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Negative regulation of RIG-I-mediated antiviral signaling by TRK-fused gene (TFG) protein

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ABSTRACT

RIG-I (retinoic acid inducible gene I)-mediated antiviral signaling serves as the first line of defense against viral infection. Upon detection of viral RNA, RIG-I undergoes TRIM25 (tripartite motif protein 25)-mediated K63-linked ubiquitination, leading to type I interferon (IFN) production. In this study, we demonstrate that TRK-fused gene (TFG) protein, previously identified as a TRIM25-interacting protein, binds TRIM25 upon virus infection and negatively regulates RIG-I-mediated type-I IFN signaling. RIG-I-mediated IFN production and nuclear factor (NF)-κB signaling pathways were upregulated by the suppression of TFG expression. Furthermore, vesicular stomatitis virus (VSV) replication was significantly inhibited by small inhibitory hairpin RNA (shRNA)-mediated knockdown of TFG, supporting the suppressive role of TFG in RIG-I-mediated antiviral signaling. Interestingly, suppression of TFG expression increased not only RIG-I-mediated signaling but also MAVS (mitochondrial antiviral signaling protein)-induced signaling, suggesting that TFG plays a pivotal role in negative regulation of RNA-sensing, RIG-I-like receptor (RLR) family signaling pathways.

1. Introduction

Production of type-I interferon (IFN) upon recognition of viral infection by host cells acts as a first line of defense against viral infection. The innate immune system utilizes a limited number of specific pattern-recognition receptors (PRRs) to detect conserved viral patterns and activate signal transduction cascades that activate IFN-mediated antiviral defense mechanisms [1–3]. Among these sensors, retinoic acid-inducible gene-I (RIG-I) and melanoma differentiation-associated gene 5 (MDA5) have emerged as key cytosolic viral RNA sensors that detect various clinically important viruses, including influenza virus, dengue virus, paramyxoviruses, and hepatitis C virus [4,5].

Recently, several tripartite motif protein family (TRIM) proteins, which are E3-ubiquitin ligases, have been implicated in innate immune signaling pathways [6]. Tripartite motif protein 25 (TRIM25), in particular, plays a critical role in RIG-I-mediated antiviral signaling. Upon recognition of viral RNA by RIG-I, TRIM25 interacts with the N-terminal caspase recruitment domains (CARDs) of RIG-I. The delivery of a K63-linked ubiquitin moiety to RIG-I by TRIM25 [7] and subsequent oligomerization of RIG-I results in RIG-I interaction with MAVS/VISA/IPS-1/Cardif, a crucial downstream adaptor protein [8–11]. Signaling molecules, such as TBK1 (TANK-binding kinase 1) complex and IKK (I kappa B kinase) complex, are recruited to MAVS (mitochondrial antiviral signaling protein) and activate interferon regulatory factor 3 (IRF3) and nuclear factor (NF)-κB transcription factors to induce IFN production. A recent study also demonstrated that TRIM25 activates RIG-I in an *in vitro*-reconstituted cell-free system [12].

Tight regulation of innate immune sensing and IFN induction is crucial for eliciting an effective immune response since excessive and prolonged production of IFN and inflammatory cytokines might be detrimental to the host. Indeed, multiple negative regulatory mechanisms by which RIG-I-mediated signaling can be modulated have been suggested. For example, LUBAC (linear ubiquitin assembly complex), comprised of HOIL-1L (heme-oxidized IRP2 ubiquitin ligase 1) and HOIP (HOIL-1-interacting protein), negatively regulates RIG-I by inhibiting TRIM25-mediated RIG-I activation [13].

TRK-fused gene (TFG) protein, which has been implicated in neuropathy [14], protein secretion and oncogenesis [15], has been identified in a high-throughput screening study as a TRIM25-interacting protein [16]. In the present study, we confirmed the interaction between TRIM25 and TFG and examined the role of TFG in the RIG-I-mediated antiviral signaling pathway. Our data suggest that TFG associates with TRIM25 and negatively regulates RIG-I-mediated type-I IFN production.

