Polyubiquitin conjugation to NEMO by triparite motif protein 23 (TRIM23) is critical in antiviral defense

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The rapid induction of type I IFN is a central event of the innate defense against viral infections and is tightly regulated by a number of cellular molecules. Viral components induce strong type I IFN responses through the activation of toll-like receptors (TLRs) and intracellular cytoplasmic receptors such as an RNA helicase RIG-I and/or MDA5. According to recent studies, the NF-κB essential modulator (NEMO, also called IKKγ) is crucial for this virus-induced antiviral response. However, the precise roles of signal activation by NEMO adaptor have not been elucidated. Here, we show that virus-induced IRF3 and NF-κB activation depends on the K(lys)-27-linked polyubiquitination to NEMO by the novel ubiquitin E3 ligase triparite motif protein 23 (TRIM23). Virus-induced IRF3 and NF-κB activation, as well as K27-linked NEMO polyubiquitination, were abrogated in TRIM23 knockdown cells, whereas TRIM23 knockdown had no effect on TNFα-mediated NF-κB activation. Furthermore, in NEMO-deficient mouse embryo fibroblast cells, IFN-stimulated response element-driven reporter activity was restored by ectopic expression of WT NEMO, as expected, but only partial recovery by NEMO K165/309/325/326/344R multipoints mutant on which TRIM23-mediated ubiquitin conjugation was substantially reduced. Thus, we conclude that TRIM23-mediated ubiquitin conjugation to NEMO is essential for TLR3- and RIG-I/MDA5-mediated antiviral innate and inflammatory responses.

innate immunity | signal transduction | virus infection

Upon viral infection, host cells recognize the viral components and activate innate immune signaling to exert antiviral responses (1–4). RIG-I and/or MDA5 sense viral dsRNA (5–8) and are recruited to another antiviral signaling adaptor, IPS-1 (also called MAVS, Cardif, or VISA) (9–12). IPS-1 directly interacts with TRAF3 and triggers auto-ubiquitination of TRAF3, which then activates TBK1 and IKKε, leading to activation of transcription factors NF-κB and IRF3 (13, 14). A recent study indicated that NEMO acts upstream of TBK1 and IKKε and is essential for virus-induced TLR3- and RIG-I/MDA5–mediated antiviral activation (15).

Because rapid induction of type I IFN expression is the key process in initiating the innate antiviral response, clarification of NEMO-mediated antiviral signaling is important for understanding innate immune signaling; however, NEMO-mediated antiviral signaling is not well elucidated. Recent studies indicate that several ubiquitin E3 ligases are involved in the regulation of innate immune signaling (16–21). We identified the ubiquitin E3 ligase TRIM23 (Triparite motif protein 23), also named ADP ribosylation factor domain protein 1 (ARD1), which was reported to have E3 ligase activity in vitro (22), that functioned as an E3 ligase for NEMO ubiquitin conjugation. TRIM23 exerts a potent antiviral state following its overexpression. Furthermore, we demonstrated that antiviral activity depends not on K(Lys)63-linked but on K27-linked polyubiquitin conjugation to multiple sites of NEMO by TRIM23 expression. Virus-induced IRF3 and NF-κB activation, as well as K27-linked NEMO polyubiquitination, were abrogated in TRIM23 knockdown cells (including primary mouse embryonic fibroblasts), whereas TRIM23 knockdown had no effect on TNFα-mediated NF-κB activation.

Results

TRIM23 Interacts with NEMO. Recent studies indicate that several ubiquitin E3 ligases are involved in the regulation of innate immune signaling (16–21). We previously reported that the E3 ubiquitin ligase RNF125 negatively regulates RIG-I signaling (17), and it has been reported that RNF125 is also a T-cell activator (23), which suggests the presence of plural functions of RNF125 in regulation of cell proliferation. To identify genes affected by RNF125, we conducted microarray analysis (mock- vs. RNF125-transfected 293T cells) and found the gene for TRIM23 up-regulated ∼3-fold (Fig. S1*A*). Through analysis of function of TRIM23, we also found that TRIM23 up-regulated the NF-κB–driven reporter gene in cells expressing NEMO. Introduction of TRIM23 slightly activated NF-κB in cells expressing endogenous NEMO and substantially activated NF-κB in cells ectopically expressing NEMO. Furthermore, we found that NEMO migrated slowly by SDS/PAGE when coexpressed with TRIM23 (Fig. S1*B*), suggesting posttranslational modification, most likely ubiquitin conjugation by TRIM23. It has been reported that TRIM23 has E3 ligase activity in vitro (22). Because ubiquitin E3 ligases generally require association with the substrate to execute its enzyme activity, we analyzed the association of TRIM23 with NEMO by GST-pulldown and coimmunoprecipitation assays. These results showed that TRIM23 interacted with NEMO directly (Fig. S1*C*). Deletion analysis of NEMO showed that both the CC1 and LZ domains of NEMO are essential for this interaction (Fig. 1 *A* and *C*). Binding analysis showed further that the TRIM23 C-terminal ARF domain interacted with NEMO CC1 and LZ domains as effectively as the full-length TRIM23, whereas the RING finger and B-box/B-box/CCD domains did not (Fig. 1 *A, B,* and *D*). Bifluorescent complementation analysis also revealed interaction of NEMO with TRIM23 in living HeLa cells (Fig. 1*E*). An interaction between endogenous NEMO and TRIM23 was also detected in 293T cells (Fig. 1*F*).

