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VIRUS-CELL INTERACTIONS

TRIM25 Is Required for the Antiviral Activity of Zinc Finger Antiviral Protein

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ABSTRACT Zinc finger antiviral protein (ZAP) is a host factor that specifically inhibits the replication of certain viruses by binding to viral mRNAs and repressing the translation and/or promoting the degradation of target mRNA. In addition, ZAP regulates the expression of certain cellular genes. Here, we report that tripartite motif-containing protein 25 (TRIM25), a ubiquitin E3 ligase, is required for the antiviral activity of ZAP. Downregulation of endogenous TRIM25 abolished ZAP's antiviral activity. The E3 ligase activity of TRIM25 is required for this regulation. TRIM25 mediated ZAP ubiquitination, but the ubiquitination of ZAP itself did not seem to be required for its antiviral activity. Downregulation of endogenous ubiquitin or overexpression of the deubiquitinase OTUB1 impaired ZAP's activity. We provide evidence indicating that TRIM25 modulates the target RNA binding activity of ZAP. These results uncover a mechanism by which the antiviral activity of ZAP is regulated.

IMPORTANCE ZAP is a host antiviral factor that specifically inhibits the replication of certain viruses, including HIV-1, Sindbis virus, and Ebola virus. ZAP binds directly to target mRNA, and it represses the translation and promotes the degradation of target mRNA. While the mechanisms by which ZAP posttranscriptionally inhibits target RNA expression have been extensively studied, how its antiviral activity is regulated is not very clear. Here, we report that TRIM25, a ubiquitin E3 ligase, is required for the antiviral activity of ZAP. Downregulation of endogenous TRIM25 remarkably abolished ZAP's activity. TRIM25 is required for ZAP optimal binding to target mRNA. These results help us to better understand how the antiviral activity of ZAP is regulated.

KEYWORDS ZAP, TRIM25, ubiquitin, RNA binding, antiviral factor, ubiquitination

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Zinc finger antiviral protein (ZAP) is a type I interferon-inducible host factor that specifically inhibits the replication of certain viruses, including HIV-1, Sindbis virus (SINV), Ebola virus, hepatitis B virus, and murine leukemia virus (1–6), as well as the retrotransposition of some retrotransposons (7, 8). ZAP binds directly to the ZAP-responsive element (ZRE) in the viral mRNA, and it represses the translation and promotes the degradation of target mRNA (6, 9–11). No obvious motifs or conserved sequences have been identified in the known ZREs. Structural analyses of the RNA-binding domain of ZAP indicate that ZAP recognizes a tertiary structure of target RNA (12). In addition to the antiviral activity, ZAP participates in posttranscriptional regulation of cellular gene expression. ZAP targets the 3′-untranslated region of TRAILR4 mRNA and promotes its degradation, thereby sensitizing cells to TRAIL-mediated apoptosis (13). ZAP interacts with Ago2 and regulates miRNA-mediated gene silencing (14).

ZAP represses target mRNA translation by interfering with the assembly of the translational initiation complex on the RNA. ZAP interacts with eIF4A and thereby disrupts the interaction between eIF4A and eIF4G (11). Furthermore, ZAP recruits the

deadenylase PARN to remove the poly(A) tail of target mRNA and recruits the RNA exosome, a 3′-5′ exoribonuclease complex, to degrade the deadenylated RNA body from the 3′ end (6, 10). ZAP also recruits the decapping complex through its cofactor p72, a DEAD box RNA helicase, to remove the cap structure (6, 15). The decapped RNA body is degraded by the 5′-3′ exoribonuclease XrnI from the 5′ end (6). ZAP-mediated translational repression precedes and is required for the degradation of target mRNA (11).

There are two isoforms of human ZAP arising from alternative splicing, which differ only in their C-terminal domains. The long isoform (ZAPL) consists of 902 amino acids and the short form (ZAPS) consists of 699 amino acids (16). In the C-terminal domain of ZAPL, there is a poly(ADP-ribose) polymerase (PARP) domain that is missing from ZAPS (16). In the N-terminal domain of ZAP, there are four CCCH-type zinc finger motifs. The N-terminal domain of 254 amino acids fused with the zeocin resistance gene product displayed the same antiviral activity as the full-length ZAPS, indicating that this domain is the major functional domain (2). Structural and functional analyses of this domain revealed that there is a large putative RNA-binding cleft on the surface of the protein, comprised of multiple positively charged residues and two cavities, which are proposed to bind to the phosphate backbone and bases of the nucleosides of target RNA, respectively (12). Posttranslational modification of ZAP has been reported to modulate ZAP's activity. Phosphorylation by glycogen synthase kinase 3β (GSK3β) enhances the antiviral activity of ZAP (17). *S*-Farnesylation of ZAPL but not ZAPS enhances the antiviral activity (18). Both ZAPL and ZAPS can be modified by poly(ADP-ribose) and such modification was proposed to participate in the regulation of microRNA-mediated gene silencing (14). Here, we report that TRIM25-mediated ubiquitination is required for the antiviral function of ZAP. In this report, unless otherwise specified, ZAPS is referred to as ZAP.

Ubiquitination is a process carried out by the ubiquitin-activating enzymes (E1s), ubiquitin-conjugating enzymes (E2s), and ubiquitin ligases (E3s) to form covalent conjunctions of a single ubiquitin molecule or ubiquitin chains to protein substrates (19, 20). Ubiquitin consists of 76 amino acids with seven lysines (K6, K11, K27, K29, K33, K48, and K63). All seven lysine residues, along with the N-terminal methionine, can be the sites linked by the C terminus of another ubiquitin, with ubiquitin-K48 and -K63 being the best-characterized residues involved in polyubiquitination (19, 20). Chains of ubiquitins are different in length, pattern, and linkage types (21). Ubiquitination was originally found to mark proteins for degradation by the proteasome (22). It was later found that ubiquitination can also modify proteins for nonproteolytic regulation of protein functions involved in a variety of biological processes (19, 20). Polyubiquitin chains of at least four K48-linked ubiquitin molecules can efficiently target a conjugated substrate protein for degradation (23). K63-linked polyubiquitin chains typically do not target proteins for proteasomal degradation but are commonly involved in nonproteolytic regulation of protein functions (24). Ubiquitin also exists in the unanchored form to bind proteins for signaling activation (25). Ubiquitination of target proteins can be reversed by deubiquitination enzymes (DUBs) (26).

Tripartite motif-containing protein 25 (TRIM25) is a member of the TRIM family, which is composed of a RING domain, one or two B-boxes, and a coiled-coil region (27). TRIM25 is an E3 ligase that can catalyze the ubiquitination or ISGylation of target proteins (28, 29). TRIM25 has been reported to be involved in the RIG-I-mediated antiviral response by inducing the K63-linked polyubiquitination of RIG-I (30). TRIM25 has also been reported to target the cell cycle for tumor cell progression in ovarian and breast cancers (28, 31). In addition, TRIM25 was reported to interact with pre-let-7 microRNA to facilitate Lin28a/TuT4-mediated uridylation of pre-let-7 microRNA (32).

Very recently, Li et al. reported that TRIM25 mediated ZAP ubiquitination and synergized with ZAP to inhibit Sindbis virus replication (33). In the present study, we confirmed their results that TRIM25 is required for the antiviral activity of ZAP. In addition, we provide evidence indicating that TRIM25 regulates the target RNA binding activity of ZAP.

RESULTS

TRIM25 is required for the antiviral activity of ZAP. To identify cellular factors involved in ZAP's antiviral activity, we set out to isolate ZAP-interacting proteins using tandem affinity purification (TAP). The full-length ZAP protein was not very stable during the purification (X. Zheng and G. Gao, unpublished data). Thus, we used the N-terminal domain of 332 amino acids of ZAP (NZAP332), which contains the major functional domain and is much more stable than the full-length protein. The NZAP332-TAP fusion protein was expressed in mammalian cells and purified by the TAP method. The associated proteins were subjected to SDS-PAGE, followed by silver staining. A band specific to NZAP332-TAP was identified as TRIM25 by mass spectrometry analysis (Zheng and Gao, unpublished). To confirm the interaction, Flag-tagged TRIM25 and myc-tagged full-length ZAP were transiently expressed in HEK293T cells. Myc-tagged tristetraprotin (TTP), which also has CCCH-type zinc finger motifs like ZAP, was used as a negative control. Immunoprecipitation results showed that TRIM25 indeed specifically interacted with ZAP but not with TTP (Fig. 1A). We failed to analyze the interactions between the endogenous TRIM25 and endogenous ZAP, although their interactions were shown by Li et al. (33). Our failure could be accounted for by the fact that our antibodies against TRIM25 and ZAP were not good enough for immunoprecipitation assays. We then analyzed the interaction between endogenous TRIM25 and overexpressed ZAPS. Data showed that immunoprecipitation of myc-tagged ZAP coprecipitated endogenous TRIM25 (Fig. 1B).