2. Materials and methods

2.1. Plasmids and reagents

GST-RIG-2CARD (RIG-IN), MAVS, and pIRES-V5-TRIM25 plasmids were previously described [17]. Human TFG cDNA was

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purchased from Open Biosystems. TFG cDNA was amplified by conventional polymerase chain reaction (PCR) and cloned into the pEF1-Myc vector for mammalian cell expression. Anti-TFG antibody was purchased from Abcam (Cat. No. ab86606).

2.2. Cells and viruses

HEK293T human embryonic kidney and Huh7 hepatocellular carcinoma cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and penicillin/streptomycin (100 U/mL). Wild-type (WT) and TRIM25-knockout mouse embryonic fibroblasts (MEFs) were previously described [7]. Sendai virus (SeV; Cantell strain) was purchased from Charles River Laboratories, Inc. Enhanced green fluorescence protein (eGFP)-fused vesicular stomatitis virus (VSV–eGFP) was described previously [7].

2.3. co-immunoprecipitation (co-IP) and immunoblot analysis

For co-IP, cells were lysed with Triton X-100 lysis buffer (25 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.5% Triton X-100). After centrifugation to remove cellular debris, protein concentration was determined using the BCA assay. Cell extracts were incubated for 12–16 h with the indicated antibodies and then incubated with protein A/G bead for 2–4 h. The immune complexes were washed with lysis buffer containing different concentrations of NaCl and analyzed by immunoblotting.

2.4. Luciferase reporter assay

All reporter assays were performed using a Dual-Luciferase Assay kit (Promega). Cells were transfected with the indicated plasmids and reporter plasmids together with the pRL-TK reporter. For SeV infection, cells were infected with SeV 24 h after transfection and further incubated for 10 h. Thereafter, lysis luciferase assays were performed according to the manufacturer's instructions.

2.5. RNA isolation and quantitative real-time reverse-transcription-polymerase chain reaction (RT-qPCR)

Expression of IFN-β was analyzed by RT-qPCR using CFX-9000 (Bio-Rad) real-time PCR. Total RNA was extracted from cells using an RNeasy RNA extraction kit (Qiagen), according to the manufacturer's instructions. cDNA was synthesized from 1 μg of total RNA using Superscript III reverse transcriptase (Invitrogen) and oligo$_{20}$(dT) primers. Quantitative PCR was performed using 2 μl of synthesized cDNA as a template. IFN-β was amplified using the primer pair 5′-GAACTTTGACATCCCTGAGGAGATT-3′ (forward) and 5′-TGCGGCGTCCTCCTTCT-3′ (reverse). IFN-β mRNA levels were normalized to those of β-actin, amplified with the primer pair 5′-TGCCGCATCCTCTTCCTC-3′ (forward) and 5′-CGCCTTCACCGTTCCAGT-3′ (reverse).

2.6. Suppression of TFG expression by shRNA

A set of small (inhibitory) hairpin RNAs (shRNAs) against TFG and control non-silencing shRNA were purchased from Open Biosystems, Inc. (Cat. No. RHS4533-EG10342). After comparing knockdown efficiency by immunoblot analysis, TRCN0000078660 (#3; antisense sequence: 5′-TAGTAGGCTGAGAAGTTTGGG-3′) and TRCN0000078662 (#5; antisense sequence: 5′-TTGGGCAGTGTAAGTTTGTGC-3′) were selected and used for further studies. For luciferase assays, shRNAs were co-transfected with the indicated plasmids. For RT-qPCR and VSV-eGFP virus replication assays, cells were transfected with the indicated shRNA and selected with puromycin (2 μg/ml) for 3 d before experiments.

