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Article

The Ubiquitin Ligase RNF5 Regulates Antiviral Responses by Mediating Degradation of the Adaptor Protein MITA

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SUMMARY

Viral infection activates transcription factors NF-κB and IRF3, which collaborate to induce type I interferons (IFNs) and elicit innate antiviral response. MITA (also known as STING) has recently been identified as an adaptor that links virus-sensing receptors to IRF3 activation. Here, we showed that the E3 ubiquitin ligase RNF5 interacted with MITA in a viral-infection-dependent manner. Overexpression of RNF5 inhibited virus-triggered IRF3 activation, *IFNB1* expression, and cellular antiviral response, whereas knockdown of RNF5 had opposite effects. RNF5 targeted MITA at Lys150 for ubiquitination and degradation after viral infection. Both MITA and RNF5 were located at the mitochondria and endoplasmic reticulum (ER) and viral infection caused their redistribution to the ER and mitochondria, respectively. We further found that virus-induced ubiquitination and degradation of MITA by RNF5 occurred at the mitochondria. These findings suggest that RNF5 negatively regulates virus-triggered signaling by targeting MITA for ubiquitination and degradation at the mitochondria.

INTRODUCTION

Viral infection triggers a series of signaling events that lead to induction of type I interferons (IFNs). Type I IFNs activate the JAK-STAT signal transduction pathways, leading to transcriptional induction of a wide range of downstream antiviral genes and subsequent innate antiviral response (Akira et al., 2006; Durbin et al., 2000; Hiscott, 2007; Honda et al., 2006). Transcriptional induction of type I IFN genes requires the coordinate activation of multiple transcription factors and their cooperative assembly into transcriptional enhancer complexes in vivo. For example, the *IFNB1* gene promoter contains conserved enhancer elements recognized by NF-κB (κB site) and phosphorylated IRF3 (ISRE site, also known as PRDIII or IRF-E). It has been shown that transcriptional activation of the *IFNB1* gene requires coordinate and cooperative assembly of an enhanceosome that contains all of these transcription factors (Honda et al., 2006; Maniatis et al., 1998).

At least two types of viral-sensing receptors are capable of triggering signaling events leading to induction of type I IFNs. One is mediated by Toll-like receptors (TLRs). For example, engagement of TLR3 by dsRNA triggers TLR-interacting factor (TRIF)-mediated induction of type I IFNs (O'Neill and Bowie, 2007; Oshiumi et al., 2003; Yamamoto et al., 2003). The second mechanism involves two RIG-I-like receptors (RLRs), RIG-I and MDA5, which function as cytoplasmic viral RNA sensors (Hornung et al., 2006; Kato et al., 2006; Pichlmair et al., 2006; Yoneyama et al., 2004). Both RIG-I and MDA5 contain two CARD modules at their N terminus and a DexD/H-box RNA helicase domain at their C terminus. The RNA helicase domains of RIG-I and MDA5 serve as intracellular viral RNA receptors, whereas the CARD modules are responsible for transmitting signals to downstream CARD-containing mitochondrion-located adaptor protein VISA (also known as MAVS, IPS-1, and Cardif) (Kawai et al., 2005; Meylan et al., 2005; Seth et al., 2005; Sun et al., 2006; Xu et al., 2005). The adaptor protein VISA further activates the IKK-protein-kinase family members. Although the canonical IKK family members IKKα and IKKβ are essential for virus-triggered NF-κB activation, the noncanonical IKK family members TBK1 and IKKε are responsible for phosphorylating and activating IRF3 and IRF7 (Fitzgerald et al., 2003; Hemmi et al., 2004; Matsui et al., 2006). Various studies have also demonstrated the involvement of several other signaling components in virus-induced activation of NF-κB and/or IRF3, including TRAF3, TRAF6, TANK, NEMO(IKKγ), TRADD, FADD, and RIP (Kawai et al., 2005; Michallet et al., 2008; Oganesyan et al., 2006; Saha et al., 2006; Xu et al., 2005; Zhao et al., 2007).