We next analyzed the effect of TRIM25 downregulation on the antiviral activity of ZAP. A short hairpin RNA (shRNA) targeting TRIM25 was designed and confirmed for its ability to efficiently downregulate the expression of TRIM25 (Fig. 1C). To confirm the specificity of the shRNA, a rescue TRIM25-expressing ($TRIM25_{res}$) plasmid was constructed in which silent mutations were introduced such that it was not downregulated by the shRNA (Fig. 1C). A vesicular stomatitis virus glycoprotein G (VSV-G)-pseudotyped retroviral vector expressing the shRNA was generated to transduce 293Trex-ZAP cells, which express ZAP in a tetracycline-inducible manner (6). The endogenous protein level of TRIM25 in the cells expressing the shRNA (293TRex-ZAP-TRIM25i) was significantly reduced compared with that in the cells expressing a control shRNA (293TRex-ZAP-Ctrli) (Fig. 1D). The cells were challenged with a replication-competent reporter Sindbis virus expressing nanoluciferase. Increased virus replication would be expected to result in increased luciferase reporter expression. ZAP's activity was indicated as fold inhibition against the virus, calculated as the luciferase activity in the absence of ZAP divided by that in the presence of ZAP. The results showed that downregulation of TRIM25 nearly abolished ZAP inhibition of virus replication (Fig. 1E, left). Noticeably, downregulation of TRIM25 did not affect tetracycline-induced ZAP expression (Fig. 1E, right). These results indicate that TRIM25 is required for the antiviral activity of ZAP against SINV.

To test whether the TRIM25 requirement of ZAP is specific to its antiviral activity against SINV and to facilitate the analyses, we used the ZAP-responsive plasmid reporter pMLV-luc, which is derived from the murine leukemia virus (MLV) vector and expresses firefly luciferase (2). The reporter was transfected into 293TRex-ZAP-Ctrli and 293TRex-ZAP-TRIM25i cells, and the antiviral activity of ZAP was indicated as fold inhibition against the reporter. Downregulation of TRIM25 nearly abolished ZAP's activity, and ectopic expression of $TRIM25_{res}$ restored the activity (Fig. 1F). To test the effect of TRIM25 downregulation on the antiviral activity of endogenous ZAP against the reporter, 293T cells stably expressing a control shRNA or the shRNA targeting TRIM25 (293T-Ctrli or 293T-TRIM25i) were transfected with the reporter together with the short interfering RNA (siRNA) targeting ZAP. In 293T-Ctrli cells, downregulation of the endogenous ZAP increased the reporter expression by about 2-fold (Fig. 1G, left). If TRIM25 is required for the endogenous ZAP to inhibit reporter expression, one would expect that downregulation of TRIM25 alone should diminish ZAP's activity and thus increased the reporter expression. Indeed, downregulation of TRIM25 increased the reporter expression by about 2.5-fold (Fig. 1G, left). Downregulation of both ZAP and

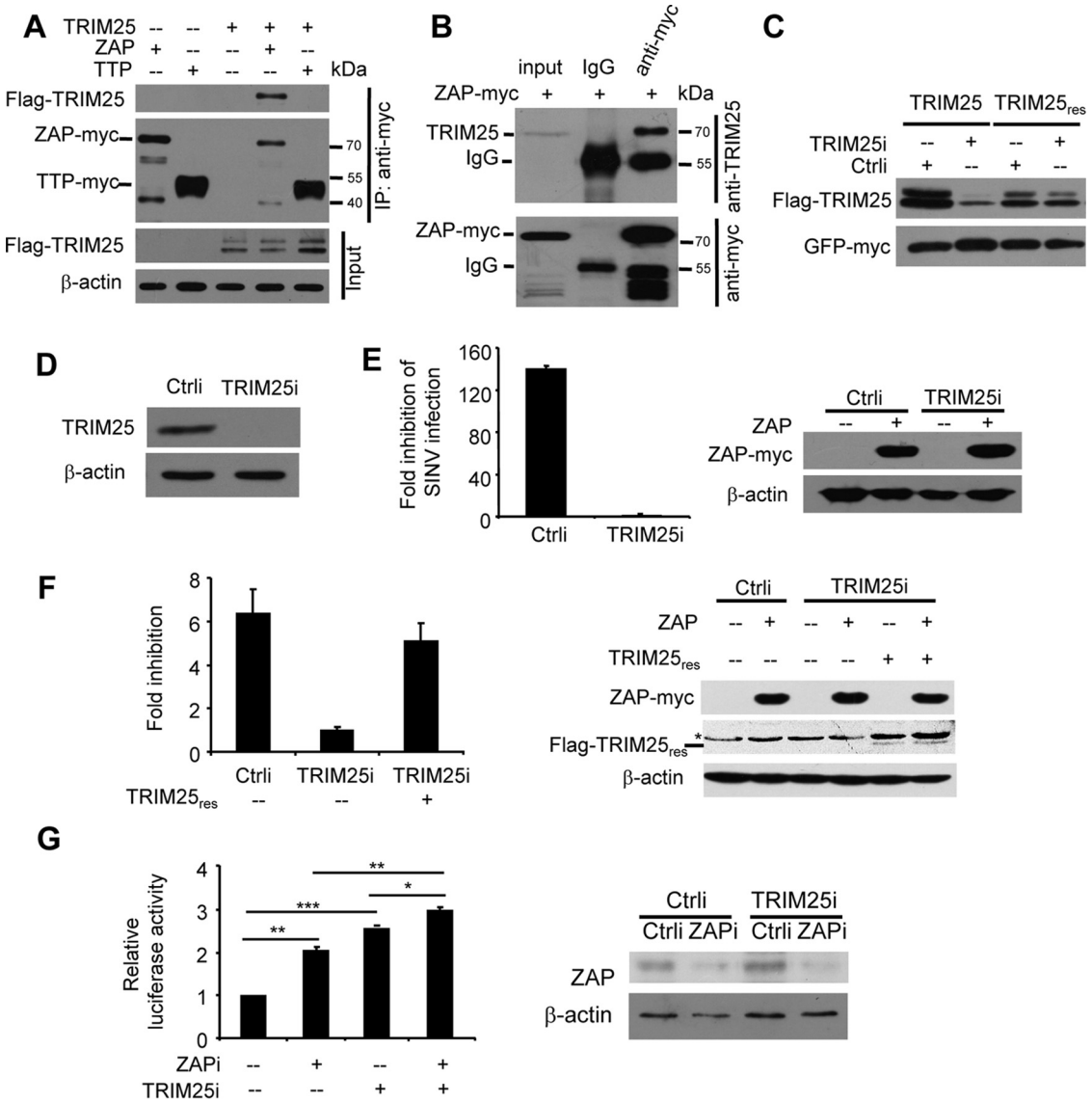

FIG 1 TRIM25 is required for the antiviral activity of ZAP. (A) Flag-tagged TRIM25 and myc-tagged ZAP or myc-tagged TTP were transiently expressed in HEK293 cells. Cell lysate was immunoprecipitated (IP) with anti-myc antibody-conjugated affinity gel and detected by Western blotting. (B) 293TRex-ZAP cells were treated with tetracycline to induce ZAP expression. The cell lysate was immunoprecipitated with an IgG antibody or an anti-myc antibody and detected by Western blotting with an antibody against TRIM25. (C) A plasmid expressing Flag-tagged TRIM25 or a rescue TRIM25-expressing ($TRIM25_{res}$) plasmid was transfected into HEK293T cells together with a plasmid expressing a control shRNA (Ctrli) or an shRNA targeting TRIM25 (TRIM25i). A plasmid expressing myc-tagged GFP was included to serve as a control for transfection efficiency and sample handling. At 48 h posttransfection, cells were lysed and protein expression was detected by Western blotting. (D) The shRNAs were stably expressed in 293TRex-ZAP cells. TRIM25 protein levels were detected by Western blotting. (E) 293Trex-ZAP-Ctrli and 293Trex-ZAP-TRIM25i cells were mock treated or treated with tetracycline to induce ZAP expression, followed by infection with a replication-competent reporter Sindbis virus expressing nanoluciferase. At 24 h postinfection, cells were lysed and the lysate was subjected to luciferase assays (left) and Western blot analyses (right). Fold inhibition was calculated as the luciferase activity in mock-treated cells divided by that in tetracycline-treated cells. Data presented are means ± standard deviations (SD) from four independent experiments. (F) 293TRex-ZAP-Ctrli and 293TRex-ZAP-TRIM25i cells were transfected with the ZAP-responsive firefly luciferase reporter pMLV-luc and the control renilla luciferase reporter pRL-TK, with or without the $TRIM25_{res}$-expressing plasmid. At 6 h posttransfection, cells were mock treated or treated with tetracycline to induce ZAP expression. At 48 h posttransfection, cells were lysed and the lysate was subjected to luciferase assays (left) and Western blotting (right). Firefly luciferase activity was normalized with renilla luciferase activity. Fold inhibition was calculated as the normalized luciferase activity in mock-treated cells divided by that in tetracycline-treated cells. Data presented are means ± SD from three independent experiments. An asterisk indicates nonspecific bands. (G) 293T-Ctrli and 293T-TRIM25i cells were transfected with a control siRNA or an siRNA targeting ZAP, together with reporters pMLV-luc and pRL-TK. At 48 h posttransfection, cells were lysed and the lysate was subjected to luciferase assays. (Left) The relative luciferase activity in the 293T-Ctrli cells transfected with the control siRNA was set to 1. (Right) The protein levels of endogenous ZAP detected by Western blotting. Data presented are means ± SD from four independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

TRIM25 increased the reporter expression by about 3-fold (Fig. 1G, left). It was confirmed that the siRNA targeting ZAP effectively reduced ZAP protein levels with or without TRIM25 downregulation (Fig. 1G, right). Noticeably, in the presence of ZAPi, TRIM25 downregulation further increased reporter expression (Fig. 1G, left). This could be plausibly explained by incomplete downregulation of ZAP. Nonetheless, the possibility cannot be excluded that TRIM25 also affects the reporter expression in a ZAP-independent manner. Collectively, these results demonstrate that TRIM25 is required for ZAP's antiviral activity.