TRIM23 Is an E3 Ligase for Conjugation of K27 Type Ubiquitin to NEMO. To examine whether TRIM23 ubiquitinates NEMO, NEMO-FLAG was coexpressed with WT TRIM23 or its E3 ligase activity-defective RING mutants (TRIM23C34A and TRIM23ΔRING). Although TRIM23 expression markedly increased the ubiquitin conjugation levels of NEMO-FLAG, neither TRIM23C34A nor TRIM23ΔRING had any effect (Fig. 2*A*). Levels of ubiquitin conjugation to NEMO were enhanced by increasing amounts of TRIM23. Under these conditions, the mRNA levels of NEMO, GAPDH, and tubulin were unchanged (Fig. 2*B*). By analyzing mutants of ubiquitin for conjugation to NEMO in a TRIM23-dependent manner, we observed that the K27-only type could be

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The authors declare no conflict of interest.

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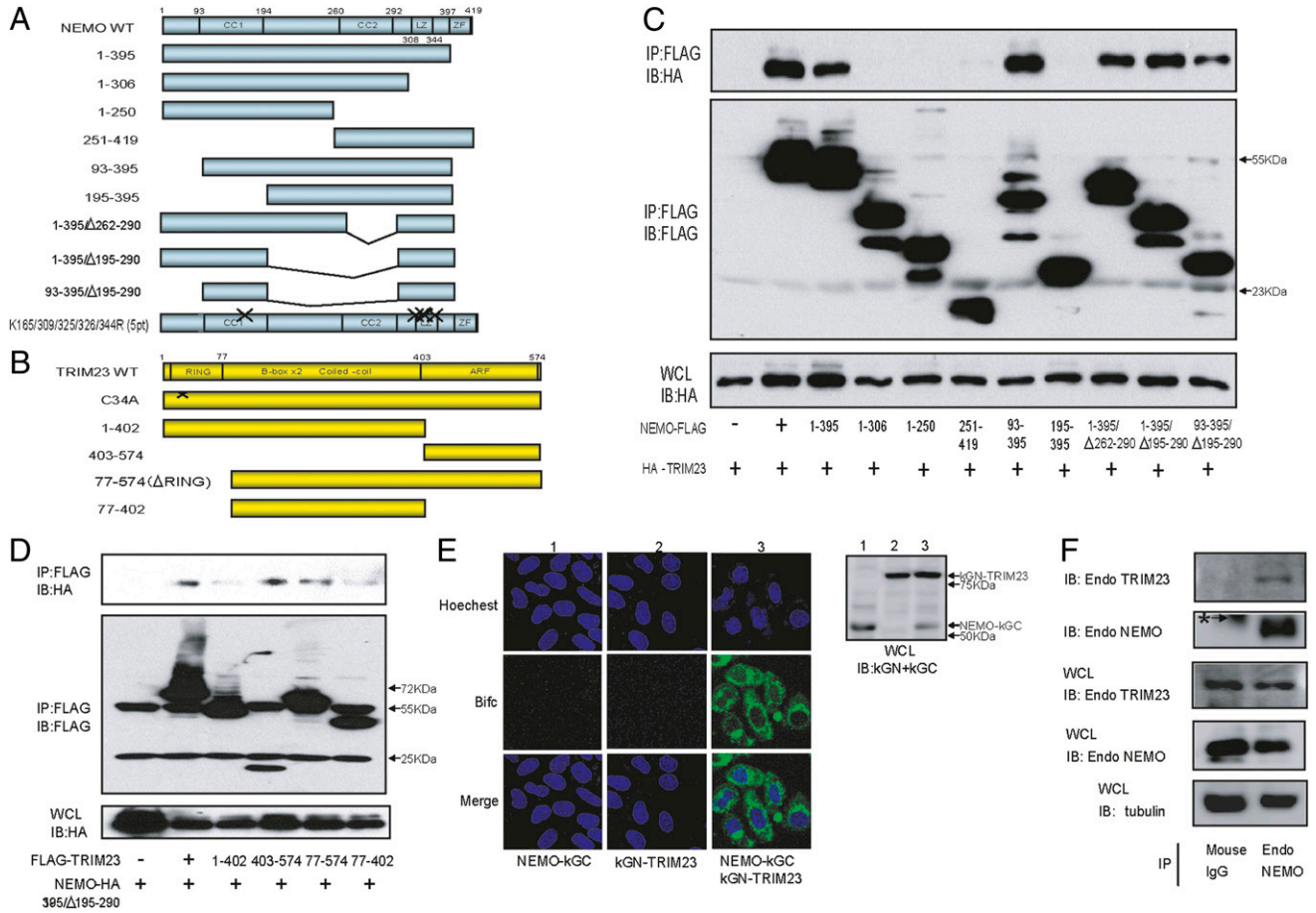

