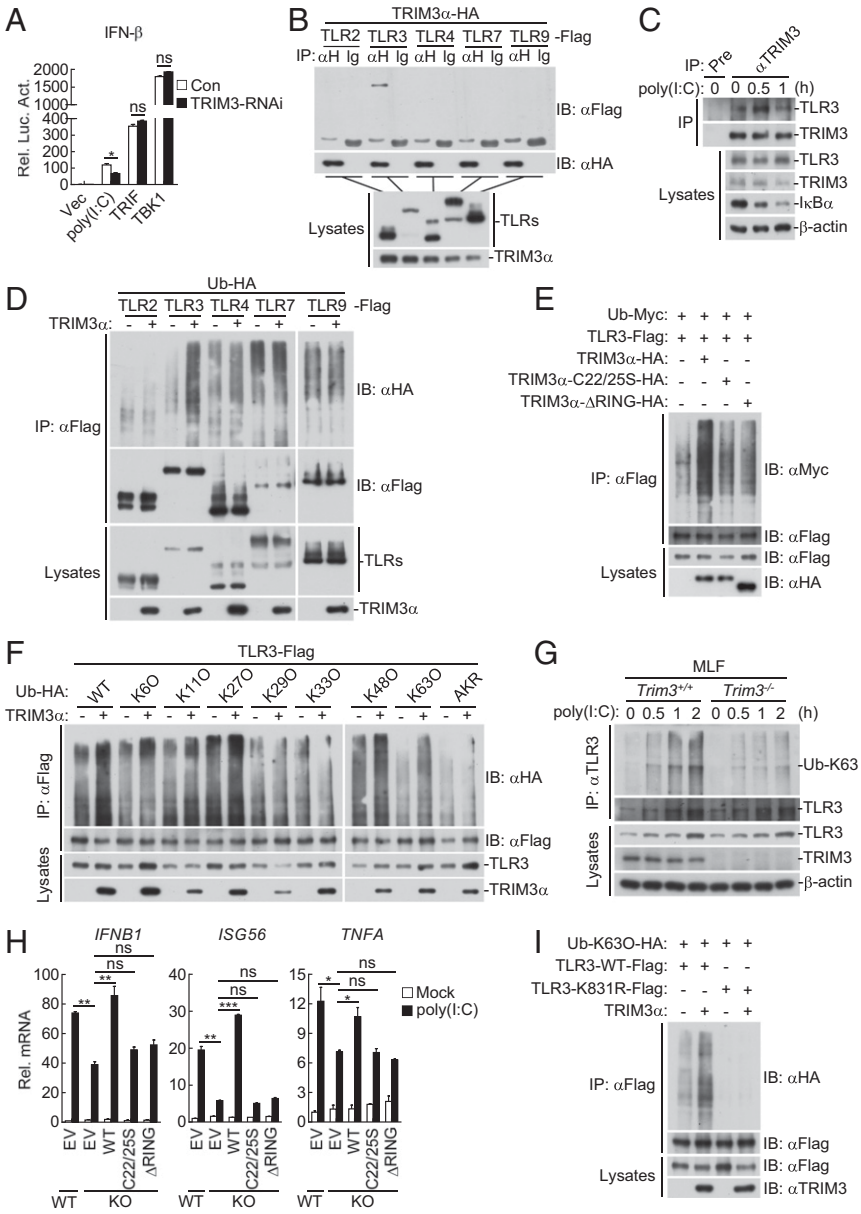

Fig. 3. TRIM3 specifically mediates K63-linked polyubiquitination of TLR3. (*A*) Effects of TRIM3 knockdown on the activation of IFN-β promoter induced by poly(I:C), TRIF, or TBK1. The control and TRIM3-RNAi HEK293-TLR3 cells (1×10^5) (TRIM3-RNAi #1 plasmid was used) were transfected with IFN-β reporter for 24 h and then treated with poly(I:C) (50 μg/mL) for 10 h or retransfected with TRIF or TBK1 plasmids for 18 h before luciferase assays. Data were analyzed by unpaired, two-tailed Student's *t* test. (*B*) TRIM3α interacts with TLR3 in mammalian overexpression system. HEK293 cells (2×10^6) were transfected with the indicated plasmids for 24 h. Coimmunoprecipitation and immunoblot analysis were performed with the indicated antibodies. (*C*) Endogenous TRIM3 is associated with TLR3. HEK293-TLR3 cells (2×10^7) were left untreated or treated with poly(I:C) (50 μg/mL) for the indicated times. Coimmunoprecipitation and immunoblot analysis were performed with the indicated antibodies. (*D*) TRIM3α mediates polyubiquitination of TLR3 but not TLR2/4/7/9. HEK293 cells (2×10^6) were transfected with the indicated plasmids for 24 h. Ubiquitination assays were performed with the indicated antibodies. (*E*) TRIM3α but not its C22/25S or ΔRING mutants mediates polyubiquitination of TLR3. Ubiquitination assays were similarly performed as in *D*. (*F*) TRIM3α mediates K63-linked polyubiquitination of TLR3. Ubiquitination assays were similarly performed as in *D*. (*G*) Effects of TRIM3 deficiency on poly(I:C)-induced K63-linked polyubiquitination of TLR3. *Trim3*$^{+/+}$ and *Trim3*$^{-/-}$ MLFs (2×10^7) were left untreated or treated with poly(I:C) (50 μg/mL) for the indicated times. Ubiquitination assays were performed with the indicated antibodies. (*H*) Reconstitution of TRIM3-deficient cells with TRIM3α but not its C22/25S or ΔRING mutants restores poly(I:C)-induced transcription of downstream genes. HEK293-TLR3 cells (2×10^5) (TRIM3-WT #2 and TRIM3-KO #2) were transduced with the indicated plasmids by retroviral-mediated gene transfer to establish the stable cell lines with G418 selection. Cells then were left untreated or treated with poly(I:C) (50 μg/mL) for 3 h before qPCR analysis. Data were analyzed by one-way ANOVA, followed by the Dunnett post hoc test. (*I*) TRIM3α does not mediate K63-linked polyubiquitination of TLR3(K831R). Ubiquitination assays were similarly performed as in *D*. Graphs show mean ± SD; $n = 3$. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, ns, not significant. IB: immunoblot, IP: immunoprecipitation, HA: human influenza virus hemagglutinin, EV: empty vector.