The E3 ligase activity of TRIM25 is required for ZAP's antiviral activity. We next analyzed whether the E3 ligase activity of TRIM25 is required for its regulation of the antiviral activity of ZAP. Two TRIM25 mutants were constructed, one with the RING domain deleted (M1) and the other with the two cysteines in the RING domain replaced with serines (M2) (Fig. 2A). The RING domain of TRIM25 is required for its E3 ligase activity, and the two cysteines in the RING domain are required for the formation of the zinc finger motif (34). Silent mutations were introduced into the expression constructs such that the expression of the mutants cannot be downregulated by the TRIM25i shRNA (Fig. 2B). We first confirmed that the two mutants lost the E3 ligase activity. Multiple cellular proteins have been reported as the substrates of TRIM25, such as RIG-I, 14-3-3δ, AMF, ATBF1, and PCNA (28, 30, 35–37). We reasoned that TRIM25 overexpression would increase the ubiquitination levels of these proteins as well as that of some unidentified cellular proteins. Thus, they can be used as indicators for the E3 ligase activity of TRIM25. HEK293T cells were transfected with plasmids expressing Flag-tagged Ube1L (E1) and Flag-tagged UbcH8 (E2), as well as Flag-tagged ISG15 or hemagglutinin (HA)-tagged ubiquitin, together with a plasmid expressing wild-type or mutant TRIM25. Overexpression of wild-type TRIM25 enhanced the ubiquitination (Fig. 2C) and ISGylation (Fig. 2D) of multiple cellular proteins. In contrast, the two mutants failed to do so (Fig. 2C and D), indicating that their E3 ligase activity was indeed lost. To test the function of these two mutants, they were expressed in 293TRex-ZAP-TRIM25i cells. While expression of wild-type $TRIM25_{res}$ restored the antiviral activity of ZAP, expression of neither of the mutants improved ZAP's activity (Fig. 2E). Furthermore, in 293TRex-ZAP cells, overexpression of wild-type TRIM25 enhanced ZAP's activity (Fig. 2F). In contrast, overexpression of either of the mutants nearly abolished ZAP's activity (Fig. 2F). A plausible explanation is that the mutants inhibited the function of endogenous TRIM25 in a dominant-negative manner. Collectively, these results strongly suggest that the E3 ligase activity of TRIM25 is required for the antiviral activity of ZAP.

Ubiquitination, but not ISGylation, is required for the antiviral activity of ZAP. TRIM25 as an E3 ligase can catalyze both ubiquitination and ISGylation. We next analyzed whether ubiquitination and/or ISGylation is required for ZAP's activity. 293TRex-ZAP cells were transfected with an siRNA targeting ubiquitin or ISG15, and the effect on the antiviral activity of ZAP against the reporter pMLV-luc was evaluated. We failed to detect endogenous ubiquitin and ISG15 proteins, possibly due to the low levels of ubiquitin and ISG15 proteins in these cells. As a surrogate, we analyzed the endogenous ubiquitin and ISG15 mRNA levels, which were indeed downregulated by the siRNAs (Fig. 3A). Downregulation of ubiquitin significantly reduced the antiviral activity of ZAP (Fig. 3B). In contrast, downregulation of ISG15 had little effect (Fig. 3B). Noticeably, under this condition, fold inhibition of reporter expression, an indicator of the antiviral activity of ZAP, varied to some extent between experiments. However, the relative reduction in fold inhibition caused by the siRNA was very consistent in all of the experiments. To reflect this consistency, relative fold inhibition was presented (Fig. 3B). Cotransfection with the siRNA of a rescue ubiquitin-expressing (Ub_{res}) plasmid that cannot be targeted by the siRNA (Fig. 3C) restored the antiviral activity of ZAP (Fig. 3D and E). These results indicate that downregulation of endogenous ubiquitin impaired the antiviral activity of ZAP. In line with these results, overexpression of ubiquitin in 293TRex-ZAP cells increased ZAP's activity (Fig. 3F and G). To test whether the enhancement effect of ubiquitin expression on ZAP's activity was mediated by TRIM25,

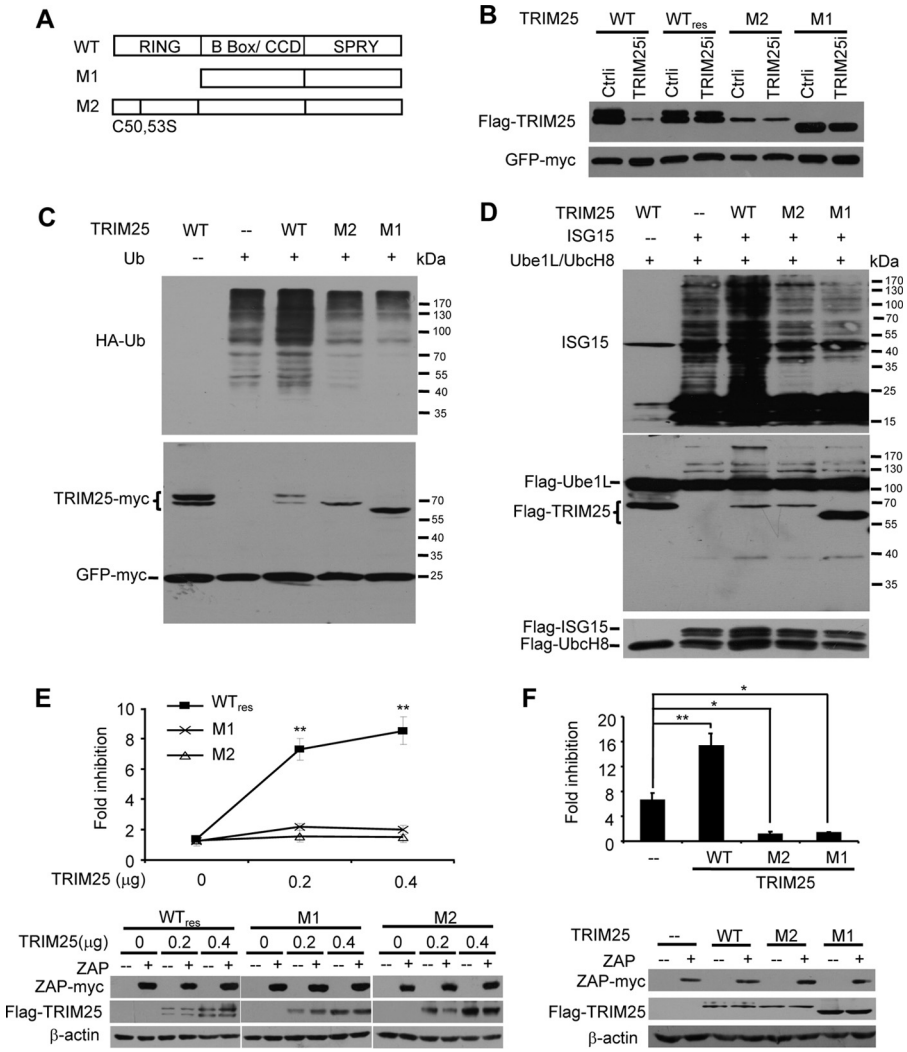

FIG 2 E3 ligase activity of TRIM25 is required for the antiviral activity of ZAP. (A) Schematic representation of TRIM25 mutants. (B) A plasmid expressing Flag-tagged wild-type (WT) or mutant TRIM25 was transfected into HEK293T cells together with a plasmid expressing the shRNA TRIM25i or Ctrli. A plasmid expressing myc-tagged GFP was included to serve as a control for transfection efficiency and sample handling. At 48 h posttransfection, cells were lysed and protein expression was detected by Western blotting. (C and D) HEK293T cells were transfected with plasmids expressing the proteins indicated. At 48 h posttransfection, cell lysates were subjected to Western blotting. Ubiquitinated (Ub) proteins were detected with an anti-HA antibody, and ISGylated proteins were detected with an antibody against ISG15. (E) 293TRex-ZAP-TRIM25i cells were transfected with increasing amounts of a plasmid expressing the TRIM25 indicated, together with reporters pMLV-luc and pRL-TK. At 6 h posttransfection, cells were mock treated or treated with tetracycline to induce ZAP expression. (Upper) At 48 h posttransfection, cells were lysed. Luciferase activities were measured and fold inhibition was calculated as described in the legend to Fig. 1E. Data presented are means ± SD from three independent experiments. (Lower) Protein expression was detected by Western blotting. (F) 293TRex-ZAP cells were transfected with a plasmid expressing the TRIM25 mutant indicated, together with reporters pMLV-luc and pRL-TK. At 6 h posttransfection, cells were mock treated or treated with tetracycline to induce ZAP expression. At 48 h posttransfection, cells were lysed. (Upper) Luciferase activities were measured and fold inhibition was calculated as described in the legend to Fig. 1E. Data presented are means ± SD from three independent experiments. (Lower) Protein expression was detected by Western blotting. *, $P < 0.05$; **, $P < 0.01$.

the TRIM25 mutant M2 was coexpressed with ubiquitin in these cells. Consistent with the above-described results (Fig. 2F), expression of M2 nearly abolished ZAP's activity (Fig. 3F and G). Coexpression of ubiquitin with M2 failed to restore ZAP's activity, implying that ubiquitin overexpression enhanced ZAP's activity through TRIM25. Taken together, these results indicate that TRIM25-mediated ubiquitination rather than ISGylation is required for ZAP's antiviral activity.