3. Results

3.1. TFG associates with TRIM25 upon virus infection

In a previous high-throughput screening study (Human Protein Reference Database; www.hprd.org) designed to identify proteins that interact with TRIM25 and are involved in RIG-I-mediated antiviral signaling [14], we detected an interaction between TRIM25 and TFG. To test whether TFG plays a role in RIG-I-mediated antiviral signaling pathway, we first confirmed the interaction of TFG with TRIM25 using a co-immunoprecipitation (co-IP) approach. These co-IP studies revealed a strong interaction between overexpressed TFG and overexpressed TRIM25 (Fig. 1A); they also demonstrated co-precipitation of endogenous TRIM25 and ectopically expressed TRIM25 (Fig. 1B). Next, we examined whether endogenous TFG interacts with endogenous TRIM25. Since Sendai virus (SeV) infection readily induces RIG-I-mediated type I IFN signaling, we performed co-IP assays with or without SeV infection. As shown in Fig. 1C, an interaction between endogenous TFG and endogenous TRIM25 was detected upon SeV infection. This interaction was not detected in the absence of SeV infection, suggesting a potential role of TFG in antiviral signaling.

3.2. Increased RIG-I-mediated antiviral signaling by suppression of TFG

To analyze the role of TFG in RIG-I-mediated antiviral signaling, we first examined endogenous TFG levels upon virus infection.

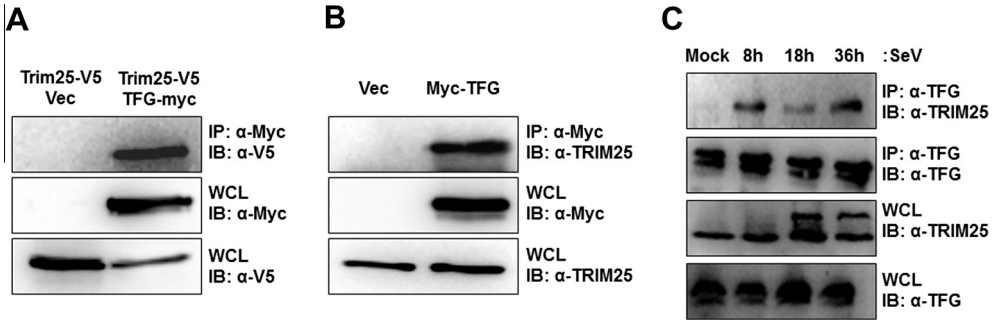

Fig. 1. TFG associates with TRIM25 upon virus infection. (A) Interaction between overexpressed TRIM25 and TFG. HEK293T cells were transfected with a control vector or Myc-TFG expression plasmid together with a V5-tagged TRIM25 expression plasmid, followed by co-IP using an anti-Myc antibody. Whole-cell lysates (WCL) and co-IP samples were analyzed by SDS–PAGE and immunoblotting (IB). (B) Interaction between endogenous TRIM25 and over-expressed TFG. HEK293T cells were transfected with a control vector or Myc-TFG expression plasmid followed by co-IP and IB using the indicated antibodies. (C) Interaction between endogenous TRIM25 and TFG upon SeV infection (50 HAU/ml). HEK293T cells were mock infected or infected with SeV for the indicated hours. Cell lysates were subjected to co-IP using an anti-TFG antibody followed by IB analysis.

Interestingly, TFG level were decreased by SeV infection, consistent with the potential involvement of TFG in antiviral signaling (Fig. 2A). To dissect the role of TFG in RIG-I-mediated type-I IFN production, we tested the role of endogenous TFG in the signaling induced by RIG-IN, a constitutively active form of RIG-I. Knockdown of TFG by two different shRNAs resulted in an increase in RIG-IN-induced IFN-β promoter activity (Fig. 2B). In addition, RIG-IN-induced NF-κB-responsive promoter activity was also increased by the suppression of TFG expression (Fig. 2C), suggesting a suppressive role of TFG in RIG-I-mediated type-I IFN signaling. To further confirm the suppressive role of TFG, we analyzed the synthesis of IFN-β mRNA upon SeV infection by RT-qPCR. Eighteen hours after infection, the level of IFN-β mRNA was higher in TFG knockdown cells than in control cells transfected with non-silencing shRNA (Fig. 2D). These results suggest a possible negative regulatory role of TFG in the RIG-I-mediated antiviral signaling pathway. Suppression of TFG in the absence of viral infection did not increase IFN-β promoter activity, indicating that TFG is not a responsible for preventing spontaneous RIG-I activation in uninfected cells (Fig. 2D and data not shown). Similar results were obtained in experiments using Huh7 cells. mRNAs for both IFN-β and IFN-stimulated gene (ISG) 15 were increased by shRNA-mediated depletion of TFG (Supplementary Fig. S1A, S1B). In contrast to knockdown of TFG, ectopic expression of TFG only marginal suppressed SeV- or RIG-IN-induced IFN-β promoter activity (Fig. 2E and F), possibly owing to very high levels of endogenous TFG in cells.

