



Zebrafish TRIM25 Promotes Innate Immune Response to RGNNV Infection by Targeting 2CARD and RD Regions of RIG-I for K63-Linked Ubiquitination

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RIG-I-like receptors (RLRs) play important roles in response to virus infection by regulating host innate immune signaling pathways. Meanwhile, the RLR signaling pathway is also tightly regulated by host and virus to achieve the immune homeostasis between antiviral responses and virus survival. Here, we found that zebrafish TRIM25 (zbTRIM25) functioned as a positive regulator of RLR signaling pathway during red spotted grouper nervous necrosis virus (RGNNV) infection. Post-RGNNV infection, zbTRIM25 expression was obviously inhibited and ectopic expression of zbTRIM25 led to enhanced expression of RLR signaling pathway-related genes. Overexpression and knockdown analysis revealed that zbTRIM25 promoted zebrafish RIG-I (zbRIG-I)-mediated IFN signaling and inhibited RGNNV replication. Mechanistically, zbTRIM25 bound to zbRIG-I; in particular, the SPRY domain of zbTRIM25 interacted with the tandem caspase activation and recruitment domains (2CARD) and repressor domain (RD) regions of zbRIG-I. zbTRIM25 promoted the K63 polyubiquitination of 2CARD and RD regions of zbRIG-I. Furthermore, zbTRIM25-mediated zbRIG-I activation of IFN production was enhanced by K63-linked ubiquitin, indicating that zbTRIM25-mediated zbRIG-I polyubiquitination was essential for RIG-I-triggered IFN induction. In conclusion, these findings reveal a novel mechanism that zbTRIM25 positively regulates the innate immune response by targeting and promoting the K63-linked polyubiquitination of zbRIG-I.

Keywords: TRIM25, RIG-I, ubiquitination, zebrafish, red spotted grouper nervous necrosis virus

INTRODUCTION

The innate immune system recognizes pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs) as against microbial pathogen invasion (1). Retinoic acid inducible gene-I (RIG-I)-like receptors (RLRs), as intracellular PRRs, composed of RIG-I, MDA5, and LGP2, recognize non-self signatures of viral RNAs in the cytosol of cells. After activated by viral RNA, RIG-I and MDA5 recruited the downstream adaptor molecule, MAVS, to their N-terminal caspase-recruitment domains (CARDs). Then, tumor necrosis factor receptor-associated factors

(TRAF) and TANK-binding kinase 1/I κ -B kinase ϵ interacted with MAVS, which in turn leads to the phosphorylation and cytoplasm-to-nucleus translocation of interferon (IFN) regulatory factor 3 (IRF3), and the activation of type I IFN. Subsequent IFNs activated a variety of IFN-stimulated genes (ISGs) to limit the virus replication (2).

Nervous necrosis virus (NNV) is a non-enveloped, single-stranded RNA virus belonging to the family *Nodaviridae*. Increasing evidence has shown that NNV can infect more than 120 fish species and causes mass mortalities of infected fish worldwide (3). It has been revealed that RLRs respond *in vivo* or *in vitro* to the stimulation of NNV and possess capacities in the induction of IFNs and ISGs in a variety of fish species. For example, in ZF4 cells, expression of RLRs was significantly enhanced post-NNV infection and RIG-I knockdown significantly restrained group II type I IFN activation (4). Our previous studies also suggested that RLR signaling pathway was activated during red spotted grouper nervous necrosis virus (RGNNV) infection in sea perch and its key components possessed anti-RGNNV activities (5, 6). However, regulation mechanisms of RLR signaling pathway during RGNNV infection is still unclear. RLR-mediated antiviral signaling pathway is tightly regulated at multiple steps in the signaling cascade. Several studies demonstrated that post-translational modifications, including ubiquitination, ISGylation, and phosphorylation, were important mechanisms that regulated the RLR signaling pathway, of which ubiquitination was a key regulatory mechanism for RLR pathway (2). For instance, RNF122 negatively regulated RLR signaling pathway by targeting RIG-I (7). MDA5 and MAVS were targeted for K48-linked ubiquitination by TRIM13 and RNF5, respectively, which induced MDA5 and MAVS degradation and RLRs signal termination (8, 9). TRIM25 E3 ubiquitin ligase induced the K63-linked ubiquitination of RIG-I, which activated RLR signaling pathway to elicit host antiviral innate immunity (10).

TRIM25, an IFN-inducible E3 ligase, is associated with all kinds of cellular processes, such as the immune response, cancer, and so on (11). It is becoming evident that TRIM25 has a dual role in RIG-I regulation, since TRIM25 not only induces K63-linked ubiquitination of RIG-I to positive regulate RLR signaling activation but also negatively regulates RIG-I activation through inhibiting HLA-F adjacent transcription 10 degradation, a negative regulator of RIG-I-mediated inflammatory response (12).

Multiple fish TRIM25 homologs have been reported, including *Rhodeus uyekii* (13), *Epinephelus coioides* (14), and *Larimichthys crocea* (15). Increasing evidence showed that fish TRIM25 was involved in antiviral immunity and played a pivotal role in RLR antiviral signaling pathway (14). However, the mechanism by which fish TRIM25 regulates RLR signaling pathway has not been explored. In the present study, zebrafish TRIM25 (zbTRIM25) was involved in RGNNV infection and was identified as a positive mediator of RLR signaling pathway by binding to and ubiquitinating the caspase activation and recruitment domain (2CARD) and repressor domain (RD) regions of RIG-I, which is different with the findings in mammals. Our findings reveal a novel mechanism of TRIM25

to activate RLR signaling pathway and will help to develop new treatments for viral nervous necrosis disease.

MATERIALS AND METHODS

Ethics Statement

All procedures with zebrafish were approved by the Ethics Committee of Sun Yat-Sen University and the methods were carried out following the approved guidelines.

Fish Strains, Cell Lines, Virus, and Reagents

Zebrafish wild-type AB line was purchased from China Zebrafish Resource Center. Fish were raised with 10 h darkness and 14 h light at 28°C and were fed with commercial pellets twice a day. All embryos were obtained by natural spawning and staged as previously reported (16).

ZBE3 cells derived from zebrafish embryos were cultured at 28°C as previously described (17). HEK 293T cells were cultured in DMEM (Invitrogen) enriched with 10% FBS (Invitrogen) at 37°C under a humidified atmosphere of air containing 5% CO₂.

RGNNV was propagated in ZBE3 cells and stored at -80°C until use.

Anti-Flag (M20008), anti-Myc (M20002), anti-His (M20001L), and anti-HA antibodies (M20013) were purchased from Abmart. Anti- α -tubulin (ab15246) and anti-GFP antibodies (G1544) were purchased from Abcam and Sigma, respectively. Goat anti-rabbit IgG-HRP, goat anti-mouse IgG-HRP, Alexa Fluor 488-labeled goat anti-mouse IgG, and Alexa Fluor 555-labeled goat anti-rabbit IgG secondary antibodies were purchased from Invitrogen.

Viral Challenge

For *in vitro* infection, ZBE3 cells were challenged with RGNNV [multiplicity of infection (MOI) = 1] for 6, 12, and 24 h, respectively. Subsequently, RNA from cells was extracted to detect the expression of *zbTRIM25* mRNA by quantitative real-time PCR (qRT-PCR).