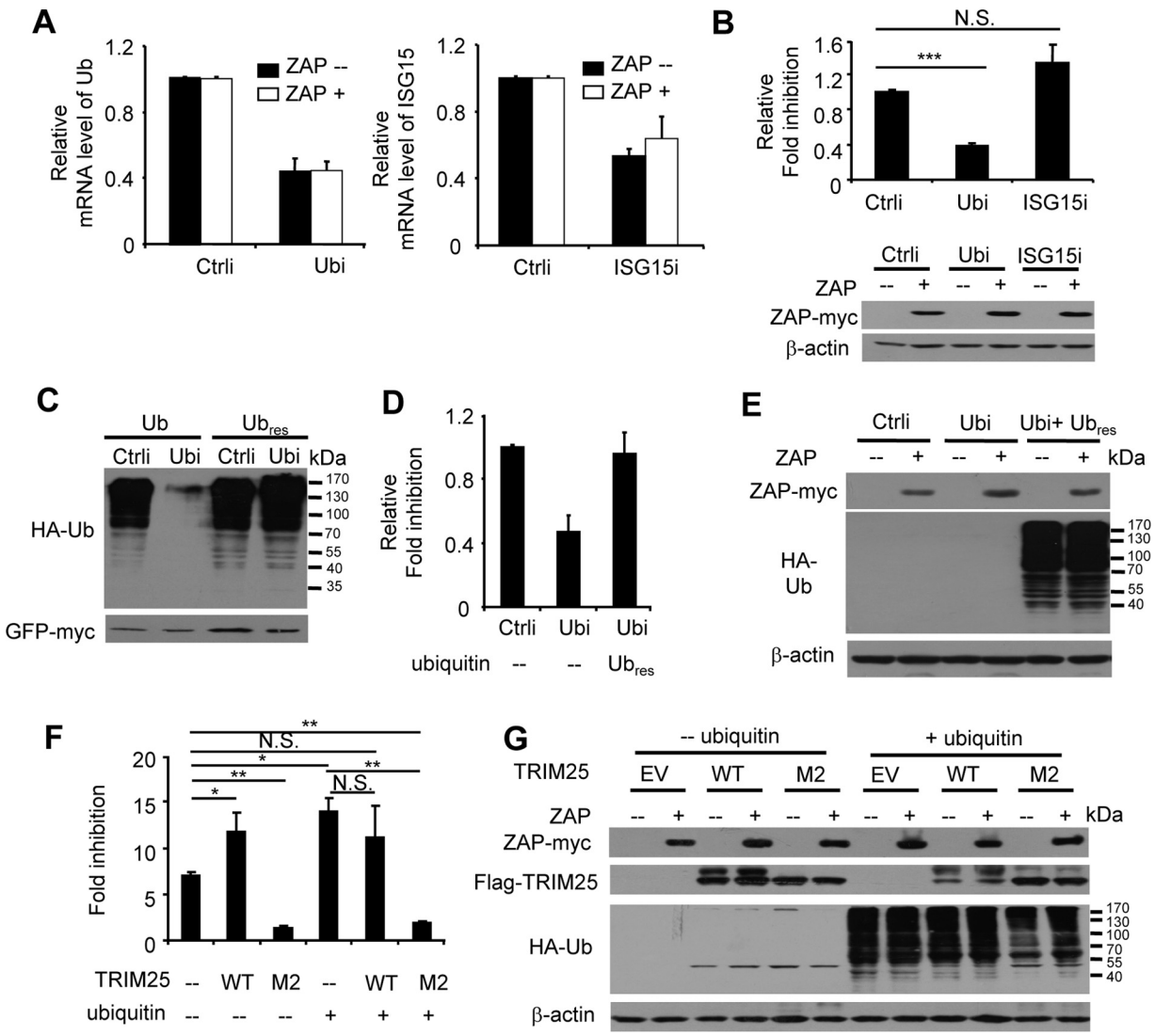

FIG 3 Ubiquitination but not ISGylation activity of TRIM25 is required for ZAP's antiviral activity. (A and B) 293TRex-ZAP cells were transfected with an siRNA targeting ubiquitin (Ubi) or ISG15 (ISG15i), together with reporters pMLV-luc and pRL-TK. At 6 h posttransfection, cells were mock treated (ZAP−) or treated with tetracycline (ZAP+) to induce ZAP expression. At 48 h posttransfection, cells were lysed. An aliquot of the cell lysate was used to measure luciferase activities, and the rest was used to extract RNA. (A) The relative mRNA levels of endogenous ubiquitin and ISG15 were measured by quantitative PCR and normalized with GAPDH mRNA levels. The relative mRNA level of ubiquitin and ISG15 in the control cells was set to 1. (B) Fold inhibition was calculated as described in the legend to Fig. 1E. (Upper) The relative fold inhibition of ZAP in the cells transfected with the control siRNA was set to 1. Data presented are means ± SD from three independent experiments. (Lower) Protein expression was detected by Western blotting. (C) 293TRex-ZAP cells were transfected with a control siRNA or the siRNA targeting ubiquitin, together with a plasmid expressing HA-tagged ubiquitin (Ub) or a rescue ubiquitin-expressing (Ub_{res}) plasmid. A plasmid expressing myc-tagged GFP was included to serve as a control for transfection efficiency and sample handling. At 48 h posttransfection, protein expression was detected by Western blotting. (D and E) 293TRex-ZAP cells were transfected with a control siRNA or an siRNA targeting ubiquitin with or without the Ub_{res}-expressing plasmid, together with reporters pMLV-luc and pRL-TK. At 6 h posttransfection, cells were mock treated or treated with tetracycline to induce ZAP expression. At 48 h posttransfection, cells were lysed. (D) Luciferase activities were measured. Fold inhibition was calculated as described in the legend to Fig. 1E. The relative fold inhibition in the cells transfected with the control siRNA was set to 1. Data presented are means ± SD from three independent experiments. (E) Protein expression was detected by Western blotting. (F and G) 293TRex-ZAP cells were transfected with plasmids expressing the proteins indicated, together with reporters pMLV-luc and pRL-TK. At 6 h posttransfection, cells were mock treated or treated with tetracycline to induce ZAP expression. At 48 h posttransfection, cells were lysed. (F) Luciferase activities were measured. Fold inhibition was calculated as described in the legend to Fig. 1E. Data presented are means ± SD from three independent experiments. (G) Protein expression was detected by Western blotting. (C, E, and G) Ubiquitinated proteins were detected with an anti-HA antibody. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; N.S., not statistically significant.

TRIM25 mediates ZAP ubiquitination. We next analyzed whether TRIM25 is a ubiquitin E3 ligase for ZAP. We previously reported that the N-terminal domain of 254 amino acids of ZAP in fusion with the zeocin resistance gene product (NZAP-Zeo) has the same antiviral activity as the full-length protein (2), indicating that this domain is