Fig. 1. Interaction between NEMO and TRIM23. (*A* and *B*) Schematic drawings of NEMO, TRIM23, and their derivatives used in this work. CC1 coiled-coil domain 1; CC2, coiled-coil domain 2; LZ, leucine zipper; ZF, zinc finger. RING finger domain, B-box-B-box/CCD (coiled-coil domain), and ARF (ADP ribosylation factor) domain in TRIM23 are shown. X indicates sites of mutagenesis described in the text. (*C*) 293T cells were cotransfected with plasmids encoding HA-TRIM23 and NEMO-FLAG or its mutants. (*D*) NEMO CC1 and LZ domains interacted with the TRIM23 ARF region in 293T cells. (*E*) HeLa cells were transfected with plasmids encoding NEMO-kGC and kGN-Mock, kGN-TRIM23 and Mock-kGC, or NEMO-kGC and kGN-TRIM23. Nuclear localizations were detected by Hoechst 33342. Bifc signal revealed TRIM23-NEMO–specific association. Expression of NEMO and TRIM23 in HeLa cells were also confirmed by immunoblot using anti-kGN and -KGC antibody. (*F*) Interaction of endogenous NEMO with endogenous TRIM23 in 293T cells 24 h after SV infection. Asterisk denotes nonspecific band.

conjugated as polyubiquitin to NEMO (Fig. 2*C*). This was further confirmed by the ubiquitin conjugation of K63R, but not of K27R mutant of ubiquitin (Fig. 2*D*). NF-κB reporter activity in cells ectopically expressing K27-only ubiquitin was higher when compared with K27R ubiquitin in NEMO and TRIM23 expressing 293T cells (Fig. 2*E*). An in vitro ubiquitination assay showed that TRIM23 could use UbcH1, 5a, 5b, 5c, and 13/Mms2 as an ubiquitin E2-conjugating enzyme (Fig. S2*A*). Among these E2 enzymes, the presence of UbcH5s could conjugate ubiquitin to NEMO at shorter time of reaction, suggesting that the presence of UbcH5s, rather than UbcH1 or Ubc13/Mms2, may have a strong ability to conjugate ubiquitin to NEMO. When using the K27-only ubiquitin mutant, only UbcH5a, 5b, and 5c showed ubiquitin conjugation activity, suggesting that these may be the major E2 enzymes functioning in vivo (Fig. S2*B*).

In the analysis using deletion mutants of NEMO, it was observed that TRIM23 could conjugate ubiquitin preferentially to the NEMO CC1 and LZ domains (Fig. S3*A*). Single point mutation of K to R in these domains of WT-NEMO was not affected by TRIM23 expression at the ubiquitin conjugation level (Fig. S3*B*). In an analysis of several NEMO mutants having mutations on plural lysine residues in these domains of NEMO, we observed that ubiquitin conjugation to NEMO K165/309/325/326/344R, NEMO-5pt, was substantially reduced when compared with that of the WT NEMO. This suggests the importance of these five lysine residues in TRIM23-dependent ubiquitin conjugation to NEMO, at least in part (Fig. 2*F*).

TRIM23 Exerts a Potent Antiviral State Following Its Overexpression. NEMO has critical roles in virus-induced innate and inflammatory responses (15). To investigate the roles of TRIM23-mediated NEMO ubiquitination, we examined IFNβ, ISRE, or NF-κB promoter-driven reporter activity by expressing TRIM23 or TRIM23C34A after treating cells with poly I:C, infection with Sendai virus (SV), or coexpressing upstream adaptor molecules of innate immunity signaling. Ectopic expression of WT TRIM23, together with plasmids encoding TLR3, RIG-I, or IPS1, upregulated ISRE reporter activity. Expression of TRIM23C34A suppressed the reporter activity (Fig. S4A). Same results were observed in both IFNβ and NF-κB reporter assays with polyI:C or SV infection (Fig. S4 *B* and *C*). Consistent with the NEMO ubiquitination level (Fig. 2*B*), NF-κB promoter activity considerably increased and decreased dose-dependently with TRIM23 and TRIM23C34A expression, respectively (Fig. S4*D*). Reduction of the reporter activity by TRIM23C34A seems to have a dominant negative effect on endogenous TRIM23, although the precise mechanism requires further clarification.

Suppression of TRIM23 Impaired K27-Linked Ubiquitin Conjugation to NEMO and Virus-Induced Antiviral Activity. To examine the physiological roles of TRIM23 in antiviral innate immunity, we analyzed IFNβ and NF-κB reporter activity in cells knocked-down of TRIM23 by specific siRNA. We also established 293T cells that were knocked down TRIM23 constitutively (Fig. 3*A*). TRIM23 knockdown impaired IFNβ reporter activity by SV infection and