3.3. TFG acts downstream of MAVS

Since TFG interacts with TRIM25, which is responsible for the ubiquitination of RIG-I, we tested whether TFG negatively regulates the RIG-I signaling pathway through suppression of RIG-I ubiquitination. However, RIG-I ubiquitination was not increased by TFG knockdown, indicating that the suppression of RIG-I signaling activity by TFG is not due to the inhibition of RIG-I ubiquitination by TRIM25 (Fig. 3A). Several previous studies have shown that ectopic expression of MAVS, a downstream adaptor protein of RIG-I, elicits strong type-I IFN responses [17]. Thus, we tested whether TFG affects MAVS-induced IFN-β promoter activity. Surprisingly, suppression of TFG resulted in activation of MAVS-induced signaling, producing a level of activation similar to that of RIG-IN-induced signaling (Fig. 3B). These results suggest that TFG may act downstream of MAVS.

3.4. Suppression of VSV replication by TFG knockdown

In order to confirm the biological role of TFG in RIG-I-mediated antiviral signaling, we examined the replication of VSV–eGFP using

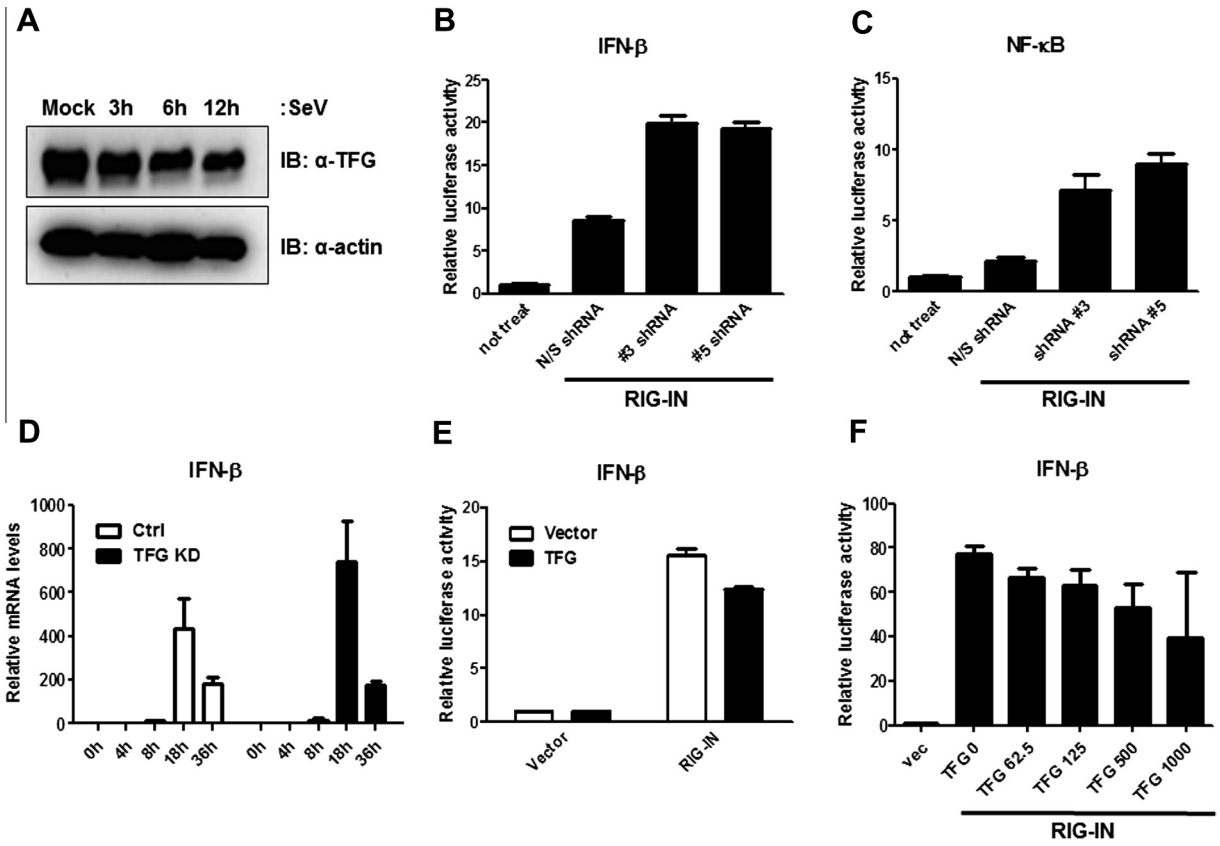

Fig. 2. Negative regulatory role of TFG in RIG-I-mediated antiviral signaling. (A) Decrease in endogenous TFG level upon SeV infection. HEK293T cells were infected with SeV for 3, 6, and 12 h. Cell lysates were subjected to SDS–PAGE and IB using anti-TFG and anti-β-actin antibodies. (B) Enhanced IFN-β promoter activity by