For *in vivo* infection, RGNNVs (10⁸ TCID₅₀/ml) were injected into the egg yolk of 50 embryos at the single-cell stage in the experimental group. In the mock group, 50 embryos were injected with DMEM. A total of 1 nl of solution was microinjected into each embryo using a microinjector. RNA from zebrafish embryo was extracted to detect the expression of *zbTRIM25* mRNA by qRT-PCR at 24 h post-injection.

Knockdown of zbTRIM25 by siRNA

zbTRIM25 siRNA (5'-GAATCCAGTTGAAGAGAAA-3') and control siRNA were synthesized by Ribobio Company (Guangzhou, China). ZBE3 cells were transfected with zbTRIM25 siRNA or control siRNA according to the manufacturer's protocol using Lipofectamine 3000 as previously described (18). Twenty-four hours after transfection, ZBE3 cells were infected with RGNNV (MOI = 1) for 24 h and total RNAs were extracted for qRT-PCR analysis.

Plasmid Construction

The ORF of zbTRIM25 (GenBank accession no. NM200175.1) was sub-cloned into *pCMV-Flag* or *pCMV-Myc* vectors (Invitrogen) to generate recombinant plasmid *pCMV-Flag-zbTRIM25* or *pCMV-Myc-zbTRIM25*, respectively. Full-length zbRIG-I and zbRIG-I deletion mutant cDNAs encoding amino acids 1–187 (zbRIG-I-2CARD), 188–937 (zbRIG-I- Δ 2CARD), 812–927 (zbRIG-I-RD), and 188–811 [zbRIG-I- Δ (2CARD+RD)] were inserted into the *pEGFP-N3* vectors. Full-length zbRIG-I was inserted into the *pET-32a(+)* (Clontech) vector to generate recombinant plasmid *pET-32a(+)-zbRIG-I*. zbTRIM25 deletion mutant zbTRIM25-SPRY and zbTRIM25- Δ SPRY were generated using the *pCMV-Flag-zbTRIM25* plasmid as a template. Primers used for amplifying these genes are listed in **Table S1**.

HA-K63Ub plasmid was purchased from Rebio (Shanghai, China).

RNA Isolation and qRT-PCR

RNA extraction and cDNA synthesis were performed using Trizol (Invitrogen) and PrimeScriptTM 1st Strand cDNA Synthesis Kit (Takara) according to the manufacturer's instructions. QRT-PCR analyses of *zbTRIM25*, *zbRIG-I*, *RNA dependent RNA polymerase (RDRP)*, RLR signaling pathway related genes (*MAVS*, *TRAF3*, *IRF3*, and *IFN 1*), and *ISG15* were performed as previously described (19). Relative expression levels of target genes were normalized to *18s rRNA* using $2^{-\Delta\Delta Ct}$ methods. Data represent the mean \pm SD from three independent experiments, each performed in triplicate. Primers sequences used for qRT-PCR are listed in **Table S1**.

Dual Luciferase Reporter Assay

HEK 293T cells, pre-seeded in 24-well plates, were transfected with 250 ng of *pGL3-DrlFN 1-pro-Luc* plasmid or *pGL3-Basic* empty vector with 25 ng of *pRL-TK* vector (Promega) together with *pCMV-Myc-zbRIG-I* or *pCMV-Myc* and *pCMV-Flag* or *pCMV-Flag-zbTRIM25* (250 ng per well) for 24 h. Then, cells were incubated with poly I:C for 48 h and lysed. Luciferase activities were measured using the dual-luciferase reporter assay system (Promega). Relative luciferase activities were expressed as the ratio of firefly to Renilla luciferase activity. The results were the representative of three independent experiments in triplicate.

HEK 293T cells, pre-seeded in 24-well plates, were transfected with 250 ng of *pGL3-DrlFN1-pro-Luc* plasmid or *pGL3-Basic* empty vector with 25 ng of *pRL-TK* vector (Promega). Meanwhile, *pCMV-Myc-zbTRIM25*, mutant *zbRIG-I* or empty control plasmids were co-transfected. After being incubated with poly I:C for 48 h, cells were lysed for luciferase assay as described above. At least three independent experiments were performed.

Immunofluorescence Labeling and Confocal Microscopy

HEK 293T cells, seeded on glass cover slips, were transfected with *pCMV-Myc-zbTRIM25* and *pCMV-Flag-zbRIG-I* plasmids. Twenty-four hours post-transfection, cells were washed with PBS three times and fixed with prechilled methanol and then permeabilized using 1% Triton X-100 in PBS for 10 min and

blocked with 5% normal goat serum for 30 min at room temperature (RT). Cells were incubated with anti-Myc and anti-Flag antibodies for 60 min at RT. Finally, cells were washed with PBS and incubated with the appropriate Alexa Fluor 488 or 555 conjugated secondary antibodies for 1 h. After cell nucleus was stained with Hoechst 33342, cells were observed by a confocal microscope (Zeiss, Germany).

Coimmunoprecipitations (Co-IP) and Western Blotting Analysis

Co-IP and Western blotting experiments were performed as described previously (18). HEK 293T cells in 75-cm² flasks were co-transfected with 10 μ g of different plasmid combinations for 48 h. Then, the cells were lysed on ice with lysis buffer for 15 min and were immunoprecipitated with the indicated antibodies. The precipitated samples and whole-cell lysates (Input) were analyzed by immunoblotting with the indicated antibodies.

His Fusion Protein Expression and Pull-Down Assays

Escherichia coli BL21(DE3) cells were transformed with *pET-32a(+)-zbRIG-I* or *pET-32a(+)* plasmids, respectively. Then, cells were grown in 50 ml of LB medium (Beyotime) containing 0.5 mM isopropyl-1-thio- β -D-galactopyranoside (IPTG) (Sigma) at 18°C overnight with shaking at 120 rpm. Cells were pelleted by centrifugation at 4,500 rpm for 30 min and lysed in 10 ml of lysis buffer (100 mM sodium phosphate, pH 8.0, 600 mM NaCl, and 0.02% Tween-20) (Beyotime) via sonication on ice. The sonicated mixture was centrifuged at 15,000 rpm at 4°C for 20 min, and then the supernatant was affinity-purified with Dynabead His-Tag magnetic beads (Invitrogen) according to the manufacturer's instruction.

His pull-down assays were performed as described previously with some modifications (20). His-zbRIG-I-magnetic beads were incubated with the lysates of HEK 293T cells transfected with *pCMV-Flag-zbTRIM25* or *pCMV-Flag* empty vectors on a roller, respectively. After incubation at 4°C overnight, the magnetic beads were washed three times with lysis buffer to remove unbound His-zbRIG-I and then analyzed via Western blotting using anti-Flag or anti-His antibodies. His tag protein alone was served as a negative control.

Ubiquitination Assays

Ubiquitination assays were performed as described previously with some modifications (21). HEK 293T cells, pre-seeded in 75-cm² flasks overnight, were co-transfected with 10 μ g of different plasmid combinations. Cells were lysed at 24 h after transfection, and then GFP-zbRIG-I mutants were immunoprecipitated with anti-GFP antibodies as described above. Immunoprecipitates or Input were analyzed by immunoblotting with the indicated antibodies.