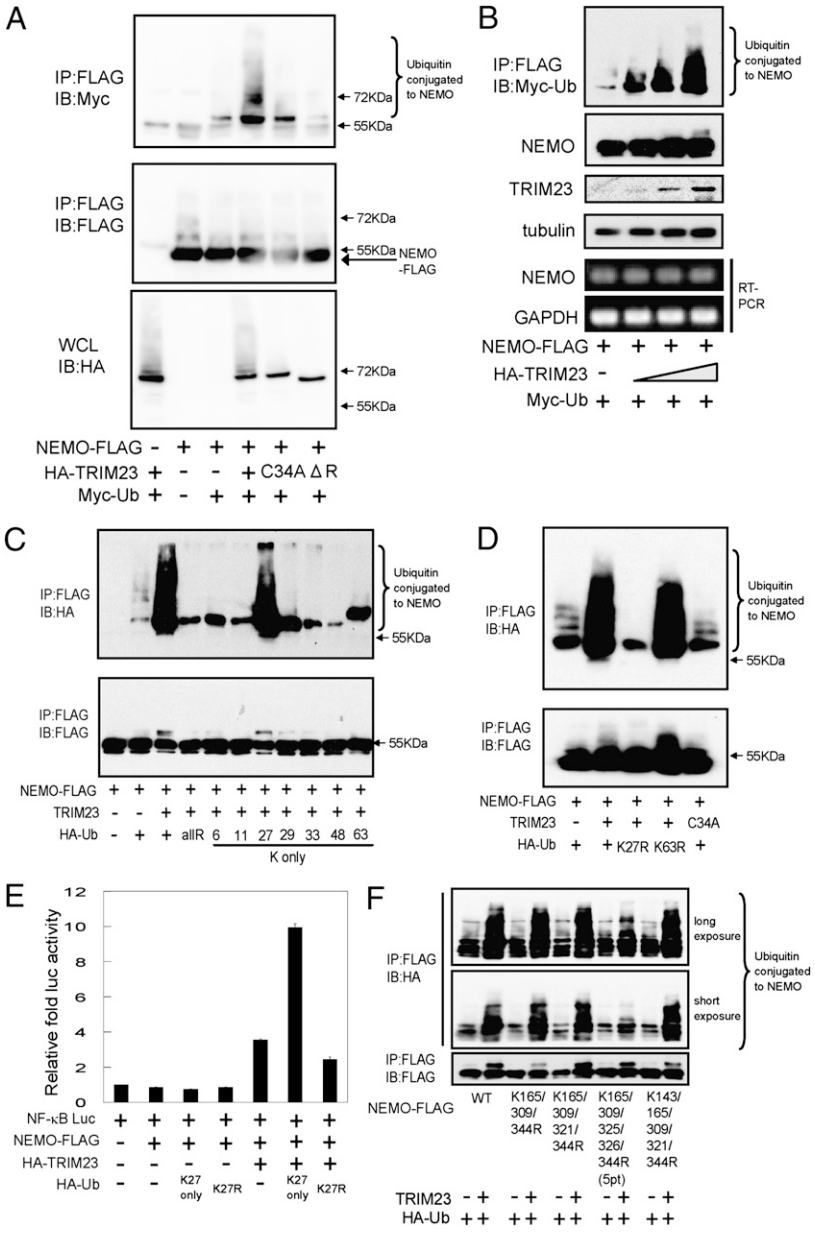

Fig. 2. TRIM23 conjugates K27-linked polyubiquitin to NEMO at multiple sites. (*A*) 293T cells were cotransfected with plasmids encoding NEMO-FLAG, HA-TRIM23, HA-TRIM23C34A (abbreviated as C34A), HA-TRIM23 ΔRING (abbreviated as ΔR), and Myc-Ub as indicated. (*B*) Ubiquitin conjugation to NEMO is enhanced dose-dependent TRIM23 expression. 293T cells were cotransfected with plasmids encoding NEMO-FLAG, Myc-Ub, or plasmid (0–2 μg) expressing HA-TRIM23. Levels of NEMO and GAPDH mRNA are also shown. (*C*) 293T cells were cotransfected with plasmids expressing NEMO-FLAG, TRIM23, and HA-Ub (WT, KallR, K6-, 11-, 27-, 29-, 33-, 48-, and 63-only). (*D*) 293T cells were cotransfected with plasmids encoding NEMO-FLAG, TRIM23, TRIM23C34A, and HA-Ub (WT, K27R, or K63R). (*E*) The K27-only ubiquitin conjugation enhanced NEMO-TRIM23-mediated NF-κB reporter activity. 293T cells were transfected with plasmids encoding NF-κB-luc, NEMO-FLAG, or HA-TRIM23, together with plasmids expressing HA-K27-only Ub or HA-K27R Ub as indicated. (*F*) NEMO KR mutants expressing in 293T cells transfected with plasmids encoding TRIM23 and HA-Ub were analyzed for their ubiquitin conjugation.

poly I:C treatment, depending on the knockdown level of TRIM23 (Fig. 3*A*). Similar results were obtained in the murine leukemic monocyte macrophage cell line Raw264.7 (Fig. S4*E*). TRIM23 knockdown also impaired NF-κB reporter activity in poly I:C treatment, but did not impair that which was activated by TNFα treatment (Fig. 3*A*). Virus-induced IFNβ mRNA and phosphorylation of STAT1 and IRF3, as well as IFN-inducible ISG15, were substantially reduced in cells expressing sh-TRIM23-1 (Fig. 3*B*). Thus, quantitative analysis indicated that the TRIM23 level was correlated with antiviral inflammatory and IFN responses (Fig. 3 *A* and *B*). TRIM23 knockdown also impaired IFNβ reporter activity activated by RIG-I and IPS-1 expression in 293T cells (Fig. 3*C*). NEMO is upstream of TBK1 (15). To investigate whether TBK1-triggered IFNβ activation is regulated by TRIM23 expression, we conducted an IFNβ luciferase reporter assay using NEMO$^{-/-}$ MEFs. As a result, TBK1-mediated IFNβ up-regulation was not affected at all in TRIM23 siRNA-treated cells (Fig. 3*D*).