shRNA-mediated TFG depletion upon SeV infection. HEK293T cells were transfected with a RIG-IN expression plasmid or control vector together with control shRNA (ctrl shRNA) or shRNAs against TFG (TFG KD #3, #5), as indicated. Cells were co-transfected with IFN-β promoter-reporter and TK-renilla reporter plasmids. Promoter activities were determined by Dual-Luciferase assay. Results of experiments, performed in triplicate, are presented as means ± standard deviation (SD). (C) Enhanced RIG-IN-mediated NF-κB signaling by depletion of TFG. Experiments were performed as in (B) except using an NF-κB reporter instead of an IFN-β promoter-reporter plasmid. (D) Increased IFN-β mRNA synthesis upon SeV infection by the depletion of TFG. Control cells and TFG-depleted cells were infected with SeV. Cells were lysed at the indicated hours after infection and analyzed for IFN-β mRNA levels by RT-qPCR, as described in Materials and methods. (E) Effect of ectopically expressed TFG on RIG-I signaling. Cells were transfected with a control vector or RIG-IN expression plasmid with or without co-transfection of a TFG expression plasmid. Reporter plasmids were co-transfected and IFN promoter activity was analyzed by luciferase assay 36 h after transfection. (F) Dose-dependent inhibition of RIG-I signaling by TFG. Cells were transfected with RIG-IN together with increasing amounts of TFG expression plasmid (0–1000 ng) using procedures similar to those in (E).