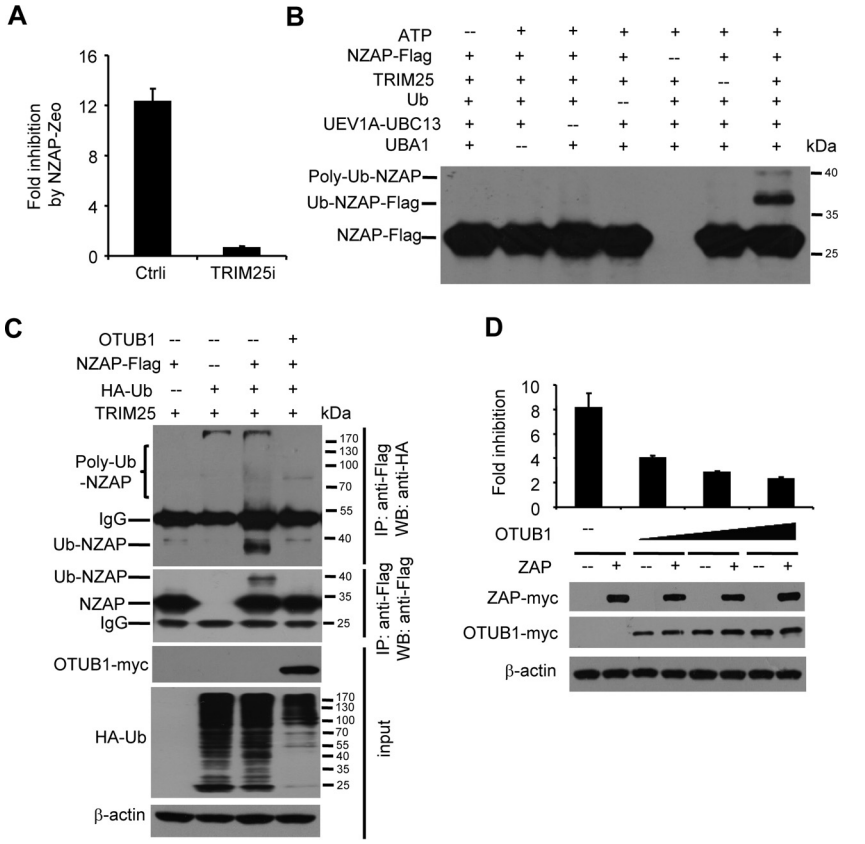

FIG 4 TRIM25 mediates ZAP ubiquitination. (A) 293TRex-NZAP-Zeo cells stably expressing TRIM25i or Ctrli were transfected with reporters pMLV-luc and pRL-TK. At 6 h posttransfection, cells were mock treated or treated with tetracycline to induce NZAP-Zeo expression. At 48 h posttransfection, cells were lysed and luciferase activities were measured. Fold inhibition was calculated as described in the legend to Fig. 1E. Data presented are means ± SD from three independent experiments. (B) Flag-tagged NZAP was bacterially expressed, partially purified, and incubated in a reaction buffer with recombinant ubiquitin and the ubiquitination enzymes indicated. NZAP proteins were detected by Western blotting using an anti-Flag antibody. (C) HEK293T cells were transfected with plasmids expressing the proteins indicated. At 48 h posttransfection, cell lysates were immunoprecipitated with an anti-Flag antibody and analyzed by Western blotting. Ubiquitinated (Ub) proteins were detected with an anti-HA antibody. (D) 293TRex-ZAP cells were transfected with increasing amounts of a plasmid expressing myc-tagged OUTB1, together with reporters pMLV-luc and pRL-TK. At 6 h posttransfection, cells were mock treated or treated with tetracycline to induce ZAP expression. (Upper) At 48 h posttransfection, cells were lysed. Luciferase activities were measured and fold inhibition was calculated as described in the legend to Fig. 1E. Data presented are means ± SD from three independent experiments. (Lower) Protein expression was detected by Western blotting.

the major functional domain. In addition, structural analyses of bacterially expressed protein of this domain revealed that it was well folded (12). To simplify the ubiquitination assay system, NZAP was used. We first confirmed that downregulation of endogenous TRIM25 abolished the antiviral activity of NZAP-Zeo (Fig. 4A). Bacterially expressed Flag-tagged NZAP protein was incubated with purified proteins that are required for ubiquitination, including ubiquitin, Uba1 (E1), an E2 complex for TRIM25 Uev1a-Ubc13 (38, 39), and TRIM25. The resulting NZAP proteins were detected by Western blotting with an anti-Flag antibody. In the presence of TRIM25, three NZAP bands were detected, corresponding to the sizes of NZAP without modification, modified with one and two ubiquitin molecules, respectively (Fig. 4B). These results imply that TRIM25 can ubiquitinate ZAP. To further substantiate the ubiquitination of ZAP, we analyzed the effect of the expression of the deubiquitinating enzyme (DUB) OTUB1 on ZAP. OTUB1 belongs to the ovarian tumor (OTU) DUB family and has been reported to suppress ubiquitination by targeting the E2 enzymes UbcH5 and Ubc13, both of which participate in TRIM25-mediated ubiquitination (38, 40, 41). Flag-tagged

NZAP, myc-tagged TRIM25, and HA-tagged ubiquitin were expressed in HEK293T cells with or without OTUB1. In the absence of OTUB1, NZAP ubiquitination was easily detected (Fig. 4C). However, in the presence of OTUB1, NZAP ubiquitination was barely detected (Fig. 4C), indicating that OTUB1 effectively suppressed TRIM25-mediated ubiquitination of NZAP. When OTUB1 was overexpressed in 293TRex-ZAP cells, the antiviral activity of ZAP was reduced in an OTUB1 dose-dependent manner (Fig. 4D). Collectively, these results indicate that TRIM25 ubiquitinates NZAP and demonstrate the importance of ubiquitination for ZAP's antiviral function.

TRIM25 is required for ZAP optimal binding to target mRNA. ZAP binding to target mRNA is a prerequisite for its inhibition of mRNA expression. We speculated that TRIM25 plays an important role in ZAP binding to target mRNA. To test this idea, we analyzed the effect of TRIM25 downregulation on ZAP binding to target RNA. 293TRex cells stably expressing a control shRNA (293TRex-Ctrli) or the shRNA targeting TRIM25 (293TRex-TRIM25i) were transfected with a plasmid expressing Flag-tagged NZAP, together with a ZAP-responsive firefly luciferase reporter, pCMV-FL-Mk, and a control renilla luciferase reporter, pRL-CMV. We chose the pCMV-FL-Mk reporter because ZAP represses the translation of this reporter mRNA without promoting its degradation (11). Downregulation of TRIM25 had little effect on the reporter mRNA levels (Fig. 5A). NZAP was immunoprecipitated, and levels of the associated reporter mRNA were analyzed by quantitative PCR. TRIM25 downregulation significantly reduced the amount of NZAP-associated FL-Mk reporter RNA (Fig. 5B). In comparison, the amount of coprecipitated control RL reporter RNA was very small with or without TRIM25 downregulation (Fig. 5B), indicating the specificity of the association of NZAP with the reporter RNAs. These results imply that TRIM25 is required for optimal ZAP binding to target RNA. To further substantiate the importance of ubiquitination for the RNA binding ability of ZAP, OTUB1 was coexpressed with NZAP and the reporters. Expression of OTUB1 reduced target RNA association with NZAP in a dose-dependent manner (Fig. 5C). In comparison, expression of OTUB1 had little effect on the nonspecific association of NZAP with the control RL reporter (Fig. 5C). Collectively, these results support the notion that TRIM25-mediated ubiquitination is important for ZAP binding to target RNA.

K63-linked but not K48-linked polyubiquitination is required for ZAP's optimal binding to target mRNA. We next asked whether polyubiquitination (and which type of polyubiquitination) is required for the antiviral activity of ZAP. Two ubiquitin mutants were constructed, one containing the K63R mutation and the other containing K48R. The mutants were overexpressed in 293TRex-ZAP cells to analyze the effect on the antiviral activity of ZAP. The results showed that overexpression of wild-type ubiquitin or the K48R mutant enhanced ZAP's activity (Fig. 6A and B). In contrast, overexpression of the K63R mutant significantly reduced ZAP's activity (Fig. 6A and B). In line with these results, in a rescue experiment, ectopic expression of wild-type ubiquitin or the K48R mutant restored the reduced antiviral activity of ZAP caused by downregulation of endogenous ubiquitin, while expression of the K63R mutant failed to do so (Fig. 6C and D). These results indicate that the K63-linked polyubiquitination is involved in the regulation of ZAP's activity. To analyze whether ZAP is modified by K63-linked polyubiquitination, HA-tagged wild-type ubiquitin and the mutants were expressed in HEK293T cells together with Flag-tagged NZAP and myc-tagged TRIM25. NZAP was immunoprecipitated and analyzed by Western blotting using an anti-HA antibody. Multiple bands were detected, which were presumably the polyubiquitinated species of NZAP (Fig. 6E). Compared with wild-type ubiquitin, expression of the K63R mutant led to reduced levels of the high-molecular-weight species of ubiquitinated NZAP, although the levels of the low-molecular-weight species were comparable (Fig. 6E). Expression of the K48R mutant generally slightly increased the polyubiquitination levels of NZAP (Fig. 6E). These results indicate that ZAP undergoes K63-linked dependent ubiquitination and that K63-linked polyubiquitination is important for ZAP's activity.

We next analyzed the effect of K63-linked polyubiquitination on ZAP's target RNA-binding activity. The wild-type or mutant ubiquitin was coexpressed with NZAP