was verified by genotyping and immunoblotting analysis (*SI Appendix*, Fig. S2*B*). The homozygous *Trim3*$^{-/-}$ mice were born at the Mendelian ratio and showed normal growth and development as their wild-type littermates. We first prepared murine primary embryonic fibroblasts (MEFs), bone marrow-derived macrophages (BMDMs), and bone marrow-derived monocytes (BMDCs) from *Trim3*$^{-/-}$ mice and their wild-type littermates and then stimulated these cells with peptidoglycan (PGN), poly(I:C), LPS, and resiquimod (R848) (ligands for TLR2, TLR3, TLR4, and TLR7/8, respectively). We found that poly(I:C)-induced but not LPS-induced transcription of *Ifnb1*, *Isg56*, *Tnfa*, and *Il6* genes was dramatically inhibited in *Trim3*$^{-/-}$ MEFs, BMDMs, and BMDCs in comparison with their wild-type counterparts (Fig. 1*E* and *SI Appendix*, Fig. S2*C*). In addition, TRIM3 deficiency had no obvious effects on PGN- or R848-induced transcription of *Tnfa* and *Il6* genes (*SI Appendix*, Fig. S2*D*). Consistently, poly(I:C)-induced phosphorylation of TBK1, IRF3, p65, and IκBα was severely impaired in TRIM3-deficient MEFs, BMDMs, and BMDCs (Fig. 1*F* and *SI Appendix*, Fig. S2*E*). However, TRIM3 deficiency had no marked effects on LPS-induced phosphorylation of TBK1, IRF3, p65, or IκBα in BMDMs (*SI Appendix*, Fig. S2*F*). These results suggest that TRIM3 deficiency specifically attenuates TLR3-mediated signaling in divergent types of primary mouse cells.

TRIM3 Deficiency Attenuates poly(I:C)-Triggered Innate Immune and Inflammatory Responses in Mice. To further investigate whether TRIM3 regulates poly(I:C)-triggered innate immune and inflammatory responses in vivo, age- and sex-matched *Trim3*$^{-/-}$ mice and their wild-type littermates were injected intraperitoneally (i.p.) with poly(I:C) or LPS. We found that poly(I:C)-induced levels of serum cytokines including IFN-β, TNFα, and IL-6 in *Trim3*$^{-/-}$ mice were lower than those of their wild-type littermates, whereas there was no significant difference in the production of these cytokines between *Trim3*$^{-/-}$ mice and their wild-type littermates when treated with LPS (Fig. 2*A*). Similarly, after injection with poly(I:C) plus D-galactosamine, compared with their wild-type littermates, *Trim3*$^{-/-}$ mice showed attenuated inflammatory damage in lungs and alleviated apoptosis in livers as revealed by immunohistochemistry and terminal-deoxynucleotidyl transferase mediated nick end labeling (TUNEL) assays (Fig. 2*B*). Consistently, *Trim3*$^{-/-}$ mice experienced a lower percentage of lethality than their wild-type littermates following administration of poly(I:C) plus D-galactosamine. In these experiments, *Trim3*$^{-/-}$ and wild-type mice were similarly susceptible to LPS-induced death (Fig. 2*C*). These results suggest that TRIM3 deficiency attenuates TLR3- but not TLR4-mediated innate immune and inflammatory responses in mice.

TRIM3 Specifically Mediates K63-Linked Polyubiquitination of TLR3 at Lysine 831. We next investigated the mechanisms underlying how TRIM3 specifically regulates poly(I:C)-triggered signaling. Reporter assays indicated that knockdown of TRIM3 inhibited poly(I:C)-, but not TRIF- or TBK1-mediated activation of IFN-β promoter, which implied that TRIM3 might act on TLR3 (Fig. 3*A*). Coimmunoprecipitation experiments indicated that TRIM3α interacted with TLR3, but not TLR2, TLR4, TLR7, or TLR9 (Fig. 3*B*). In addition, endogenous TRIM3 was weakly associated with TLR3 in unstimulated HEK293-TLR3 cells, and their association was enhanced at 0.5 h and subsequently reduced at 1 h after poly(I:C) stimulation (Fig. 3*C*). Domain-mapping experiments indicated that the NHL domain of TRIM3α was essential for its interaction with TLR3 (*SI Appendix*, Fig. S3*A*). These results suggest that TRIM3 specifically associates with TLR3.