Statistics Analysis

All statistics were calculated using SPSS version 20. Differences between control and treatment groups were assessed by one-way ANOVA. $P < 0.05$ is considered

statistically significantly different. $P < 0.01$ was considered highly significant.

RESULTS

zbTRIM25 Expression Is Down-Regulated During RGNNV Infection *in vitro* and *in vivo*

As shown in **Figure 1A**, mRNA level of *zbTRIM25* was down-regulated within 24 h after RGNNV infection. Meanwhile, we also investigated the expression of *zbTRIM25* in RGNNV-infected zebrafish embryos at 24 h, and the results were concordant with ZBE3 cells (**Figure 1B**). These data indicated a potential role of *zbTRIM25* in innate immune response to RGNNV infection.

zbTRIM25 Positively Regulates RLR Signaling Pathway in Zebrafish

In mammals, TRIM25 has been suggested to promote IFN- β production by functioning as a key upstream activator of RIG-I to activate the RLR signaling pathway (10). To investigate whether *zbTRIM25* regulated RLR signaling pathway in zebrafish, the expression of several RLR signaling pathway-related genes was measured in *zbTRIM25* overexpressing ZBE3 cells. As shown in **Figure 2A**, overexpression of *zbTRIM25* markedly enhanced the expression of *RIG-I*, *MAVS*, *TRAF3*, *IRF3*, *IFN 1*, and *ISG15* during RGNNV infection. Similar results were detected in *zbTRIM25* overexpressing ZBE3 cells treated with poly I:C (**Figure 2B**). Furthermore, co-expression of *zbTRIM25* with *zbRIG-I* induced a dose-dependent increase in IFN activation compared with the *zbRIG-I* overexpression alone (**Figure 2C**). Overexpression of *zbTRIM25* dose-dependently inhibited RGNNV replication (**Figure 2D**). On the contrary, knockdown of *zbTRIM25* using siRNA increased the level of *RDRP* in RGNNV-infected ZBE3 cells (**Figures 2E,F**). All these results demonstrate that *zbTRIM25* is a positive regulator of RLR signaling pathway

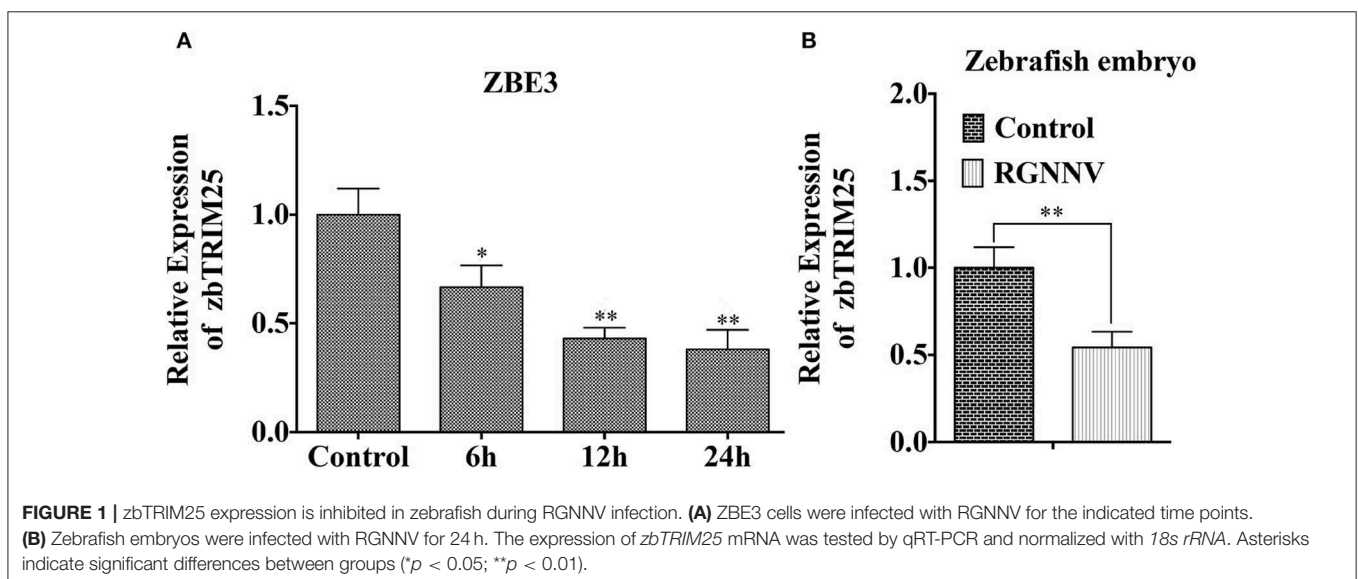
and functions as an antiviral factor during RGNNV infection in zebrafish.

zbTRIM25 Interacts With zbRIG-I

To elucidate the mechanism by which *zbTRIM25* participates in RLR signaling pathway in zebrafish, the interaction of *zbTRIM25* and *zbRIG-I* was investigated. We co-expressed *Myc-zbTRIM25* and *Flag-zbRIG-I* plasmids in HEK 293T cells, and immunofluorescence imaging showed *zbTRIM25* and *zbRIG-I* colocalized in the cytoplasm of HEK293T cells (**Figure 3A**). Co-IP against the Myc tag revealed that *zbTRIM25* could interact with full-length *zbRIG-I* but not with Flag-vector (**Figure 3B**). His pull-down analysis showed that *zbTRIM25* was directly bound to *zbRIG-I* (**Figure 3C**). All these data suggest that *zbTRIM25* interacts with *zbRIG-I*.

SPRY Domain of zbTRIM25 Interacts With 2CARD and RD Regions of zbRIG-I

To identify the region involved in the *zbRIG-I*/*zbTRIM25* interaction, firstly, *zbRIG-I* deletion mutants [*pEGFP-zbRIG-I-2CARD*, *pEGFP-zbRIG-I- Δ 2CARD*, *pEGFP-RIG-I-RD*, and *pEGFP-RIG-I- Δ (2CARD+RD)*] were constructed and co-transfected with *Flag-zbTRIM25* in HEK 293T cells (**Figure 4A**). *zbRIG-I-2CARD*, *zbRIG-I- Δ 2CARD*, and *zbRIG-I-RD* could bind to *zbTRIM25* individually (**Figures 4B–D**); however, *zbRIG-I- Δ (2CARD+RD)* failed to co-precipitate with *zbTRIM25* (**Figure 4E**). These results indicate that *zbRIG-I* binds to *zbTRIM25* through its N-terminal 2CARD region and the C-terminal RD region. Furthermore, we constructed two truncations of *zbTRIM25* (*zbTRIM25-SPRY* and *zbTRIM25- Δ SPRY*) co-transfected with *zbRIG-I-2CARD* or *zbRIG-I-RD* in HEK 293T cells, respectively (**Figure 4F**). We found that the SPRY domain of *zbTRIM25* interacted with 2CARD and RD regions of *zbRIG-I* (**Figures 4G–J**). Collectively, these results indicate that the SPRY domain of *zbTRIM25* is responsible for its interaction with 2CARD and RD regions of *zbRIG-I*.