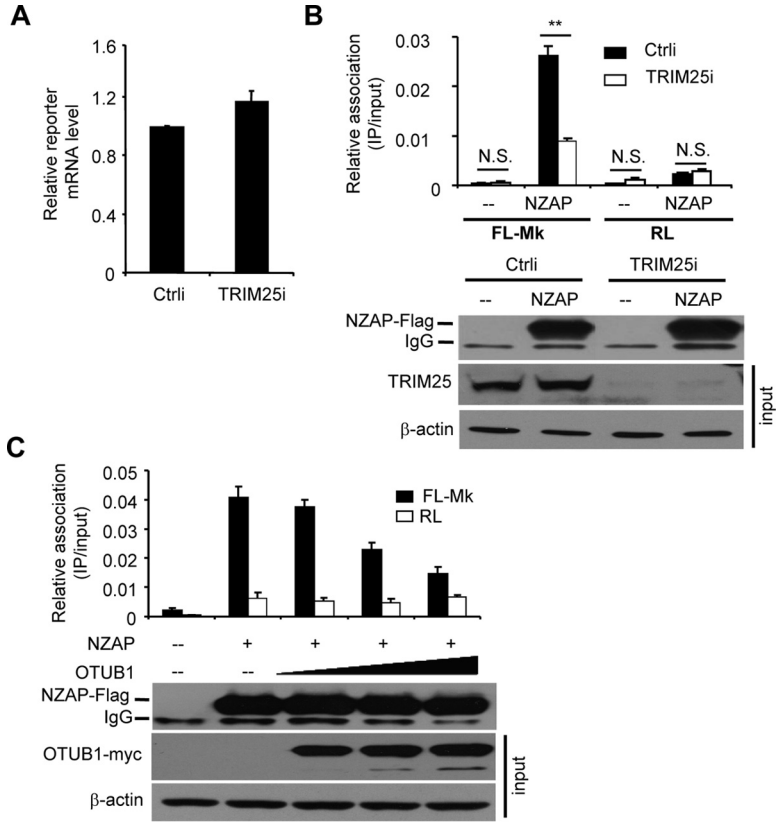

FIG 5 TRIM25 is required for ZAP optimal binding to target mRNA. (A) 293TRex cells stably expressing TRIM25i or Ctrli were transfected with the ZAP-responsive firefly luciferase reporter pCMV-FL-Mk and control renilla luciferase reporter pRL-CMV. At 48 h posttransfection, cells were lysed for RNA extraction. MK-fLuc mRNA levels were measured by quantitative PCR and normalized to the RL mRNA levels. Data presented are means ± SD from three independent experiments. (B) 293TRex cells stably expressing TRIM25i or Ctrli were transfected with reporters pCMV-FL-Mk and pRL-CMV, with or without a plasmid expressing Flag-tagged NZAP. At 48 h posttransfection, cells were lysed. An aliquot of the cell lysate was used to extract total RNA, and the rest of the lysates were immunoprecipitated with an anti-Flag antibody. Reporter mRNA levels in total cell lysate and in the precipitates were analyzed by quantitative PCR. (Upper) Relative association of reporter mRNA with NZAP was calculated as the reporter mRNA level in the precipitates divided by that in the total cell lysate. (Lower) That equivalent amounts of NZAP were immunoprecipitated and that the endogenous TRIM25 protein levels were downregulated were confirmed by Western blotting. Data presented are means ± SD from three independent measurements, representative of three independent experiments. (C) 293TRex cells were transfected with reporters pCMV-FL-Mk and pRL-CMV, together with a plasmid expressing Flag-tagged NZAP and increasing amounts of a plasmid expressing myc-tagged OTUB1. At 48 h posttransfection, cells were lysed. An aliquot of the cell lysate was used to extract total RNA, and the rest was immunoprecipitated with an anti-Flag antibody. (Upper) Relative association of reporter mRNA with NZAP was calculated as described in the legend to panel B. (Lower) That equivalent amounts of NZAP were immunoprecipitated and OTUB1 expression in the total cell lysate were confirmed by Western blotting. Data presented are means ± SD from three independent measurements, representative of three independent experiments. **, $P < 0.01$; N.S., not statistically significant.

and the reporters. Relative association of NZAP with target RNA in the presence of ubiquitin or the mutants was assayed as described above. The results showed that expression of wild-type ubiquitin and the K48R mutant increased target RNA association with NZAP (Fig. 6F and G). In contrast, expression of the K63R mutant caused a significant reduction in target RNA association with NZAP (Fig. 6F and G). Taken together, these results indicate that K63-linked polyubiquitination is important for ZAP's binding to target RNA.

DISCUSSION

ZAP specifically inhibits the expression of certain viral mRNAs as well as certain cellular mRNAs. The inhibitory activity of ZAP is dictated by its ability to bind to target

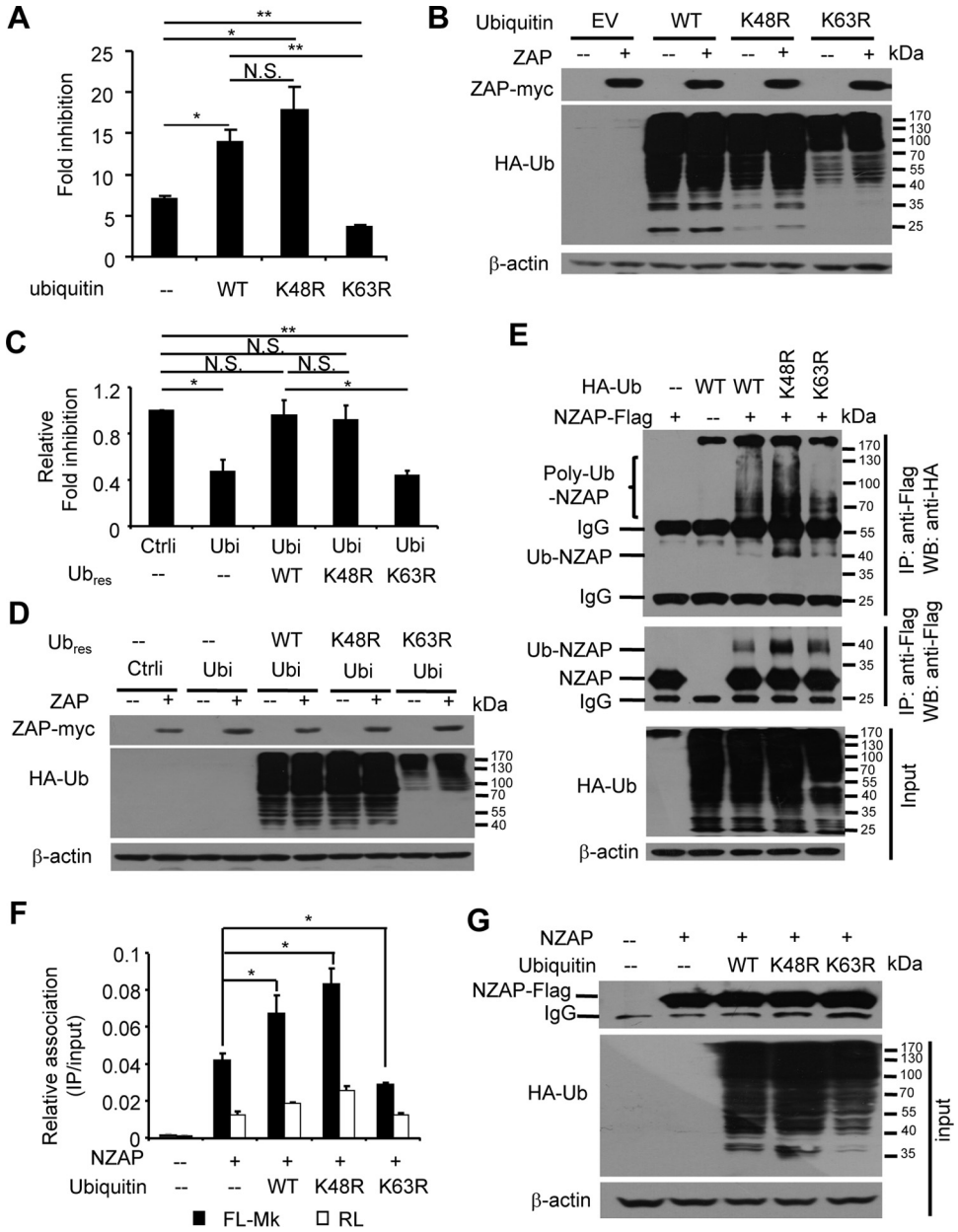

FIG 6 K63-linked polyubiquitination is required for ZAP optimal binding to target mRNA. (A and B) 293TRex-ZAP cells were transfected with a plasmid expressing the HA-tagged wild-type (WT) or the indicated mutant ubiquitin, together with reporters pMLV-luc and pRL-TK. At 6 h posttransfection, cells were mock treated or treated with tetracycline to induce ZAP expression. At 48 h posttransfection, cells were lysed. (A) Luciferase activities were measured and fold inhibition was calculated as described in the legend to Fig. 1E. Data presented are means ± SD from three independent experiments. (B) Protein expression was detected by Western blotting. (C and D) 293TRex-ZAP cells were transfected with a control siRNA or the siRNA targeting ubiquitin with or without the Ub_{res}-expressing plasmid indicated, together with reporters pMLV-luc and pRL-TK. At 6 h posttransfection, cells were mock treated or treated with tetracycline to induce ZAP expression. At 48 h posttransfection, cells were lysed. (C) Luciferase activities were measured and fold inhibition was calculated as described in the legend to Fig. 1E. The relative fold inhibition in the cells transfected with the control siRNA was set to 1. Data presented are means ± SD from three independent experiments. (D) Protein expression was detected by Western blotting. (E) HEK293T cells were transfected with plasmids expressing the proteins indicated. At 48 h posttransfection, cell lysates were immunoprecipitated with an anti-Flag antibody and analyzed by Western blotting. (F and G) 293TRex cells were transfected with reporters pCMV-FL-Mk and pRL-CMV, together with a plasmid expressing Flag-tagged NZAP and a plasmid expressing HA-tagged wild-type (WT) or the indicated mutant ubiquitin. At 48 h posttransfection, cells were lysed. An aliquot of the cell lysate was used to extract total RNA, and the rest was immunoprecipitated with an anti-Flag antibody. (F) Relative association of reporter mRNA with NZAP was calculated as described in the legend to Fig. 5B. Data presented are means ± SD from two independent measurements, representative of two independent experiments. (G) That equivalent amounts of NZAP were immunoprecipitated and Ub expression in the total cell lysate were confirmed by Western blotting. Ubiquitinated proteins were detected with an anti-HA antibody. *, $P < 0.05$; **, $P < 0.01$.

mRNA. How this activity is regulated is largely unknown. Here, we show that downregulation of endogenous TRIM25 abolished the antiviral activity of ZAP (Fig. 1). TRIM25 mediates ZAP ubiquitination both *in vitro* and *in vivo* (Fig. 4). Downregulation of endogenous ubiquitin (Fig. 3B) or overexpression of the deubiquitinase OTUB1 (Fig. 4D) significantly impaired the antiviral activity of ZAP. In line with these results, overexpression of ubiquitin enhanced ZAP's activity (Fig. 3F and 6A). However, overexpression of the K63R ubiquitin mutant, which cannot form K63-linked polyubiquitin chains, reduced ZAP's activity (Fig. 6A). These results indicate that TRIM25-mediated K63-linked polyubiquitination is important for the antiviral activity of ZAP. Furthermore, we provide evidence implicating that TRIM25 modulates the target RNA binding activity of ZAP (Fig. 5). During the course of this work, Li et al. reported that TRIM25-mediated ubiquitination was essential for ZAP (33). Our results reported here are consistent with theirs.