Furthermore, upon SV infection, we did not observe TRIM23-mediated ISRE reporter enhancement in TBK1/IKKi double knockout MEFs (TBK1$^{-/-}$IKKi$^{-/-}$), although the activity was significantly enhanced in WT MEFs (Fig. S4*F*). These results indicate that the TRIM23-NEMO complex is important for antiviral responses, and that TRIM23 does not exert its function directly on downstream molecules of NEMO, such as TBK1, in antiviral signaling.

IRF3 dimerization and IRF3 phosphorylation promoted by IPS-1 expression or SV infection were impaired in TRIM23 knockdown 293T cells (Fig. 3*E*). In 293T cells, the effect of knockdown of TRIM23 was more clearly observed than the effect of overexpression of TRIM23, suggesting that endogenous TRIM23 in 293T cells was high and can sufficiently function at physiological levels for antiviral responses in this cell line.

To further examine the physiological relevance of TRIM23 in antiviral responses, we measured the amount of IFNβ in culture medium by ELISA using primary WT and TRAF6$^{-/-}$ MEFs. TRIM23 knockdown decreased IFNβ production with SV infection in both cells (Fig. 3*F*). The level of phosphorylated STAT1 was up-regulated by the ectopic expression of TRIM23, and down-regulated by the expression of TRIM23C34A in WT as well as TRAF6$^{-/-}$MEFs (Fig. S5*A*). These results suggest that TRIM23 is more important than TRAF6 for virus-induced IFNβ production, which is in line with works reported by other groups (24–26). Importantly, we observed that SV-induced endogenous NEMO ubiquitination diminished in TRIM23 knockdown 293T cells (Fig.