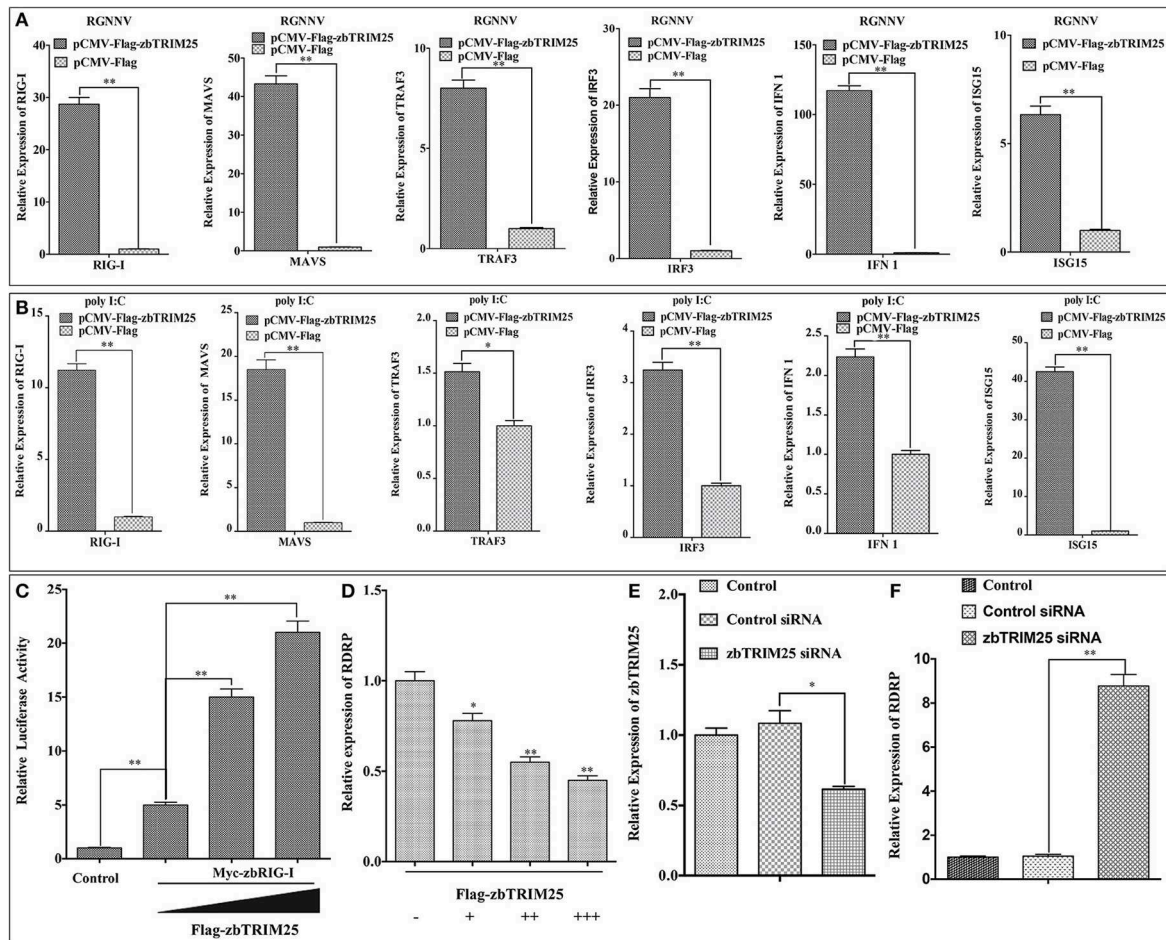


FIGURE 2 | zbTRIM25 potentiates RLR signaling pathway. **(A,B)** ZBE3 cells were transfected with pCMV-Flag-zbTRIM25 or pCMV-Flag vector for 24 h. After treated with RGNV **(A)** or poly I:C **(B)**, the transcript levels of *RIG-I*, *MAVS*, *TRAF3*, *IRF3*, *IFN 1*, and *ISG15* mRNA in ZBE3 cells were analyzed. **(C)** HEK 293T cells were transfected with pCMV-Myc or pCMV-Myc-zbRIG-I together with pCMV-Flag or increasing amount of pCMV-Flag-zbTRIM25 as well as *DrIFN1 pro-Luc* and *pRL-TK*. Luciferase activities were measured and normalized to the amount of Renilla Luciferase activities. **(D)** ZBE3 cells were transfected with pCMV-Flag and increasing amount of pCMV-Flag-zbTRIM25 for 24 h, and then infected with RGNV for 24 h. QRT-PCR analysis was performed for *RDRP*. **(E,F)** ZBE3 cells were either not transfected (Control) or transfected with 100 nM Control siRNA (NC) or 100 nM zbTRIM25 siRNA. Cells were then subcultured for 24 h and infected with RGNV for 24 h. Cells were harvested subsequently. Levels of *zbTRIM25* and *RDRP* mRNA were analyzed by qRT-PCR and normalized with *18s rRNA*. Data represent the mean + SD ($n = 3$). Asterisks indicate significant differences between groups (* $p < 0.05$; ** $p < 0.01$).

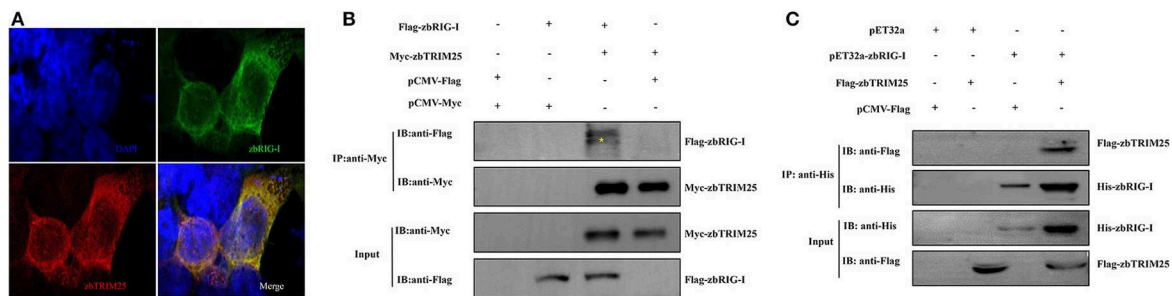


FIGURE 3 | zbTRIM25 interacts with zbRIG-I. **(A)** HEK 293T cells were transfected with pCMV-Flag-zbRIG-I and pCMV-Myc-zbTRIM25 plasmids for 24 h, and then cells were immunostained with anti-Myc and anti-Flag antibodies and analyzed by fluorescence microscopy. All nuclei were stained with Hoechst 33342. **(B)** HEK 293T cells were transfected with plasmids as indicated for 24 h. After treated with poly I:C for 24 h, cells were lysed, and the cell lysates were either analyzed directly by using anti-Myc and anti-Flag antibodies via Western blotting (Input) or subjected to immunoprecipitation using anti-Myc antibodies. The precipitates (IP) were analyzed by Western blotting with anti-Myc and anti-Flag antibodies, respectively. **(C)** The purified His-zbRIG-I proteins were incubated with the lysates of HEK 293T cells transfected with the indicated plasmids, respectively. The pulled down proteins and original cell lysates were examined by Western blotting with indicated antibodies.