Since TRIM3 is an E3 ligase, we next explored whether TRIM3 ubiquitinates TLR3. As shown in Fig. 3*D*, TRIM3α specifically enhanced the polyubiquitination of TLR3 but not the other examined TLRs including TLR2, TLR4, TLR7, or TLR9. In addition, only TRIM3α but not its enzymatic inactive mutants C22/25S or ΔRING enhanced polyubiquitination of TLR3 (Fig. 3*E*). Cotransfection experiments with linkage-specific ubiquitins showed that TRIM3α mediated K63-linked polyubiquitination of TLR3 (Fig. 3*F*). Endogenous ubiquitination assays indicated that poly(I:C)-triggered K63-linked polyubiquitination of TLR3 was impaired in *Trim3*$^{-/-}$ murine lung fibroblasts (MLFs) (Fig. 3*G*). These results suggest that TRIM3 mediates K63-linked polyubiquitination of TLR3 upon poly(I:C) stimulation.

To further determine whether the E3 ubiquitin ligase activity of TRIM3 is necessary for its regulation of TLR3-mediated signaling, we generated TRIM3 knockout HEK293-TLR3 cells by the CRISPR/Cas9 strategy with a single guide RNA targeting the exon 1 of *TRIM3*. Two TRIM3 knockout clones (TRIM3-KO #1 and #2) and two wild-type clones (TRIM3-WT #1 and #2) were randomly selected for the following experiments. Expression of TRIM3 in these clones was examined by immunoblots (*SI Appendix*, Fig. S3*B*). Compared with TRIM3-WT, knockout of TRIM3 inhibited poly(I:C)-induced transcription of *IFNB1*, *ISG56*, and *TNFA* genes (*SI Appendix*, Fig. S3*B*). Reconstitution of TRIM3-KO cells with TRIM3α but not its enzymatic inactive mutants C22/25S or ΔRING restored poly(I:C)-induced transcription of *IFNB1*, *ISG56*, and *TNFA* genes as well as phosphorylation of TBK1, p65, and IκBα (Fig. 3*H* and *SI Appendix*, Fig. S3*C*). These results suggest that the E3 ubiquitin ligase activity of TRIM3 is indispensable for its regulation of TLR3-mediated signaling.

To map potential residues of TLR3 that are modified by TRIM3α, we constructed two truncation mutants of TLR3 and found that TRIM3α mediated polyubiquitination of TLR3(723-904) but not TLR3(22-708) (*SI Appendix*, Fig. S4*A*). We next individually mutated all 15 lysine residues within TLR3(723-904) to arginine. Reporter assays indicated that knockdown of TRIM3 inhibited activation of NF-κB and ISRE mediated by all TLR3 mutants except K831R. In addition, TLR3(K831R) activated NF-κB and ISRE to a much lower level compared with that of wild-type TLR3 upon poly(I:C) stimulation (*SI Appendix*, Fig. S4*B*). Consistently, polyubiquitination of all TLR3 mutants except K831R was enhanced by TRIM3α (*SI Appendix*, Fig. S4*C*). Moreover, TRIM3α failed to mediate K63-linked polyubiquitination of TLR3(K831R) (Fig. 3*I*). These results suggest that TRIM3 specifically mediates K63-linked polyubiquitination of TLR3 at K831.

TRIM3 Is Indispensable for TLR3 Trafficking. Previous studies have shown that TRIM3 is extensively located in the early endosomes, Golgi apparatus, and cytosol (25, 30). Our confocal microscopy experiments showed that in unstimulated cells, TRIM3α was mainly located in the Golgi apparatus but not visibly colocalized with the ER, late endosomes, recycling endosomes, or lysosomes (*SI Appendix*, Fig. S5*A*), whereas TLR3 was mostly localized in the ER and minimally found in early endosomes and lysosomes (*SI Appendix*, Fig. S5*B*). Upon poly(I:C) stimulation, TLR3 and TRIM3α were colocalized in the early endosomes (*SI Appendix*, Fig. S5*B*). Since TLR3 encounters internalized dsRNA and then get activated in the endolysosomes (31, 32), we speculated that TRIM3 might be involved in the trafficking of TLR3 to endolysosomes via endosomes.