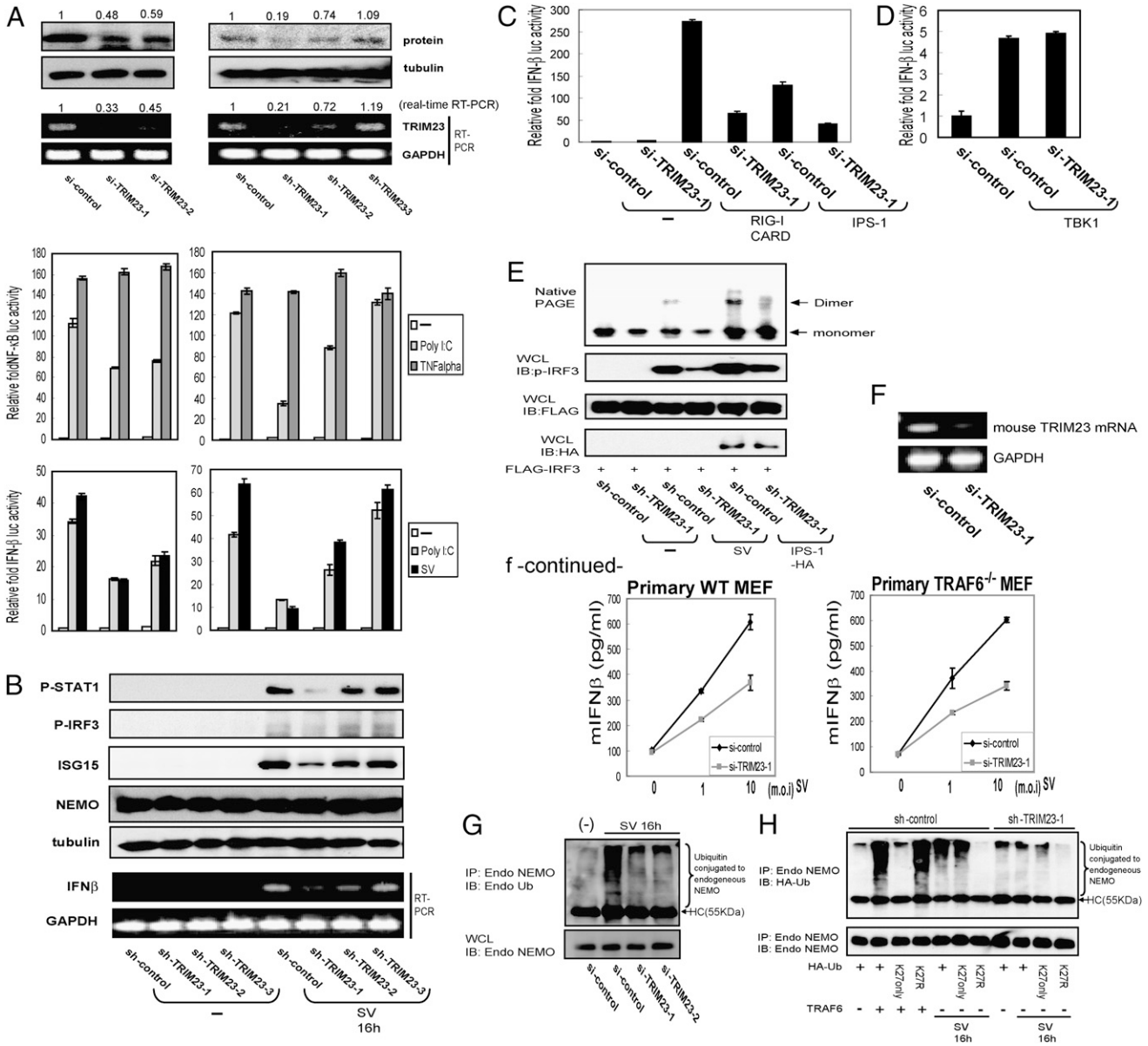

Fig. 3. TRIM23 knockdown impaired both ubiquitin conjugation to NEMO and antiviral responses. (*A*) Efficiency of TRIM23 knockdown using siRNA and shRNA in 293T cells (*Top*). Si-TRIM23-1 and sh-TRIM23-1 significantly suppressed levels of TRIM23. Protein and mRNA levels of TRIM23 were also quantified by ImageJ software and real-time RT-PCR, respectively (*Top*). The mRNA levels of TRIM23 were also visualized by RT-PCR (*Top*). Under these conditions, NF-κB (*Middle*) and IFNβ (*Bottom*) luciferase activities were measured after treatment with PolyI:C (20 μg/mL), TNFα (10 ng/mL), or infected with SV (m.o.i. 10). (*B*) Control and TRIM23 knockdown 293T cells were infected with or without SV (m.o.i 10) for 16 h. Cell lysates were subject to Western blot with the indicated antibodies. The levels of IFNβ and GAPDH mRNA were also analyzed. (*C*) 293T cells were treated with si-TRIM23-1 or control si-RNA. At 48 h after siRNA transfection, cells were further transfected with plasmids encoding IFNβ-Luc, RIG-I CARD, or IPS-1. At 72 h after siRNA transfection, luciferase activity was measured. (*D*) NEMO$^{-/-}$ MEFs were treated with si-TRIM23-1 or control si-RNA. At 48 h after siRNA transfection, cells were further transfected with plasmids encoding IFNβ-Luc and TBK1. At 72 h after siRNA transfection, luciferase activity was measured. (*E*) 293T cells treated with sh-TRIM23-1 were transfected with plasmids encoding FLAG-IRF3 or IPS-1-HA, and then cells were mock infected or infected with SV for 16 h as indicated. Dimer formation of IRF3 was analyzed. Phosphorylated IRF3 and the total amount of IRF3 in cell lysates as well as IPS-1-HA are also shown. (*F*) Suppression of IFNβ production in WT and TRAF6$^{-/-}$ MEFs treated with si-TRIM23-1. The mRNA levels of murine TRIM23 were visualized by RT-PCR (*Upper*). IFNβ in culture medium was analyzed by ELISA (*Lower*). (*G*) 293T cells were treated with control siRNA, si-TRIM23-1, or si-TRIM23-2. At 48 h after the transfection, cells were mock infected or infected with SV (m.o.i. 10). At 16 h after infection, cell lysates were analyzed for ubiquitin conjugation of NEMO. (*H*) TRAF6 did not enhance K27-linked ubiquitin conjugation to NEMO, but SV infection did. However, this conjugation was abrogated by TRIM23 knockdown. The sh-control and sh-TRIM23-1–treated cells were transfected with plasmids encoding HA-Ub WT, K27-only, or K27R. Cells were then either transfected with plasmids encoding TRAF6 or infected with SV for 16 h.

3*G*). Ubiquitin conjugation to endogenous NEMO occurred in K27-only type after SV infection, which was reduced in TRIM23 knockdown 293T cells. In contrast, the ectopic expression of TRAF6 enhanced K27R and not the K27-only type (Fig. 3*H*). Endogenous ubiquitin conjugation to endogenous NEMO was up-regulated upon viral infection, and TRIM23 knockdown impaired this. Nonetheless, the protein level of TRIM23 and the interaction between TRIM23 and NEMO were not affected by viral infection (Fig. S5*B*). Considering that some E2 ubiquitin conjugating enzymes are IFN inducible and that several E2 enzymes and E4 enzymes affect ubiquitin assembly (27–30), it may be that TRIM23-mediated ubiquitin conjugation to NEMO is regulated

by a specific E2 (such as UbcH5, as suggested in an in vitro assay in this study) and/or E4 enzyme(s) that respond to viral infection. Indeed, in a preliminary experiment, the association of NEMO and UbcH5 was increased by Sendai virus infection (Fig. S5*B*). Furthermore, knockdown of UbcH5 not UbcH1 or Ubc13 substantially impaired TRIM23-mediated ubiquitin conjugation to NEMO (Fig.S5*C*). Moreover, TRIM23 conjugates UbcH5s slightly stronger than UbcH1 or Ubc13 in 293T cells (Fig.S5*D*).

To investigate whether TRIM23-mediated NEMO polyubiquitination influences antiviral responses, we measured ISRE and NF-κB luciferase reporter activity in NEMO$^{-/-}$ MEFs transiently expressing WT-NEMO, NEMO-5pt, or vector alone. The efficiency of transfection was the same as judged from Western blots of NEMO (Figs. 4 *A* and *B*). After SV infection, both ISRE and NF-κB luciferase activities in cells expressing NEMO-5pt deteriorated when compared with WT-NEMO expression (Figs. 4 *A* and *B, Left*). Ubiquitin conjugation to NEMO-5pt by TRIM23 was reduced when compared with WT-NEMO (Figs. 2*F* and 4*C*). Despite a significant reduction in its level of ubiquitination, NEMO-5pt interacted with TRIM23 (Fig. S6) as efficiently as WT-NEMO, suggesting that the reduction of activation of downstream reporter genes by NEMO-5pt was attributed to a lower level of ubiquitin conjugation. In contrast, NF-κB luciferase activity in cells expressing WT-NEMO and NEMO-5pt by ectopic expression of TRAF6 in NEMO$^{-/-}$ MEFs did not show significant difference (Fig. 4*B, Right*). Ubiquitin conjugation to WT-NEMO and NEMO-5pt in cells expressing TRAF6 was almost the same level (Fig. 4*C*). Ectopic expression of TRIM23 alone or the expression of the deletion mutant of NEMO, which cannot interact with TRIM23 and therefore lack ubiquitin conjugation, in NEMO$^{-/-}$ MEFs did not activate ISRE- and NF-κB reporter genes upon SV infection (Fig. S7). Thus, it is likely that ubiquitin conjugation to NEMO by TRIM23 is a requisite for the activation of ISRE- and NF-κB reporter genes in SV infection.