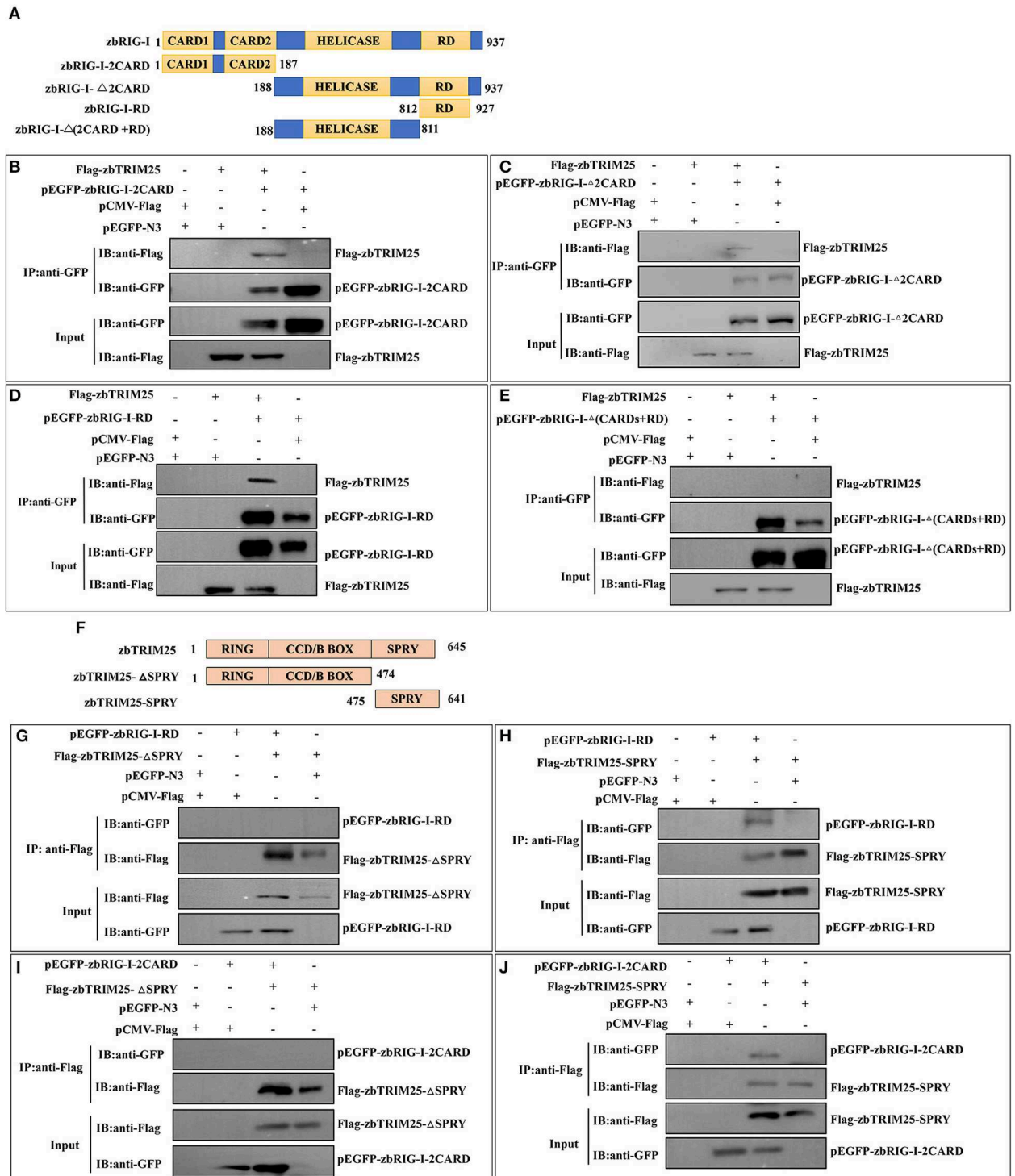
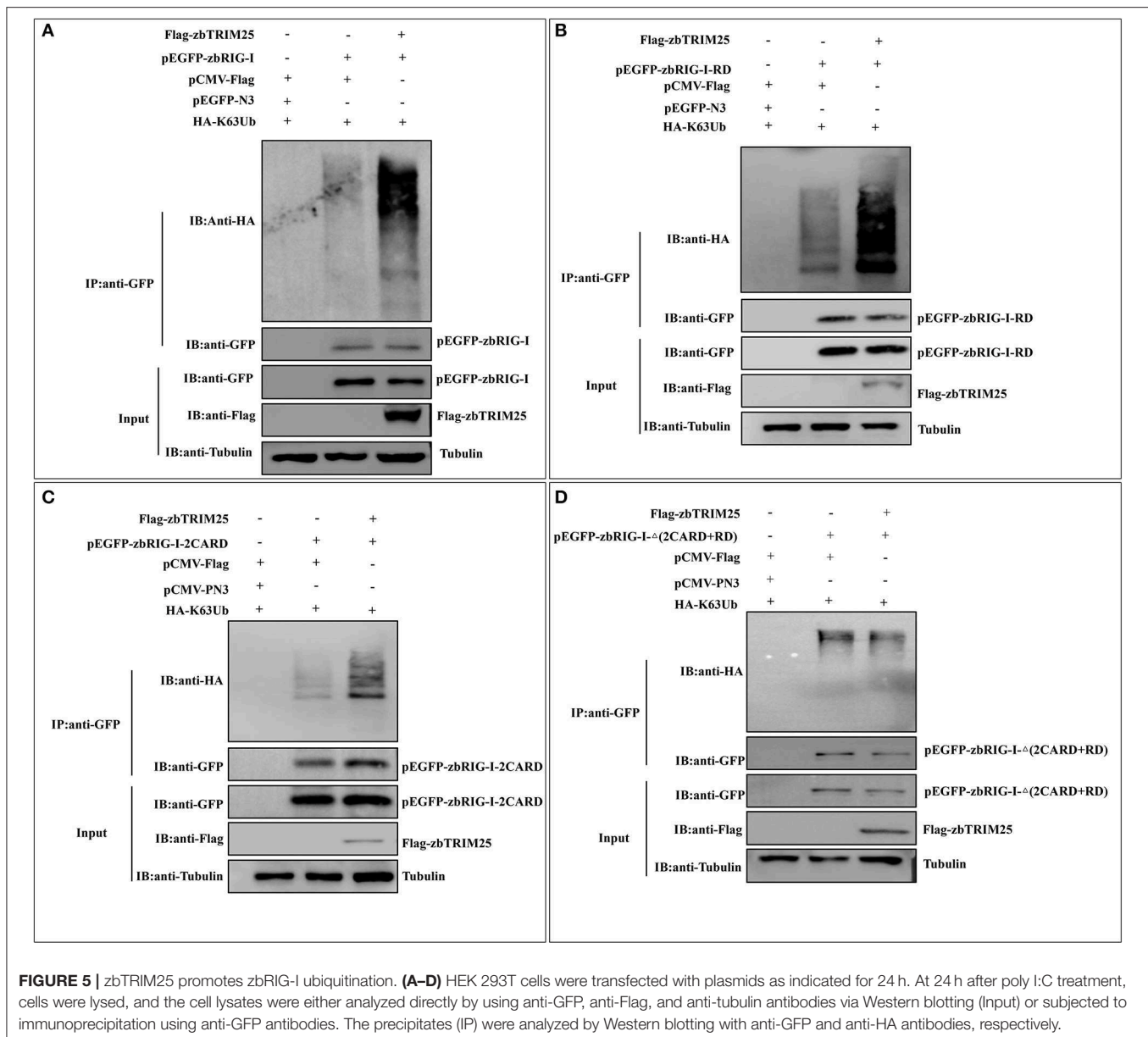