We prepared BMDMs from *Trim3*$^{-/-}$ mice and their wild-type littermates and performed confocal microscopy experiments to investigate the role of TRIM3 in the trafficking of TLR3. Endogenous markers for the ER (KDEL), Golgi apparatus (GM130), early endosomes (RAB5A), and endolysosomes (LAMP1) were stained. The results indicated that TLR3 was mainly located in the ER in rest state and transported through the Golgi apparatus to early endosomes at ∼20 min, subsequently to endolysosomes at ∼40 min after poly(I:C) stimulation in wild-type BMDMs. Interestingly, the trafficking of TLR3 from the ER to Golgi apparatus following poly(I:C) stimulation was not affected, whereas the trafficking of TLR3 from the Golgi apparatus to early endosomes and further to endolysosomes was impaired, resulting in accumulation of TLR3 in the ER and Golgi apparatus in *Trim3*$^{-/-}$ BMDMs (Fig. 4*A*). Statistical

analysis of multiple colocalization images confirmed such results (Fig. 4*B*). It has been demonstrated that TLR3 is ultimately translocated to the endolysosomes where it is proteolytically cleaved to activate downstream signaling (31, 32). Therefore we investigated if TRIM3 affects TLR3 cleavage. As shown in *SI Appendix*, Fig. S5*C*, knockout of TRIM3 attenuated poly(I:C)-induced cleavage of TLR3 and reconstitution of TRIM3-KO cells with TRIM3α but not its C22/25S mutant restored poly(I:C)-induced cleavage of TLR3. These results suggest that TRIM3 and its enzymatic activity are indispensable for the trafficking of TLR3 from the Golgi apparatus to endolysosomes and its subsequent activation.

To further explore the role of TRIM3-mediated polyubiquitination of TLR3 at K831 in its trafficking and activation, we constructed HEK293 cells stably expressing TLR3 or TLR3(K831R) by retroviral transduction. Confocal microscopy indicated that the trafficking of TLR3(K831R) was impaired and TLR3(K831R) was accumulated in the ER and Golgi apparatus compared with wild-type TLR3 (*SI Appendix*, Fig. S6 *A* and *B*). In addition, poly(I:C)-triggered cleavage of TLR3 was attenuated in HEK293-TLR3(K831R) cells (Fig. 4*C*). Consistently, poly(I:C)-induced phosphorylation of TBK1, p65, and IκBα was also markedly inhibited in HEK293-TLR3(K831R) cells (Fig. 4*D*). These results suggest that TRIM3-mediated K63-linked polyubiquitination of TLR3 at K831 is required for the trafficking of TLR3 from the Golgi apparatus to endolysosomes and the subsequent activation of downstream signaling.

TRIM3 Promotes the Recognition and Sorting of Polyubiquitinated TLR3 by the ESCRT Complexes. Previously, it has been demonstrated that the ESCRT complexes are important for sorting of ubiquitinated membrane proteins to the endolysosomes (17, 33). We next investigated whether the ESCRT complexes play a part in the trafficking of TLR3 to endolysosomes. We constructed