TRIM23 Knockdown Cells Produce More Virus. To corroborate that TRIM23 is involved in virus-mediated innate signaling, we infected vesicular stomatitis virus (VSV) at various multiplicity of infection (m.o.i.) to WT and TRIM23 knockdown primary MEF cells. Silencing of endogenous TRIM23 resulted in higher number of cells killed compared with the control cells (Fig. 4*D, Left*). Quantification of virus titration showed that the yield of infectious virus in TRIM23 knockdown cells was ~1 log higher than that in WT MEF cells (Fig. 4*D, Right*). Similar results were obtained in 293T cells, in which experiment the effect of IPS1 expression was also shown (Fig. S8). Thus, we conclude that TRIM23 plays important roles in facilitating TLR3- and RIG-I/MDA5-mediated antiviral innate signaling.

Discussion

Here, we demonstrated an essential function of TRIM23 in antiviral activity. TRIM23 induces K27-linked polyubiquitination of NEMO, leading to the activation of downstream signaling for antiviral function. Virus-induced production of endogenous IFN and IFN-inducible gene expression, as well as K27-linked NEMO polyubiquitination, were substantially impaired by TRIM23 knockdown, which was not limited to cultured cell lines but was also seen in primary WT and TRAF6$^{-/-}$ MEFs. These results clearly indicate that TRIM23 plays important roles in antiviral signaling in physiologically immunocompetent cells.

Upon viral infection, TRAF3 auto-ubiquitination is required for IRF3 activation, but there is no direct evidence that TRAF3 conjugates ubiquitin to NEMO. In this study, we showed that TRIM23 directly interacted with NEMO and conjugated ubiquitin to it. On the other hand, we also observed interaction between TRAF3 and TRIM23 in coimmunoprecipitation assays (Fig. S9*A*). For this reason, we speculate that TRIM23 sits between TRAF3 and NEMO (Fig. S9*B*). However, how TRAF3 transmits antiviral signals to the TRIM23-NEMO complex should be examined in the future.

We showed that TRIM23-mediated conjugation of K27-type uniquitin to NEMO in 293T cells occurred as efficient as the conjugation of WT ubiquitin (Fig. 2*C*). Furthermore, we showed that ubiquitin conjugation to endogenous NEMO was higher in cells ectopically expressing K27-only ubiquitin than in cells