FIGURE 4 | Physical interaction of zbTRIM25 with zbRIG-I. **(A)** Schematic representation of full-length zbRIG-I and zbRIG-I deletion mutants. **(B–E)** Interactions of GFP-tagged zbRIG-I-2CARD **(B)**, zbRIG-I-Δ2CARD **(C)**, zbRIG-I-RD **(D)**, or zbRIG-I-Δ(CARDS+RD) **(E)** with Flag-tagged zbTRIM25 were examined using immunoprecipitation assays. HEK 293T cells were transfected with plasmids as indicated for 24 h. After treated with poly I:C for 24 h, cells were lysed, and the cell lysates were either analyzed directly by using anti-GFP and anti-Flag antibodies via Western blotting (Input) or subjected to immunoprecipitation using anti-GFP antibodies. The precipitates (IP) were analyzed by Western blotting with anti-GFP and anti-Flag antibodies, respectively. **(F)** Schematic illustration of zbTRIM25 truncations. Interactions of GFP-zbRIG-I-RD with Flag-zbTRIM25-ΔSPRY **(G)** or Flag-zbTRIM25-SPRY **(H)**, GFP-zbRIG-I-2CARD with Flag-zbTRIM25-ΔSPRY **(I)**, or Flag-zbTRIM25-SPRY **(J)** were examined using immunoprecipitation assays. HEK 293T cells were transfected with plasmids as indicated for 24 h. Cells were lysed after treated with poly I:C for 24 h. Immunoprecipitation and immunoblotting were performed with indicated antibodies.



zbTRIM25 Ubiquitinates Both 2CARD and RD Regions of zbrIG-I

To investigate whether the E3 ligase activity of zbTRIM25 is involved in the regulation of zbrIG-I, the ubiquitination of zbrIG-I was tested in zbTRIM25 overexpressing cells. We found that zbTRIM25 markedly promoted the K63 polyubiquitination of zbrIG-I (Figure 5A). Furthermore, HEK 293T cells were transfected with Flag-tagged zbTRIM25, zbrIG-I deletion mutants [*GFP-zbrIG-I-2CARD*, *GFP-zbrIG-I-Δ(2CARD+RD)*, and *GFP-zbrIG-I-RD*], and HA-tagged K63 ubiquitin, and our results showed that zbTRIM25 obviously enhanced the ubiquitination of zbrIG-I-2CARD and zbrIG-I-RD (Figures 5B,C), but not zbrIG-I-Δ(2CARD+RD) (Figure 5D).

These data suggest that zbTRIM25 ubiquitinates both N-terminal 2CARD and C-terminal RD regions of zbrIG-I.

zbTRIM25-Mediated Ubiquitination of zbrIG-I 2CARD and RD Regions Is Important for IFN Inducing

It has been reported that ubiquitination of RIG-I by TRIM25 is vital for IFN signaling. Thus, the effect of zbTRIM25-mediated zbrIG-I ubiquitination on zbrIG-I's IFN-inducing activities was assessed. Our results showed that ectopic expression of zbrIG-I-2CARD and zbrIG-I-RD could enhance IFN promoter activity (Figure 6A), and this activation was markedly enhanced by zbTRIM25 overexpression (Figures 6B,C). Furthermore,