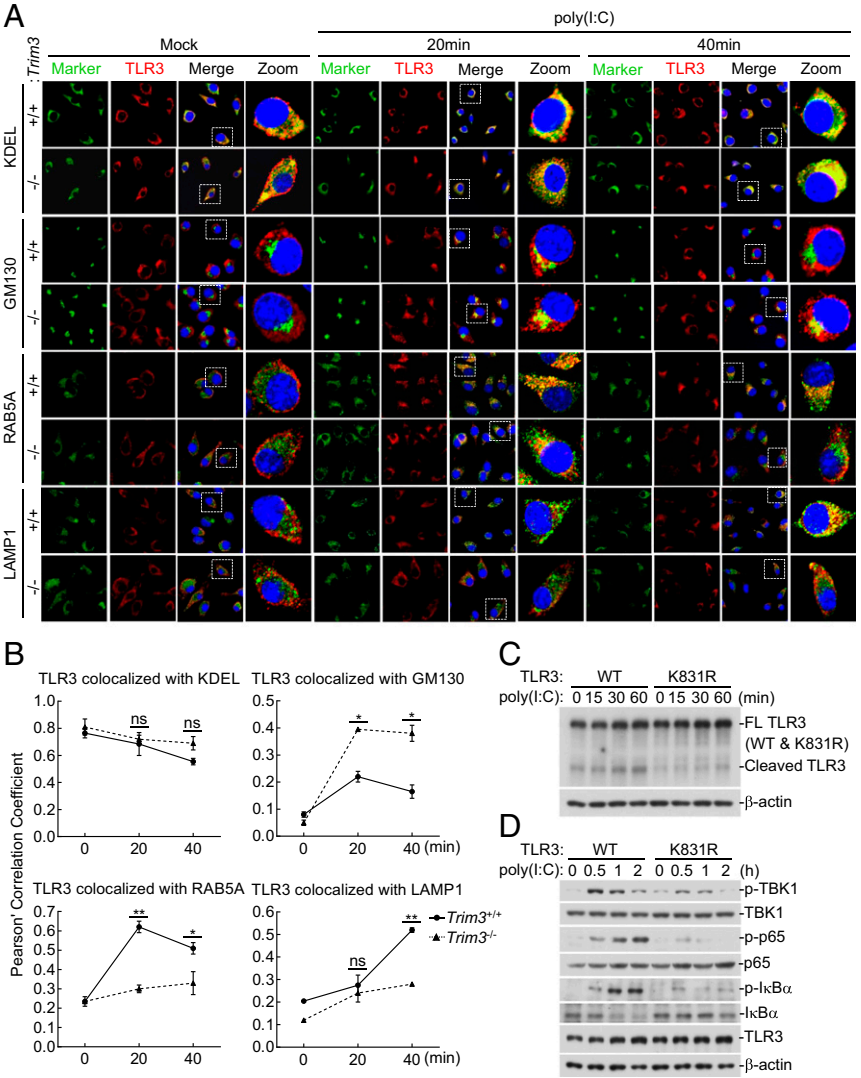

Fig. 4. TRIM3 deficiency impairs TLR3 trafficking. (*A*) TLR3 trafficking is impaired in *Trim3*$^{-/-}$ BMDMs. *Trim3*$^{+/+}$ and *Trim3*$^{-/-}$ cells (1×10^5) were left untreated or treated with poly(I:C) (50 μg/mL) for the indicated times, then fixed with 4% paraformaldehyde and stained with anti-TLR3 plus anti-KDEL, anti-GM130, anti-RAB5A, or anti-LAMP1 antibodies before confocal microscopy. The areas in the dashed boxes on the *Left* are enlarged on the *Right*. (*B*) Quantitative analysis of colocalization of TLR3 with KDEL, GM130, RAB5A, or LAMP1. Statistical analysis was based on at least two different colocalization images (covering dozens of cells) using the ImageJ software. Data were analyzed by unpaired, two-tailed Student's *t* test. Graphs show mean ± SD; $n = 2$. $^*P < 0.05$, $^{**}P < 0.01$, ns, not significant. (*C*) Poly(I:C)-induced cleavage of TLR3 is impaired when K831 is mutated to arginine. HEK293-TLR3 and HEK293-TLR3(K831R) cells (2×10^5) were left untreated or treated with poly(I:C) (50 μg/mL) for the indicated times before immunoblot analysis. FL: full length. (*D*) Poly(I:C)-induced phosphorylation of TBK1, p65, and IκBα is impaired when K831 is mutated to arginine. HEK293-TLR3 and HEK293-TLR3(K831R) cells (2×10^5) were left untreated or treated with poly(I:C) (50 μg/mL) for the indicated times before immunoblot analysis.

RNAi plasmids specifically targeting HRS, STAM1, STAM2, TSG101, and VPS36, the core components of the ESCRT-0, ESCRT-I, and ESCRT-II complexes that are responsible for recognition and binding of ubiquitinated cargoes (*SI Appendix*, Fig. S7*A*). Confocal microscopy and immunoblot results indicated that knockdown of these components impaired trafficking of TLR3 from the ER to endolysosomes as well as its proteolytic cleavage following poly(I:C) stimulation (Fig. 5*A* and *SI Appendix*, Fig. S7*B*). Knockdown of these components also attenuated transcription of *IFNB1*, *ISG56*, and *TNFA* genes induced by poly(I:C) but not SeV, and reconstitution of the knockdown cells with plasmids encoding these components restored poly(I:C)-induced transcription of downstream genes (Fig. 5*B* and *SI Appendix*, Fig. S7 *C* and *D*). Consistently, poly(I:C)-induced phosphorylation of TBK1, p65, and IκBα was also inhibited by knockdown of these components (Fig. 5*C*). These results suggest that the ESCRT complexes participate in the trafficking of TLR3 upon poly(I:C) stimulation.

Next, we investigated whether TRIM3-mediated polyubiquitination of TLR3 is important for its trafficking by the ESCRT complexes. Both coimmunoprecipitation and in vitro glutathione S-transferase (GST) pull-down assays showed that TLR3 interacted with the core ESCRT components such as HRS, STAM1, STAM2, and VPS36 (Fig. 6*A* and *SI Appendix*, Fig. S8*A*). However, TRIM3α specifically