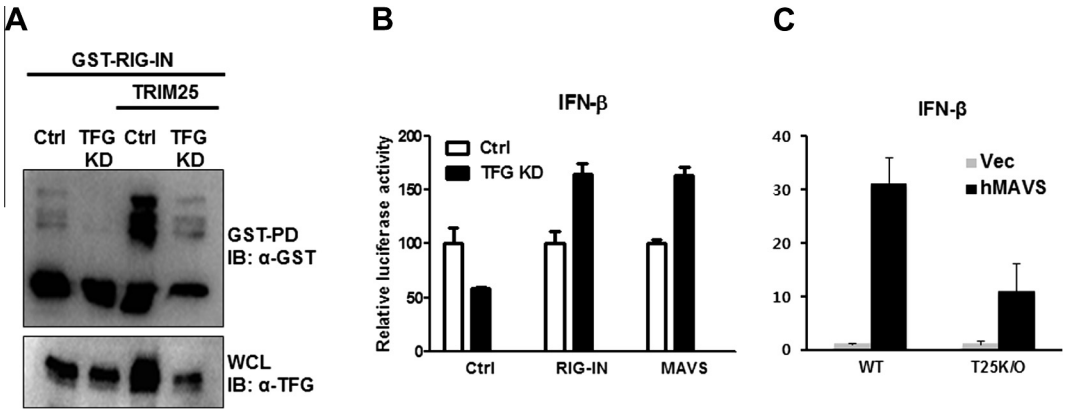

Fig. 3. Regulation of MAVS-induced IFN-β signaling by TFG. (A) HEK293T cells were transfected with GST-RIG-IN and V5-TRIM25 together with control shRNA (ctrl shRNA) or shRNAs against TFG. Cells were lysed and subjected to GST pull-down and IB 36 h after transfection. (B) HEK293T cells were transfected with control vector, RIG-IN or MAVS expression plasmid together with control shRNA or shRNA targeting TFG (#5). Cells were co-transfected with IFN-β promoter-reporter and TK-renilla reporter plasmids. Promoter activities were determined by Dual-Luciferase assay. Results of experiments, performed in triplicate, are presented as a percentage relative to vector control samples. (C) WT MEFs and TRIM25-deficient MEFs were transfected with control vector or MAVS expression plasmid together with IFN-β promoter-reporter and TK-renilla reporter plasmids. IFN-β promoter activities were analyzed as described in (B).

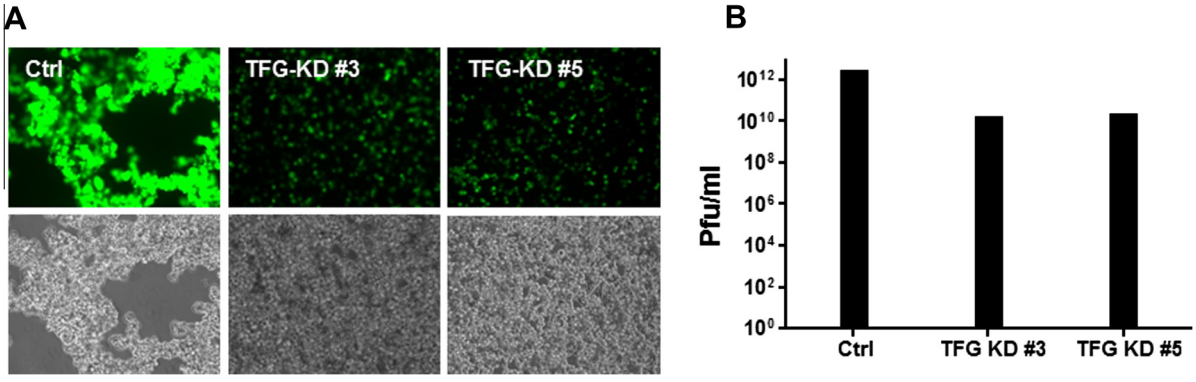

Fig. 4. (A) VSV–eGFP viral replication in TFG-depleted cells. HEK293T cells harboring control shRNA (Ctrl) or shRNAs targeting TFG (TFG KD #3, #5) were infected with VSV–eGFP (multiplicity of infection: 0.001). Photos were taken 24 h post infection. (B) Viral titers were assessed by collecting supernatants from infected cells 48 h after infection and performing plaque assays.

cells in which TFG expression was suppressed by two different shRNAs. VSV–eGFP replication was markedly decreased in cells transfected with TFG shRNAs compared to cells transfected with control non-silencing shRNA (Fig. 4A and B). This result further supports the hypothesis that RIG-I-mediated antiviral signaling is negatively regulated by TFG.