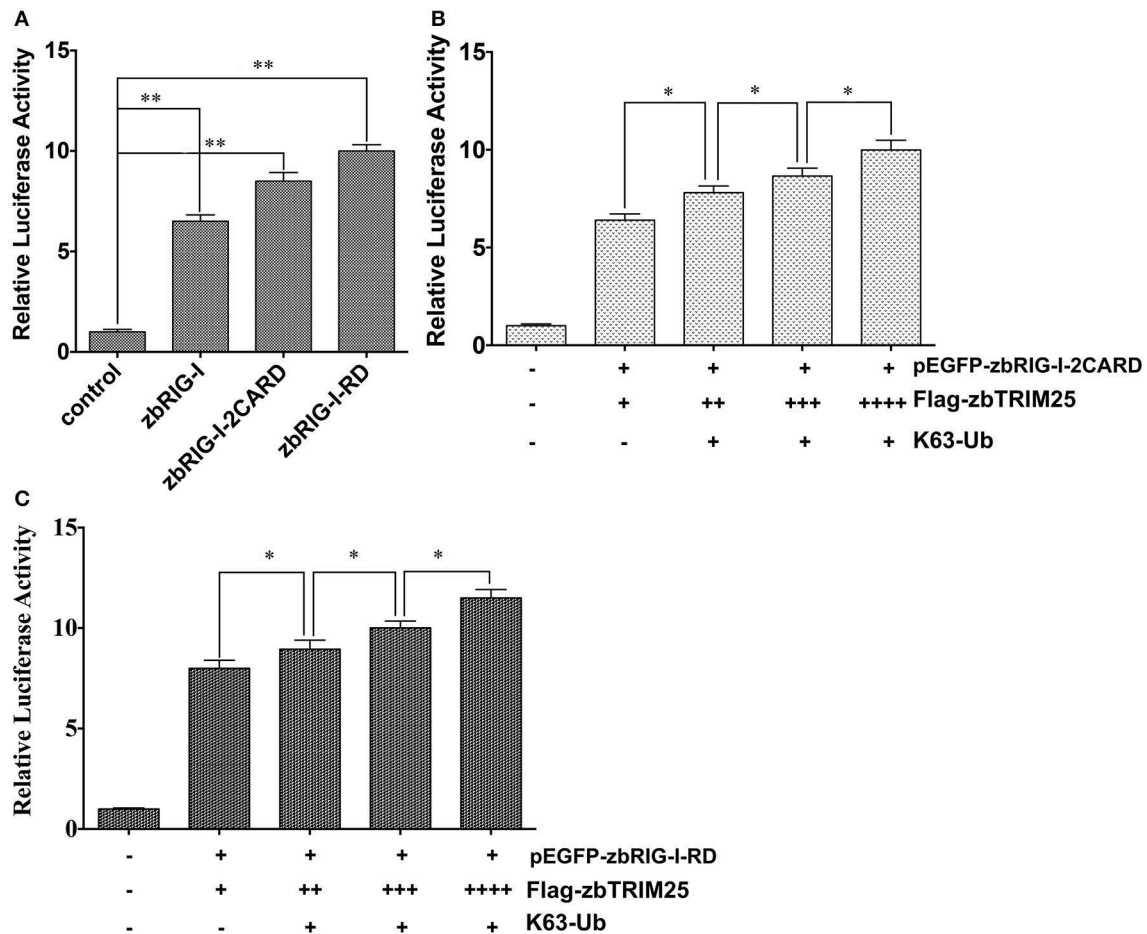


FIGURE 6 | zbTRIM25-mediated K63 ubiquitination in 2CARD and RD regions of zbRIG-I is important for IFN induction. **(A)** HEK 293T cells were transfected with pEGFP-zbRIG-I, pEGFP-zbRIG-I-2CARD, and pEGFP-zbRIG-I-RD as well as *DrlFN1 pro-Luc* and *pRL-TK* vectors. **(B,C)** HEK 293T cells were transfected with pEGFP-zbRIG-I-2CARD or pEGFP-zbRIG-I-RD together with HA-K63Ub and increasing amount of pCMV-Flag-zbTRIM25 as well as *DrlFN1 pro-Luc* and *pRL-TK* plasmids. Luciferase activities were measured and normalized to the amount of Renilla Luciferase activities. Data represent the mean + SD ($n = 3$). Asterisks indicate significant differences between groups (* $p < 0.05$; ** $p < 0.01$).

overexpression of K63-linked ubiquitin dose-dependently increased the promotion effect of zbTRIM25 on zbRIG-I-2CARD and RD mediated IFN 1 promoter activation (**Figures 6B,C**). These data confirm the importance of zbTRIM25-mediated K63 ubiquitination in the N-terminal 2CARD region and C-terminal RD region of zbRIG-I for zbRIG-I-mediated IFN induction.

DISCUSSION

RLR signaling pathway plays crucial roles in recognizing viral infections and initiating the antiviral immune response. RIG-I, as an important component of RLR signaling pathway, can detect viral dsRNAs in the cytoplasm and induce type I IFN production and the secretion of pro-inflammatory cytokines to suppress virus spread during virus infection (22). Multiple studies have demonstrated that the ubiquitination of RIG-I plays an important role in the RIG-I-mediated antiviral signaling

pathway. For instance, TRIM25, TRIM4, and MEX3C positively regulate RIG-I-mediated signaling by targeting RIG-I for K63-linked polyubiquitination (23, 24). TRIM25, well-known as an ubiquitin E3 ligase and an ISG15 E3 ligase, is widely involved in the regulation of innate immunity (10, 25). In mammals, previous reports showed that TRIM25 enhanced RLRs antiviral pathway by binding viral RNA-activated RIG-I to induce its K63-linked polyubiquitination and subsequent IFNs and ISGs production (26). In teleost fish, several TRIM25 homologs were reported to play a pivotal role in innate immunity (14, 15); however, the mechanisms by which fish TRIM25 modulates the innate immune response against viruses remain elusive. Here, we found that zbTRIM25 positively regulated RLR signaling pathway and facilitated zbRIG-I-mediated IFN 1 promoter activation, and overexpression of zbTRIM25 inhibited RGNNV infection, indicating the conservative antiviral properties of TRIM25 in fish and mammals.

Several reports showed that TRIM25 was involved in the regulation of antiviral innate immunity by targeting RIG-I (10, 27). The mammal RIG-I protein contains two N-terminal CARD-like domains, a C-terminal RD region and an RNA helicase region (28). In zebrafish, RIG-Ia (an insertion variant of RIG-I) and RIG-Ib (the typical RIG-I) were identified as two transcripts of RIG-I, and overexpression of RIG-Ib in cultured fish cells, but not RIG-Ia, activated zebrafish type I IFN and induced antiviral response (29). Thus, in this report, we investigated the interaction of zbTRIM25 and zbRIG-I (RIG-Ib), and our results showed that zbTRIM25 was directly associated with zbRIG-I and especially the 2CARD or RD region of zbRIG-I was sufficient for its interaction with zbTRIM25. TRIM25 is characterized by an N-terminal region containing a catalytic RING domain, one or two B-box domains, a coiled-coil dimerization domain, and a C-terminal SPRY domain (30). Among these domains, SPRY was associated with protein-protein interactions and/or RNA binding (31). Gack et al. reported that the C-terminal SPRY domain of TRIM25 interacted with the first CARD of RIG-I, but not the helicase region and RD of RIG-I, and this interaction delivered the K63-linked ubiquitin moieties to the second CARD of RIG-I, which facilitated the dimerization of RIG-I and subsequent interaction with MAVS to induce antiviral signal transduction (10). We further investigate whether the SPRY domain of zbTRIM25 was responsible for its interaction with zbRIG-I. Unlike previous studies, we found that the SPRY domain of zbTRIM25 interacted not only with 2CARD but also with RD regions of zbRIG-I. In non-infected cells, RD covered the RNA-binding and helicase domains and CARDS folded over one another, which made RIG-I to exist in an auto-repressed conformation. Upon virus infection, viral RNAs interacted with the RD and the helicase domain of RIG-I, which in turn exposed the CARDS for MAVS interaction, thereby triggering antiviral responses (32, 33). Considering the interaction between RD of RIG-I and viral RNAs, we speculated that the interaction of zbTRIM25 and zbRIG-I RD might inhibit zbRIG-I sensing of viral RNAs. Meanwhile, it has been known that CARD domains of RIG-I are widely involved in its interaction with other proteins, such as MAVS, TRIM40, and virus proteins (27, 34, 35). Thus, the interaction of zbTRIM25 and zbRIG-I RD might also make room for other proteins to bind to 2CARD of zbRIG-I, zbTRIM25, and other proteins and will work cooperatively in regulation of RLR signaling pathway. The differences between the findings for zbTRIM25 and TRIM25 in mammals indicate that zbTRIM25 may regulate RLR signaling pathway in various ways.

Ubiquitination is a vital post-translational modification for the modulation of RIG-I activity. Several E3 ubiquitin ligases that mediate K63-linked ubiquitination of RIG-I for its activation have been identified. For instance, MEX3C overexpression caused the K63-linked ubiquitination of RIG-I-2CARD but not RIG-I- Δ 2CARD, and lysines 48, 99, and 169 of RIG-I were required for RIG-I ubiquitination by MEX3C (23). RNF135 mediated the K63-linked polyubiquitination of RIG-I-RD, and lysines 849 and 851 residues of RIG-I were crucial for RNF135-mediated ubiquitination (36). In contrast to RNF135, TRIM25 mediated the K63-linked polyubiquitination