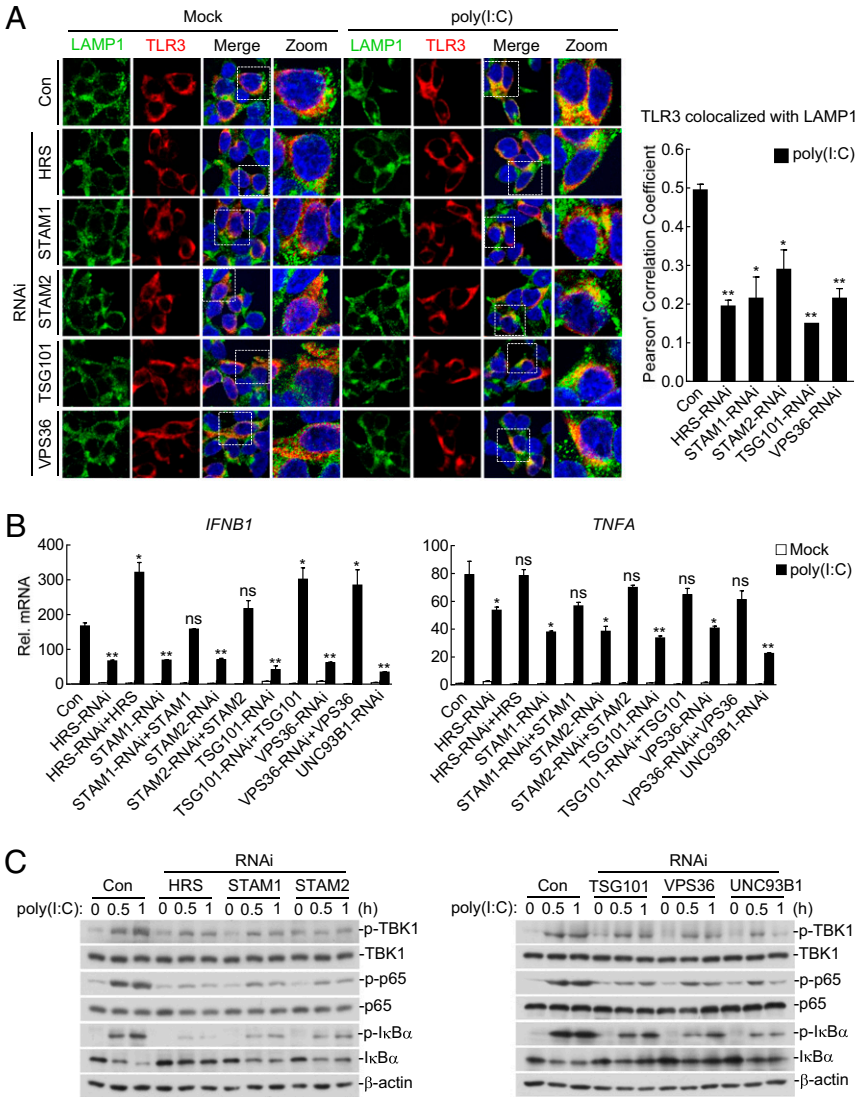

Fig. 5. Knockdown of HRS, STAM1, STAM2, TSG101, or VPS36 attenuates TLR3-mediated signaling. (*A*) Effects of HRS, STAM1, STAM2, TSG101, or VPS36 knockdown on poly(I:C)-triggered colocalization of TLR3 with LAMP1 (*Left*). The control and RNAi HEK293-TLR3 cells (1×10^5) were left untreated or treated with poly(I:C) (50 μg/mL) for 1 h, then fixed with 4% paraformaldehyde and stained with anti-TLR3 and anti-LAMP1 antibodies before confocal microscopy. Quantitative analysis of colocalization of TLR3 with LAMP1 (*Right*). Statistical analysis was based on at least two different colocalization images (covering dozens of cells) using the ImageJ software. (*B*) Effects of HRS, STAM1, STAM2, TSG101, or VPS36 knockdown as well as reconstitution of the knockdown cells with these components on poly(I:C)-induced transcription of downstream genes. These RNAi HEK293-TLR3 cells were transduced with control or the corresponding plasmids by retroviral-mediated gene transfer to establish the stable cell lines with G418 selection. The control and indicated RNAi as well as reconstituted HEK293-TLR3 cells (2×10^5) were left untreated or treated with poly(I:C) (50 μg/mL) for 3 h before qPCR analysis. UNC93B1 served as a positive control molecule in this experiment. (*C*) Effects of HRS, STAM1, STAM2, TSG101, or VPS36 knockdown on poly(I:C)-triggered phosphorylation of TBK1, p65 and IκBα. The control and RNAi HEK293-TLR3 cells (2×10^5) were left untreated or treated with poly(I:C) (50 μg/mL) for the indicated times before immunoblot analysis. Data in *A* and *B* were analyzed by one-way ANOVA, followed by the Dunnett post hoc test. The first column serves as the control to analyze the statistical differences. Graphs show mean ± SD; $n = 3$. $^*P < 0.05$, $^{**}P < 0.01$, ns, not significant.

interacted with HRS, but not STAM1, STAM2, TSG101, or VPS36 (Fig. 6*B*). Endogenous coimmunoprecipitation showed that the interaction between TRIM3 and HRS was enhanced following poly(I:C) stimulation (Fig. 6*C*). Domain-mapping experiments indicated that the Filamin domain of TRIM3α as well as the FYVE domain of HRS were necessary for their interaction (*SI Appendix*, Fig. S8*B*). Interestingly, TRIM3α but not its C22/25S or ΔFilamin mutants enhanced the interaction of TLR3 with HRS, TSG101, and VPS36 (*SI Appendix*, Fig. S9*A*). In *Trim3*$^{-/-}$ cells, poly(I:C)-induced sequential associations of TLR3 with HRS, TSG101, and VPS36 were markedly impaired (Fig. 6*D*). Moreover, TRIM3α promoted the interaction of TLR3 but not its K831R mutant with HRS, STAM1, STAM2, TSG101, and VPS36 (*SI Appendix*, Fig. S9*B*). These results suggest that TRIM3 plays an essential role for the recognition and binding of polyubiquitinated TLR3 by the ESCRT complexes.

As shown in Fig. 6*E*, TRIM3α or HRS alone promoted the proteolytic cleavage of TLR3, which was further enhanced by their cotransfection. Moreover, upon poly(I:C) stimulation, double knockdown of TRIM3 and HRS inhibited recruitment of TRIF, RIP1, and TBK1 more dramatically than individual knockdown of TRIM3 or HRS (Fig. 6*F* and *SI Appendix*, Fig. S9*C*). These results suggest that TRIM3 promotes the recognition and sorting of polyubiquitinated TLR3 by the ESCRT complexes to facilitate the activation of TLR3-mediated signaling.