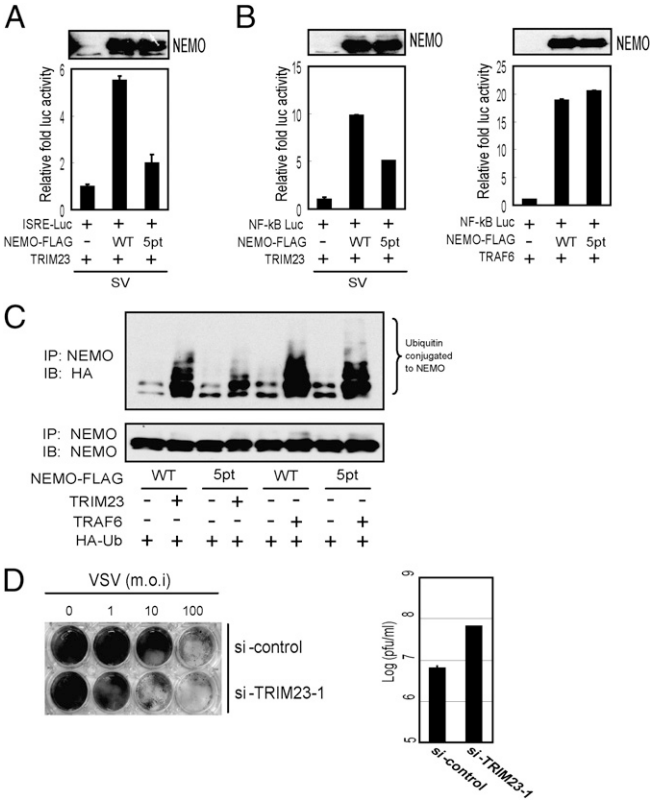

Fig. 4. Ubiquitin conjugation to NEMO by TRIM23 affects host defense. (*A*) NEMO$^{-/-}$ MEFs were transfected with plasmids encoding ISRE-Luc, TRIM23, and WT-NEMO or NEMO-5pt. At 8 h after transfection, cells were infected with SV for 16 h. (*B*) NEMO$^{-/-}$ MEFs were transfected with plasmids encoding NF-κB–Luc, TRIM23, and WT-NEMO or NEMO-5pt. At 8 h after transfection, cells were treated with SV for 16 h (*Left*). NEMO$^{-/-}$ MEFs were transfected with plasmids encoding NF-κB-Luc, TRAF6, and WT-NEMO or NEMO-5pt (*Right*). Cells were harvested 24 h after transfection, and luciferase activity was measured. The amount of NEMO in cell lysates, as an indicator of transfection efficiency, was measured. (*A* and *B*). (*C*) 293T cells were transfected with plasmids encoding TRIM23, TRAF6, FLAG-NEMO, or NEMO-5pt as indicated. (*D*) TRIM23 knockdown primary MEF cells allow virus production at a higher level than control cells. The primary MEFs were treated with control si-RNA or si-TRIM23-1 as indicated. At 60 h after transfection, VSV was infected with m.o.i. as indicated. Cells were stained at 24 h after infection (*Left*). Data are representative of two experiments. At 12 h after infection, virus titer was measured according to $TCID_{50}$ protocol (*Right*). Data are mean ± SD ($P < 0.001$, Student's *t* test, $n = 3$).

expressing K27R ubiquitin upon viral infection (Fig. 3*H*). Moreover, TRIM23 could conjugate K27-only ubiquitin to NEMO in vitro using UbcH5s as E2 enzyme (Fig. S2*B*). Importantly, knockdown of UbcH5s suppressed ubiquitin conjugation of NEMO in vivo, but knockdown of UbcH1 and Ubc13 did not (Fig. S5*C*), which may support an important role of UbcH5s in vivo. However, this does not discriminate against the possibility that other E2 enzymes also are involved in ubiquitin conjugation to NEMO in a concerted manner. Although it is strongly suggested that TRIM23 conjugates K27-type ubiquitin to NEMO, it remains further to analyze whether TRIM23 alone or TRIM23 together with other factor(s) exerts selecting activity of K27-type ubiquitin conjugation to NEMO. Recently, K63 type of ubiquitin was shown to be conjugated to NEMO upon VSV infection by in vitro ubiquitin conjugation analysis (31). However, this paper did not show the data that indicate lack of K63 ubiquitin conjugation in the lysate derived from uninfected cells. Thus, it is not clear whether K63 type ubiquitin conjugation to NEMO by VSV infection reflects physiological relevance.

There are several different ubiquitin modifications to NEMO to exert NF-κB regulation, Lys(K)-63–linked NEMO ubiquitination by several cellular events for NF-κB activation (32–35), and

LUBAC-mediated linear ubiquitin conjugation to NEMO in TNFα-mediated NF-κB activation in Ubc13-independent manner (36). Moreover, recently, it was reported that bacterial E3 ligase IpaH9.8 promotes K27-linked ubiquitin conjugation to NEMO and facilitates degradation of NEMO with unidentified molecule(s) activated by NOD1 signaling (37). Although both E3 ligases, TRIM23, and bacterially encoded IpaH9.8 conjugate K27-linked ubiquitin to NEMO, the outcomes are totally different. Because these ligases conjugate K27-linked ubiquitin to different lysine residues of NEMO, it is suggested that K27 ubiquitin conjugation exerts variety roles to NEMO with yet unclarified mechanisms.

We conducted microarray analysis as a primary tool for searching E3 ligases involved in innate immunity. Up-regulation of TRIM23 by ectopic expression of RNF125 in 293T cells suggests the presence of mutual interaction in expression of these genes. Moreover, these proteins may cooperatively function to antiviral signaling, although precise functional interaction between these proteins is not clear.

Additional works, including analysis of TRIM23-deficient mice, may reveal the specific role(s) of TRIM23 in the innate immune response to viral infection. As many viruses have evolved tactics to escape host immunity, it is likely that some viruses may target TRIM23 to establish successful infection. This suggests the potential application of TRIM23 for therapeutic and diagnostic purposes.

Materials and Methods

Cell Culture, Transfection, and Luciferase Reporter Assays. Details of cell culture, transfection, and luciferase reporter assays can be found in *SI Materials and Methods.*

Antibodies and Reagents. Antibodies and reagents are described in *SI Materials and Methods.*

Western Blotting and Immunoprecipitation. Details of Western blotting and immunoprecipitation are provided in *SI Materials and Methods.*

Knockdown. Knockdown details are given in *SI Materials and Methods.*

Microarray. Microarray details can be found in *SI Materials and Methods.*

Visual Analysis of Interaction. Details of visual analysis of interaction and bifluorescent complementation (Bifc) can be found in *SI Materials and Methods.*

ELISA. Culture medium from WT and TRAF6$^{-/-}$ MEFs were collected for the analysis of IFN-β production using mouse-specific ELISA kits (PBL Biomedical Laboratories).

cDNA Construction Details of cDNA construction can be found in *SI Materials and Methods.*

In Vitro Ubiquitination Assay. Assays details have been described previously (17). K27-only ubiquitin recombinant protein was purchased from Boston Biochem.

Assay of IRF3 Dimerization Details regarding assay of IRF3 dimerization are provided in *SI Materials and Methods.*

$TCID_{50}$ Assay. Approximate viral titers were calculated by 50% Tissue Culture Infectious Dose ($TCID_{50}$) assay. Further details can be found in *SI Materials and Methods.*

Statistical Methods. Statistical significance was determined by Student's *t* test.

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