4. Discussion

Tight regulation of immune signaling pathways, including RIG-I-mediated antiviral signaling, is required to prevent destructive and detrimental immunopathology by excessive production of IFNs and proinflammatory cytokines. Indeed, the RIG-I/MAVS signaling pathway has been implicated in autoimmune and inflammatory diseases, such as rheumatoid arthritis, type-I diabetes, and systemic lupus erythematosus [18–21]. Thus, it is not surprising that RIG-I-mediated antiviral signaling is regulated by multiple feedback inhibitory mechanisms. Several proteins and protein complexes, including LUBAC, the autophagy-related (ATG)5-ATG12 complex, the ubiquitin-like protein ISG15, NLRX1 (NLR family member X1), and the E3 ubiquitin ligase RNF125 (ring finger protein 125) have been implicated in the negative regulation of RIG-I signaling [13,22–27]. These regulators utilize a variety of mechanisms to accomplish this, including inhibition of RIG-I ubiquitination through TRIM25 inhibition [13], proteasomal degradation of RIG-I [24,27], and inhibition of the interaction between RIG-I and MAVS [25].

In the present study, we demonstrated a negative regulatory role of TFG in RIG-I-mediated antiviral signaling. TFG was initially identified in high-throughput screens as a TRIM25-interacting protein, an interaction that was confirmed in the present study by co-IP. Interestingly, a separate high-throughput screen using affinity-capture mass-spectrometry revealed that TFG interacts with TRAF3, an E3 ubiquitin ligase critical for RIG-I signaling [28]. Thus, we initially tested whether the level of endogenous TFG was changed by viral infection. Endogenous TFG was clearly reduced upon SeV infection, suggesting the possible involvement of TFG in RIG-I signaling. Subsequent studies clearly demonstrated that shRNA-mediated TFG knockdown resulted in an increase in RIG-I-mediated antiviral signaling, suggesting a negative regulatory role of TFG. Although the level of endogenous TFG was reduced upon viral infection, the basal level of IFN-β promoter activity was not increased by TFG depletion (Figs. 2D and 3B). This indicates that TFG does not prevent spontaneous activation of RIG-I; instead, it acts as a negative feedback regulator to suppress viral infection-induced RIG-I signaling. Consistent with these results, endogenous TFG interacted with TRIM25 upon SeV infection, further supporting the hypothesis that TFG acts as a negative feedback regulator in the RIG-I signaling pathway. Furthermore, VSV replication was greatly suppressed by TFG downregulation, possibly due to the high level of RIG-I-mediated antiviral signaling and IFN production.

K63-linked ubiquitination of RIG-I induced by TRIM25 ubiquitin E3 ligase is essential for RIG-I signal transduction activity [7]. Thus,

to understand the mechanism by which TFG exerts its regulatory function, we tested whether TFG affects TRIM25-mediated RIG-I ubiquitination. However, RIG-I ubiquitination was not increased upon TFG knockdown, ruling out this mechanism. Surprisingly, depletion of TFG increased MAVS-induced IFN signaling, indicating that TFG acts downstream of MAVS to suppress the RIG-I signaling pathway. Having demonstrated an interaction between TFG and TRIM25, we tested whether TRIM25 also plays a role downstream of MAVS. We found that TRIM25 is required for the full activation of MAVS-induced IFN signaling, but the mechanism underlying this additional role of TRIM25 downstream of MAVS remains to be elucidated. Although we demonstrated that TFG interacts with TRIM25 and suppresses RIG-I-mediated antiviral signaling, it is not clear that the interaction itself is responsible for the suppressive action of TFG. Another possible mechanism is that TFG suppresses RIG-I signaling through its interaction with TRAF3. Thus, further investigation is required to establish the mechanism by which TFG regulates RIG-I signaling.

In conclusion, we showed that TFG interacts with key signaling molecules of the RIG-I pathway and acts as a negative feedback regulator. In addition, both TFG and TRIM25 act downstream of MAVS to regulate RIG-I signaling.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.06.061.

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