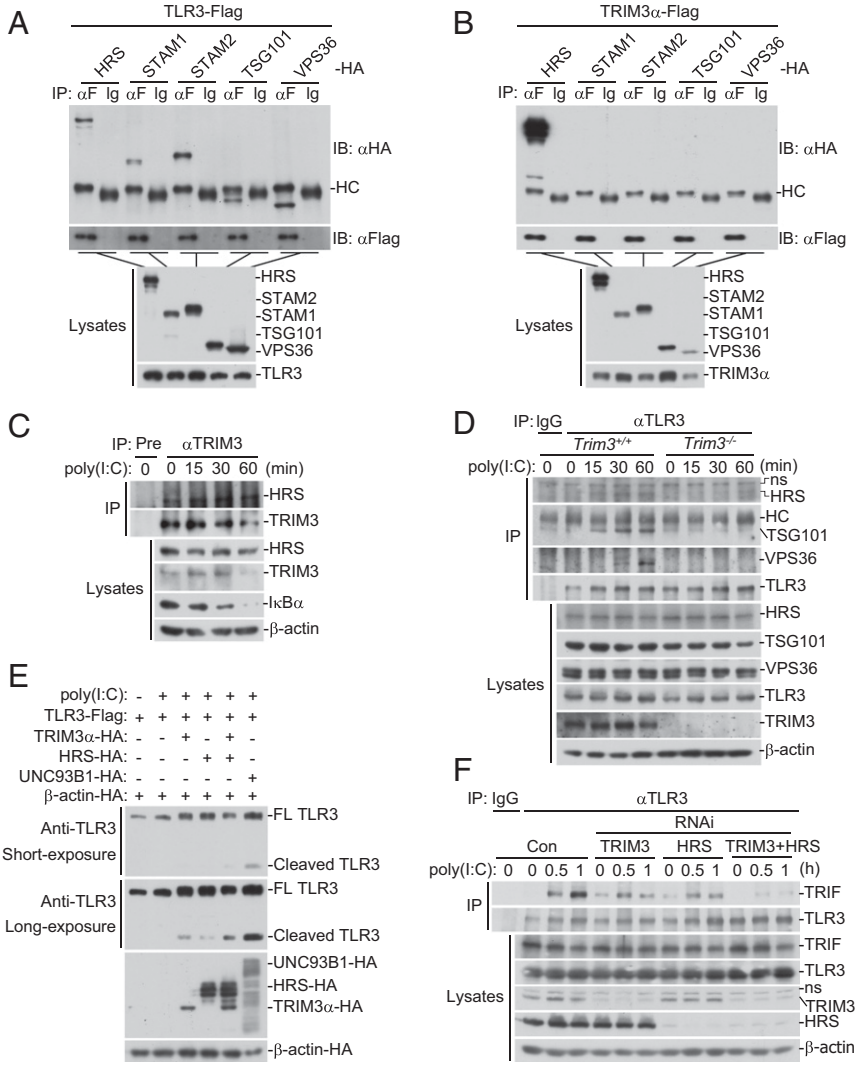

Fig. 6. TRIM3 promotes the recognition and sorting of polyubiquitinated TLR3 by the ESCRT complexes. (*A*) TLR3 interacts with HRS, STAM1, STAM2, TSG101, and VPS36. HEK293 cells (2×10^6) were transfected with the indicated plasmids for 24 h. Coimmunoprecipitation and immunoblot analysis were performed with the indicated antibodies. (*B*) TRIM3α interacts with HRS. Coimmunoprecipitation and immunoblot analysis were similarly performed as in *A*. (*C*) Endogenous TRIM3 is associated with HRS. HEK293-TLR3 cells (2×10^7) were left untreated or treated with poly(I:C) (50 μg/mL) for the indicated times. Coimmunoprecipitation and immunoblot analysis were performed with the indicated antibodies. (*D*) Effects of TRIM3 deficiency on poly(I:C)-induced association of TLR3 with HRS, TSG101, and VPS36. *Trim3*$^{+/+}$ and *Trim3*$^{-/-}$ MLFs (2×10^7) were left untreated or treated with poly(I:C) (50 μg/mL) for the indicated times. Coimmunoprecipitation and immunoblot analysis were performed with the indicated antibodies. (*E*) Effects of TRIM3α or HRS on poly(I:C)-induced cleavage of TLR3. HEK293 cells (2×10^5) were transfected with the indicated plasmids for 24 h and then left untreated or treated with poly(I:C) (50 μg/mL) for 12 h. Immunoblot analysis was performed with the indicated antibodies. (*F*) Effects of individual- or double-knockdown of TRIM3 and HRS on TLR3-mediated recruitment of TRIF. HEK293-TLR3 cells were transduced with control or the indicated RNAi plasmids by retroviral-mediated gene transfer to establish the stable cell lines (TRIM3-RNAi #1 plasmids were used). The control and RNAi HEK293-TLR3 cells (2×10^7) were left untreated or treated with poly(I:C) (50 μg/mL) for the indicated times. Coimmunoprecipitation and immunoblot analysis were performed with the indicated antibodies. IB: immunoblot, IP: immunoprecipitation, HA: human influenza virus hemagglutinin.