of RIG-I-2CARD, but not RIG-I- Δ 2CARD, and the lysine 172 residue of RIG-I was critical for efficient TRIM25-mediated ubiquitination and the ability of RIG-I to activate antiviral signal transduction (10). Our results indicated that zbTRIM25 mediated K63-linked polyubiquitination of both 2CARD and RD regions of zbRIG-I, which is distinct from the findings in mammals that TRIM25 only targeted and promoted the K63-linked polyubiquitination of RIG-I 2CARD. In addition, our reporter analysis showed that overexpression of zbRIG-I-2CARD led to the activation of IFN 1 promoter, which is similar with other reports (37). Overexpression of zbRIG-I-RD also resulted in the activation of IFN 1 promoter. Furthermore, K63-linked ubiquitin is essential for the zbTRIM25-mediated enhancement of zbRIG-I 2CARD and RD-dependent IFN 1 promoter activation. zbRIG-I possessed capacities in the induction of IFNs and ISGs to enhance the antiviral response (38). Taken together, these findings suggest that zbTRIM25-mediated ubiquitination of 2CARD and RD regions of zbRIG-I is crucial for its antiviral innate immune response. However, due to the lack of TRIM25 or RIG-I-knockout zebrafish, we cannot assess the impact of the zebrafish TRIM25/RIG-I pathway at the *in vivo* level. A recent study demonstrated that zebrafish RNF135 also interacted with and ubiquitinated zbRIG-I (39). Further studies will be performed to determine the precise architecture of the zebrafish TRIM25/RNF135/RIG-I protein complex and the mechanism by which zbTRIM25 and zbRNF135 worked together to regulate ubiquitination of zbRIG-I.

It was known that several virus proteins could positively or negatively regulate RLR signaling pathway by targeting its key components or regulatory proteins (22). For instance, paramyxovirus V proteins interacted with the RIG-I/TRIM25 regulatory complex and inhibited RIG-I signaling (27). Influenza A virus NS1 protein bound to TRIM25 to block ubiquitination of the RIG-I (40). Severe acute respiratory syndrome nucleocapsid inhibited TRIM25-mediated RIG-I ubiquitination, causing the inhibition of IFN production (41). The RGNNV genome encodes a structural (capsid protein, CP) and a nonstructural (RNA-dependent RNA polymerase, RdRp) protein (3). Huang et al. reported that RDRP from OGNNV induced IFN by activating IRF3, the key regulatory component of RLRs-IFN signaling (42), indicating that RDRP might be a positive RLR signaling pathway. Whether RDRP targets the key components of RLR signaling pathway to exert its positive regulation role is a question that deserves further research. In addition, some miRNAs could target critical regulatory proteins of RLR pathway for immune evasion (43, 44); whether RGNNV infection-related miRNA was also involved in the regulation of RLR signaling pathway needs to be further investigated.

In summary, zbTRIM25 is identified as a positive regulator of RLR signaling pathway by targeting zbRIG-I. The SPRY domain of zbTRIM25 is required for its interaction with 2CARD and RD regions of zbRIG-I. zbTRIM25 promotes K63 polyubiquitination of both zbRIG-I 2CARD and RD regions, which subsequently induces the activation of downstream signaling event via MAVS and thereby inhibits viral infection (Figure 7). These findings

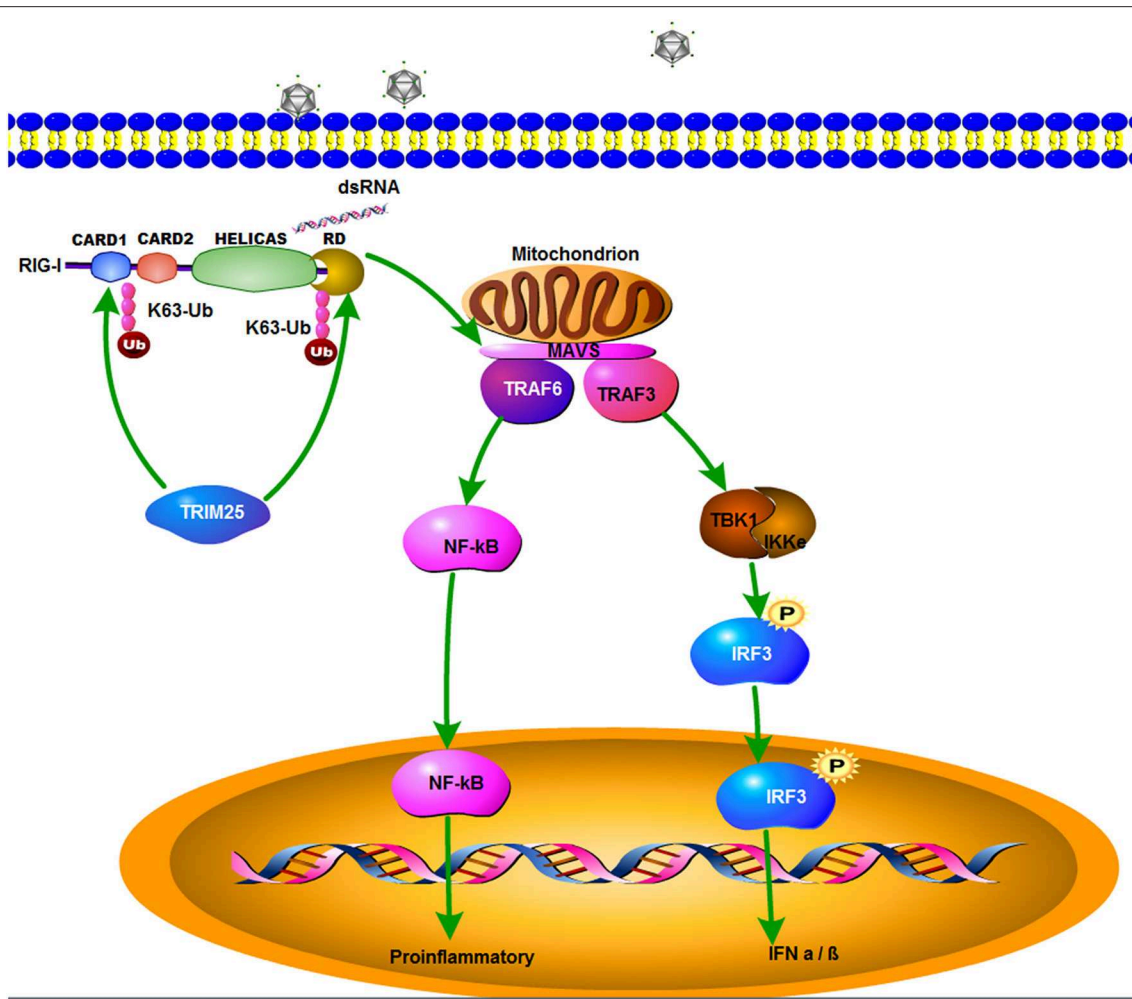


FIGURE 7 | A schematic model of zbTRIM25-mediated RLR signaling pathway during RGNNV infection. zbTRIM25 interacts with and catalyzes the K63 polyubiquitination of 2CARD and RD regions of zbRIG-I, which subsequently induces the activation of downstream signaling event via MAVS, and thereby inhibits viral infection.

represent a new mechanism underlying the regulation of RLR signaling pathway.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

The animal study was reviewed and approved by the Ethics Committee of Sun Yat-Sen University.

AUTHOR CONTRIBUTIONS

YJ and KJ performed all experiments with assistance from YX, WZ, PJ, and WL analyzed data. KJ and MY conceived the study

and designed experiments. KJ and MY wrote the manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2019.02805/full#supplementary-material>

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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