How TRIM25-mediated ubiquitination regulates the antiviral activity of ZAP is not fully understood. TRIM25 ubiquitinates NZAP *in vitro*. In the paper by Li et al., seven lysine residues (K226R, K296R, K314R, K401R, K416R, K448R, and K629R) were identified to be ubiquitinated (33). Mutation of all of these residues abolished ZAP ubiquitination but did not affect its antiviral activity. Here, we used NZAP-Zeo to simplify the ubiquitination assays. Downregulation of TRIM25 abolished the antiviral activity of NZAP-Zeo, suggesting that TRIM25 modulates the antiviral activity of ZAP and NZAP-Zeo by the same mechanism. The K226 residue was also identified as a ubiquitination site by mass spectroscopy in our hands, and replacement of this residue with arginine did not affect the antiviral activity of NZAP-Zeo (Zheng and Gao, unpublished). One possible explanation for these results is that the ubiquitination of ZAP itself is not required for its antiviral activity. Instead, the ubiquitination of protein(s) in its interactome is required for ZAP's activity. Another possible explanation is that some unidentified lysine residues are the ubiquitination sites of ZAP that are important for its activity. Loss of ubiquitination of the mutant ZAP in the paper by Li et al. could not exclude the possibility that ubiquitination of the mutant ZAP was below the detection limit (33). There are 13 lysine residues in NZAP, with 4 (K76, K89, K107, and K151) located in the putative RNA-binding cleft and 9 (K12, K119, K122, K131, K154, K197, K222, K226, and K248) outside the cleft (12). Replacement of each of the nine lysines with an arginine outside the RNA-binding cleft did not significantly affect the antiviral activity either (Zheng and Gao, unpublished). These results argue for the possibility that the ubiquitination of ZAP itself is not required for its antiviral activity, although the possibility cannot be excluded that ubiquitination at multiple sites of ZAP is required. Further investigation is needed to understand how TRIM25-mediated ubiquitination regulates the antiviral activity of ZAP.

We provide evidence showing that TRIM25 and polyubiquitination are important for ZAP binding to target RNA: downregulation of endogenous TRIM25 (Fig. 5B) or overexpression of OTUB1 (Fig. 5C) or the K63R ubiquitin mutant (Fig. 6F) significantly reduced ZAP association with target RNA. Noticeably, downregulation of TRIM25 nearly abolished the antiviral activity of ZAP (Fig. 1), but it reduced ZAP association with target RNA only to some extent (Fig. 5B). One possible explanation is that in the RNA binding assay, there was still some nonspecific association between ZAP and target RNA when TRIM25 was downregulated. This speculation is supported by the fact that nonspecific association between ZAP and the control reporter, RL, was detected (Fig. 5B). Moreover, structure-function analyses of ZAP revealed that ZAP functions as a dimer, with one RNA-binding site on the surface of each molecule (12). Two ZAP-binding motifs simultaneously binding to a ZAP dimer are required for target RNA to be functionally responsive to ZAP. TRIM25 might be required for proper binding of ZAP to target RNA. Furthermore, ZAP interacts with eIF4A to inhibit the assembly of translation initiation complex on target mRNA (11) and recruits the mRNA degradation machinery to promote target mRNA degradation (6). TRIM25-mediated ubiquitination might affect these processes. In addition, the possibility cannot be excluded that TRIM25 also regulates ZAP activity in a ubiquitin-independent manner.

In addition to inhibiting target mRNA expression through the N-terminal domain, ZAP was recently reported to promote the degradation of influenza viral proteins through the PARP domain (42). Whether TRIM25-mediated ubiquitination also affects this activity of ZAP awaits further investigation.

In summary, we report that downregulation of endogenous TRIM25 remarkably abolished the antiviral activity of ZAP. We show that TRIM25-mediated ubiquitination is required for optimal ZAP binding to target RNA. These results uncover a mechanism of how the antiviral activity of ZAP can be regulated.

MATERIALS AND METHODS

Plasmids. The coding sequence of TRIM25 was PCR amplified from a human cDNA library and cloned into the expression vector pCMV-HA-Flag (10) to express Flag-tagged TRIM25. The TRIM25 M1 mutant was constructed by deleting the RING domain. The M2 mutant was generated by replacing Cys50 and Cys53 with serines. The coding sequences of TRIM25 WT_{res}, M1 and M2, which cannot be targeted by the shRNA targeting TRIM25, were generated by introducing silent mutations (5′-AAAGTCGAACAACTGCAGCAG-3′; mutations are underlined) into the pCMV-HA-Flag expression constructs. To express myc-tagged $TRIM25_{res}$, M1 and M2, the coding sequences, were PCR amplified and cloned into the expression vector pcDNA4-myc-His, which was modified from pcDNA4/TO/myc-HisB (Invitrogen) by deleting the Tet operon sequence. Sequences of the primers are the following: for pCMV-HA-Flag-TRIM25 and M2, 5′-CCGGAATTCATGGCAGAGCTGTGCCCCCT-3′ (forward primer [FP]) and 5′-CCGCTCGAGCTACTTGGGGGAGCAGATGG-3′ (reverse primer [RP]); for pCMV-HA-Flag-M1, 5′-CCGGAATTCATGCTGCACAAGAACACGGT-3′ (FP) and 5′-CCGCTCGAGCTACTTGGGGGAGCAGATGG-3′ (RP); for pcDNA4-myc-His-$TRIM25_{res}$ and M2, 5′-CCGGAATTCGCCACCATGGCAGAGCTGTGCCCCCTGGCCG-3′ (FP) and 5′-ATAAGAATGCGGCCGCACTTGGGGGAGCAGATGGAGAGTGT-3′ (RP); for pcDNA4-myc-His-M1, 5′-CCGGAATTCGCCACCATGTACCAGGCGCGACCGCAGCT-3′ (FP) and 5′-ATAAGAATGCGGCCGCACTTGGGGGAGCAGATGGAGAGTGT-3′ (RP).

ZAP-expressing plasmid pcDNA4/TO/myc-ZAP, ZAP-responsive reporters pMLV-luc and pCMV-FL-Mk, and renilla luciferase control reporters pRL-TK and pRL-CMV have been previously described (2, 9, 11). The coding sequence of TTP was cloned from the plasmid pcDNA4/TO/myc-TTP (9) into the expression vector pcDNA4-myc-His using restriction sites AflII and NotI.

To generate a plasmid expressing an shRNA targeting TRIM25, oligonucleotides were synthesized, annealed, and cloned into pSUPER-Retro (Oligoengine). The control shRNA has been described previously (15). Sequences of the oligonucleotides are the following: TRIM25i-FP, 5′-GATCCCCGGTGGAGCAGCTACAACAATTCAAGAGATTGTTGTAGCTGCTCCACCTTTTTA-3′; TRIM25i-RP, 5′-AGCTTAAAAAGGTGGAGCAGCTACAACAATCTCTTGAATTGTTGTAGCTGCTCCACCGGG-3′.

The coding sequences of ISG15, UbcH8, and Ube1L were PCR amplified from a cDNA library and cloned into expression vector pCMV-HA-Flag. The coding sequence of OTUB1 was PCR amplified from a cDNA library and cloned into expression vector pcDNA4-myc-His. The coding sequences of ubiquitin and the K48R and K63R mutants were PCR amplified from plasmids previously reported (43) and cloned into the expression vector pCMV-HA to express HA-tagged proteins. The rescue plasmid expressing the wild-type ubiquitin or the mutants that cannot be targeted by siUbiquitin was generated by overlapping PCR to introduce silent mutations (5′-GATCAACAACGCTTAATATTC-3′). Sequences of the primers are the following: pCMV-HA-Ub FP, 5′-CCGGAATTCCGATGCAGATCTTCGTGAAAAC-3′; Ub_{res} middle FP, 5′-AAAGAAGGCATCCCCCCCGATCAACAACGCTTAATATTCGCAGGCAAGC-3′; Ub_{res} middle RP, 5′-GCTTGCCTGCGAATATTAAGCGTTGTTGATCGGGGGGGATGCCTTCTTT-3′; K48R Ub_{res} middle FP, 5′-AAAGAAGGCATCCCCCCCGATCAACAACGCTTAATATTCGCAGGCA-3′; K48R Ub_{res} middle RP, 5′-TGCCTGCGAATATTAAGCGTTGTTGATCGGGGGGGATGCCTTCTTT-3′; pCMV-HA-Ub RP, 5′-CCGCTCGAGTTAGCCACCCCTCAGACGCA-3′; for pCMV-HA-Flag-ISG15, 5′-GGAAGATCTTCATGGGCTGGGACCTGACGGT-3′ (FP) and 5′-ATAAGAATGCGGCCGCTTAGCTCCGCCCGCCAGGCT-3′ (RP); for pCMV-HA-Flag-UbcH8, 5′-CCGGAATTCATGATGGCGAGCATGCGAGT-3′ (FP); and 5′-ATAAGAATGCGGCCGCTTAGGAGGGCCGGTCCACTC-3′ (RP); for pCMV-HA-Flag-Ube1L, 5′-CCGGAATTCATGGATGCCCTGGACGCTTC-3′ (FP) and 5′-ATAAGAATGCGGCCGCTCACAGCTCATAGTGCAGAG-3′ (RP); for pcDNA4-myc-His-OTUB1, 5′-CGGGGTACCGCCACCATGGCGGCGGAGGAACCTCA-3′ (FP) and 5′-CGCGGATCCTTTGTAGAGGATATCGTAGT-3′ (RP).