Discussion

TLR3, an endosomal TLR, recognizes viral dsRNA and plays a pivotal role in host antiviral immune response. Multiple post-translational modifications of TLR3 are important for its functions. For examples, *N*-linked glycosylation of the luminal domain of TLR3 is necessary for its bioactivity (34). Src- and BTK-mediated phosphorylation of the cytosolic domain of TLR3 at Y759 is critical for its ability to activate downstream signaling (35, 36). RNF170-mediated K48-linked polyubiquitination of TLR3 at K766 promotes its degradation and negatively regulates TLR3-mediated innate immune response (37). However, post-translational modifications that regulate the trafficking of TLR3 remain largely unknown. In this study, we found that overexpression of TRIM3 positively regulated TLR3-mediated signaling. TRIM3-deficient mice exhibited lower levels of inflammatory response following poly(I:C) but not LPS stimulation. In addition, TRIM3 mediated K63-linked polyubiquitination of TLR3, which was essential for TLR3 proper sorting by the ESCRT complexes to endolysosomes and its subsequent activation for innate antiviral response.

Previously, it has been demonstrated that the ER-localized protein UNC93B1 is responsible for exit of the nucleotide-sensing TLRs from the ER and proper translocation to the endolysosomes (38). Our experiments indicated that TRIM3 was localized in the Golgi apparatus in unstimulated cells, and poly(I:C) stimulation caused the colocalization of TLR3 and TRIM3 in the early endosomes. Most importantly, TRIM3 deficiency did not affect TLR3 trafficking from the ER to Golgi apparatus, but impaired its trafficking from the Golgi apparatus to endosomes. These results suggest that TRIM3 is required for TLR3 trafficking from the Golgi apparatus to endosomes.

It has been demonstrated that ubiquitinated TLR7/9 are sorted by the ESCRT-0 complex to endolysosomes for proteolytic cleavage to activate TLR7/9-mediated signaling (21). Our various biochemical experiments indicated that TRIM3 mediated K63-linked polyubiquitination of TLR3 at K831, which was increased following poly(I:C) stimulation. Mutation of TLR3 K831 to arginine impaired its ability to mediate poly(I:C)-triggered innate immune response. TLR3 was associated with HRS of the ESCRT-0 complex, and the association was increased following poly(I:C) stimulation. Interestingly, TRIM3 but not its enzymatic inactive mutant enhanced the association of TLR3 with HRS, whereas TRIM3 deficiency impaired their association. In addition, wild-type TLR3 but not its K831R mutant interacted with HRS in the presence of TRIM3. Consistently, other core components of the ESCRT complexes including STAM1, STAM2, TSG101, and VPS36 were also specifically associated with TLR3. These results suggest that TRIM3-mediated K63-linked polyubiquitin moieties of TLR3 are recognized by the ESCRT complexes, which results in the sorting of TLR3 to the endolysosomes and subsequent activation of downstream signaling.

Our results indicated that TRIM3-mediated polyubiquitination of TLR3 and its subsequent trafficking from the Golgi apparatus to endosomes were induced by poly(I:C) stimulation. Previously, it has been demonstrated that TLR3 transits continually to the endosomal compartments at a low level even in the absence of stimulation (4, 6, 8, 10). This phenomenon has also been inferred from our current study (Fig. 4*C* and *SI Appendix*, Figs. S5*C* and S7*B*). However, whether the basal trafficking and processing of TLR3 require TRIM3 needs additional investigation in the future. In light of these studies, we propose that TRIM3-mediated polyubiquitination and sorting of TLR3 represent a positive feedback mechanism on TLR3-mediated innate immune response. The signal triggered by TLR3 activation for TRIM3-mediated activity needs to be further investigated in future studies. Nevertheless, our findings provide insights into how the trafficking and activation of TLR3 are regulated for efficient innate immune response to extracellular and endosomal viral dsRNA.

Materials and Methods

Mice and Reagents. All animal experiments were approved by the Animal Care Committees of Wuhan Institute of Virology of the Chinese Academy of Sciences. The information on reagents, antibodies, constructs, PCR primers, and RNAi target sequences are described in *SI Appendix, Materials and Methods*.

Methods. The methods for reporter assays; qPCR; coimmunoprecipitation and immunoblot analysis; generation of MEFs, BMDMs, and BMDCs; ELISA; CRISPR/Cas9 knockout; establishment of stable cell lines; ubiquitination assays; confocal microscopy; in vitro GST pull-down assays; and statistical analysis are presented in *SI Appendix, Materials and Methods*.

Data Availability. All relevant data, associated protocols, and materials are in the paper and *SI Appendix*.

ACKNOWLEDGMENTS. This study was supported by the Strategic Priority Research Program (XDB29010302, awarded to Y.-Y.W.), the National Natural Science Foundation of China (31621061, awarded to Y.-Y.W.), and Key Research Programs of Frontier Science (2017YFA0505800, awarded to Y.-Y.W.). We thank Xue-Fang An and Fan Zhang from the Center for Animal Experiment at Wuhan Institute of Virology for their help with animal experiments. We thank Ding Gao and Juan Min from the Center for Instrumental Analysis and Metrology at Wuhan Institute of Virology for their help with confocal and immunohistochemistry experiments.

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