To generate the construct expressing NZAP-Flag, the coding sequence of the zeocin resistance gene in pcDNA4TO/myc-NZAP-Zeo (6) was replaced with the coding sequence of the Flag tag. The sequence of NZAP-Flag then was cloned into pcDNA4-myc-His to generate pcDNA4-NZAP-Flag.

To express recombinant GST-TRIM25 and GST-NZAP-Flag, coding sequences of TRIM25 and NZAP-Flag were PCR amplified from pCMV-HA-Flag-TRIM25 and pcDNA4-NZAP-Flag, respectively, and cloned into pGEX-5X-3 (GE Healthcare). GST-TRIM25 and GST-NZAP-Flag were expressed in *Escherichia coli* and purified by following the handbook for the glutathione *S*-transferase (GST) fusion protein system (Amersham Biosciences). GST was removed using PreScission protease to produce TRIM25 and NZAP-Flag proteins. Purified proteins His-Uba1, His-Ubc13, His-Uev1a, and His-ubiquitin have been reported previously (44).

Cell culture. All cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) supplemented with 10% fetal bovine serum (FBS) (Invitrogen). 293TRex-ZAP and 293TRex-NZAP-Zeo cells, which express myc-tagged ZAP and NZAP-Zeo, respectively, in a tetracycline-inducible manner have been previously described (6). Transfection was performed using Neofectin by following the manufacturer's instructions (NeoBiolab).

To generate a retroviral vector expressing an shRNA targeting TRIM25 or a control shRNA, 293T cells were transfected with pSUPER-Retro-TRIM25i or pSUPER-Retro-Ctrli, together with plasmids expressing VSV-G and MLV-Gag-pol. At 48 h posttransfection, culture supernatants were harvested and used to transduce 293T, 293TRex, 293TRex-ZAP, or 293Trex-NZAP-Zeo cells. The cells were selected with puromycin (200 ng/ml) and resistant cells were pooled.

Luciferase activities were measured using the Dual-Luciferase reporter assay system (Promega) for firefly luciferase and renilla luciferase. The antiviral activity of overexpressed ZAP was indicated by fold inhibition. Following Sindbis virus infection or transfection of the luciferase reporters, 293Trex-ZAP cells were mock treated or treated with tetracycline to induce ZAP expression. Luciferase activities were measured and firefly luciferase activity was normalized by the renilla luciferase activity. Fold inhibition was calculated as the luciferase activity in the absence of ZAP divided by the luciferase activity in the presence of ZAP.

All siRNAs were obtained from GenePharma and transfected into cells using Lipofectamine 2000 according to the manufacturer's instructions. The target sequences of siRNAs are the following: control siRNA, 5′-UUCUCCGAACGUGUCACGUTT-3′; siZAP, 5′-GAUUCUUUAUCUGAUGUCATT-3′; siUbiquitin, 5′-CCAGCAGAGGCTCATCTTT-3′; and siISG15, 5′-GCAACGAAUUCCAGGUGUC-3′.

Sindbis virus infection. The production of Sindbis virus was previously reported (1). Briefly, the cells were infected with the virus for 1 h at a multiplicity of infection (MOI) of 0.01 in DMEM supplemented with 1% FBS. Cells were washed twice with 1× phosphate-buffered saline (PBS) and cultured in DMEM with 2% FBS. At 24 h postinfection, cells were lysed and the cell lysates were subjected to luciferase assay and Western blotting.

Quantitative PCR. mRNA levels of ubiquitin, ISG15, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and reporters pCMV-FL-Mk and pRL-CMV were analyzed using quantitative PCR. PCRs were performed using SYBR green PCR master mix (Tiangen) using the following conditions: 94°C for 5 min and then 40 cycles at 94°C for 30 s, 55°C for 30 s, and 68°C for 30 s. The experiments were performed in duplicate for each data point. Sequences of primers are the following: qUbiquitin FP, 5′-GGTGAGCT TGTTTGTGTCCCTGT-3′; qUbiquitin RP, 5′-TCCACCTCAAGGGTGATGGTC-3′; qISG15 FP, 5′-CTCTGAGCATC CTGGTGAGGAA-3′; qISG15 RP, 5′-AAGGTCAGCCAGAACAGGTCGT-3′; qFL-Mk FP, 5′-TGAGGCACTGGGCA GGTGTC-3′; qFL-Mk RP, 5′-ATGCAGTTGCTCTCCAGCGG-3′; qRL FP, 5′-TGAGGCACTGGGCAGGTGTC-3′; qRL RP, 5′-ATGAAGGAGTCCAGCACGTTC-3; qGAPDH FP, 5′-CCTGGCCAAGGTCATCCATG-3′; and qGAPDH RP, 5′-CTCCTTGGAGGCCATGTGGG-3′.

Ubiquitination assays. To assay ubiquitination of NZAP in cells, 293T cells were transfected with plasmids expressing NZAP-Flag, ubiquitin, and ubiquitination enzymes. At 48 h posttransfection, cells were lysed in 100 μl of lysis buffer (1% SDS, 150 mM NaCl, 10 mM Tris-HCl, pH 8.0) supplemented with 2 mM sodium orthovanadate, 50 mM sodium fluoride, protease inhibitors, and RNase A. The lysate was boiled for 10 min and then sonicated, followed by addition of 900 μl of dilution buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 2 mM EDTA, and 1% Triton X-100) and incubation at 4°C for 30 to 60 min with rotation. The lysate was then clarified by centrifugation and incubated with an anti-Flag antibody and protein G agarose resin for 2 h at 4°C. The immunoprecipitates were washed 3 times with PBS, resuspended in loading buffer, and analyzed by SDS-PAGE and Western blotting.

To assay ubiquitination of NZAP *in vitro*, bacterially expressed purified NZAP-Flag, TRIM25, His-ubiquitin, His-Uba1 (E1), and His-Ubc13-Ueva1 (E2 complex) were incubated in a reaction buffer (50 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, 1 mM dithiothreitol [DTT], and 2 mM ATP) at room temperature for 1 h. The reaction products were analyzed by SDS-PAGE and Western blotting using an anti-Flag antibody.

RNA immunoprecipitation. To evaluate the effect of TRIM25 downregulation on ZAP association with target RNA, 293TRex-TRIM25i or 293TRex-Ctrli cells were transfected with plasmids expressing NZAP-Flag and reporters pCMV-FL-MK and pRL-CMV. To evaluate the effect of the overexpression of OTUB1 or ubiquitin on ZAP association with target RNA, 293TRex cells were transfected with plasmids expressing NZAP-Flag, reporters pCMV-FL-MK and pRL-CMV, and the effector. At 48 h posttransfection, cells were lysed in 500 μl of RLN buffer (50 mM Tris-HCl, pH 8.0, 140 mM NaCl, 1.5 mM $MgCl_2$, and 0.5% NP-40) supplemented with RNase inhibitors, 1 mM DTT, and protease inhibitors. The lysate was clarified by centrifugation. One-fifth of the lysate was used to extract RNA. Heparin and tRNA were added to the rest of the cell lysate, followed by incubation with an anti-Flag antibody and protein G agarose resin for 2 h at 4°C. The immunoprecipitates were washed 4 times with binding buffer (10 mM Tris-HCl, pH 7.6, 50 mM NaCl, 1 mM EDTA, and 10 μM $ZnCl_2$) and resuspended in TRIzol for RNA extraction. RNA levels were measured by quantitative PCR.

Antibodies. All of the antibodies were obtained commercially: anti-myc monoclonal antibody (Santa Cruz Biotechnology), anti-myc agarose affinity gel (Sigma-Aldrich), anti-β-actin monoclonal antibody (Sigma-Aldrich), anti-TRIM25 antibody (Santa Cruz Biotechnology), anti-ZAP monoclonal antibody (Thermo Fisher Scientific), anti-Flag monoclonal antibody (Sigma-Aldrich), anti-ISG15 monoclonal antibody (Abmart), and anti-HA monoclonal antibody (ZSGB-Bio).

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We have no conflicts of interest to declare.

X.Z., X.W., F.T., Q.W., Z.F., and G.G. designed research; X.Z., X.W., F.T., and Q.W. performed research; X.Z., X.W., F.T., Q.W., Z.F., and G.G. analyzed data; and X.Z., X.W., and G.G. drafted the manuscript.

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