Recently, we and others identified a new adaptor protein called MITA (or STING), which plays a critical role in virus-induced type I IFN expression (Ishikawa and Barber, 2008; Zhong et al., 2008). Overexpression of MITA activates IRF3, whereas knockdown or deletion of MITA inhibits virus-triggered and VISA-mediated IRF3 activation, induction of type I IFNs, and cellular antiviral response. MITA is found to localize to the outer membrane of mitochondria (Jin et al., 2008; Zhong et al., 2008) or the endoplasmic reticulum (ER) (Ishikawa and Barber, 2008). It has been demonstrated that MITA acts as an adaptor to recruit TBK1 and IRF3 to the VISA-associated complex after viral infection (Zhong et al., 2008).

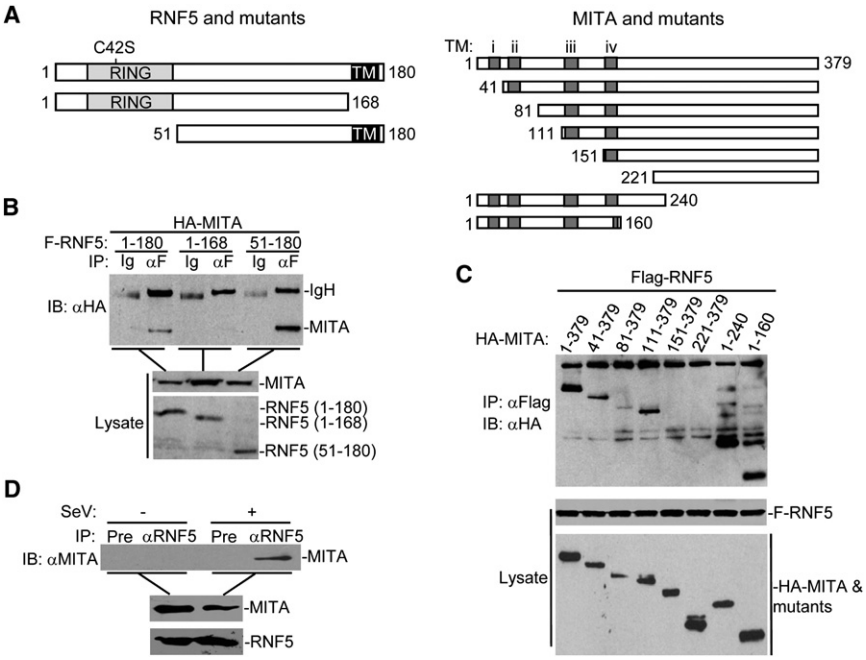

Figure 1. RNF5 Interacts with MITA

(A) A schematic presentation of full-length RNF5, MITA and their mutants. RING, ring-finger domain; TM, transmembrane domain.

(B) RNF5 interacts with MITA through its transmembrane domain. 293 cells (2×10^6) were transfected with the indicated plasmids (5 μg each). Coimmunoprecipitations were performed with anti-Flag or control IgG. Immunoblot analysis was performed with anti-HA (upper panel). Expression of the proteins were analyzed by immunoblot analysis of the lysates with anti-HA and anti-Flag (lower panels).

(C) MITA is associated with RNF5 through its third transmembrane domain. Experiments were performed similarly as in (B).

(D) Effect of viral infection on endogenous MITA-RNF5 interaction. 293 cells (5×10^7) were left uninfected or infected with SeV for 6 hr. The cells were lysed and the lysates were immunoprecipitated with anti-RNF5 or control serum. The immunoprecipitates were analyzed by immunoblotting with anti-MITA (upper panel). The expression levels of the endogenous proteins were detected by immunoblot analysis with anti-MITA and anti-RNF5 (lower panels). For (B)–(D), the experiments were performed for three times with similar results.

In this report, we found that the E3 ubiquitin ligase ring finger protein 5 (RNF5, also called RMA1) (Kyushiki et al., 1997; Matsuda et al., 2001) interacted with and ubiquitinated MITA after viral infection. Ubiquitination of MITA by RNF5 led to its degradation and inhibition of virus-induced IRF3 activation, *IFNB1* expression, and cellular antiviral response. Our findings identified a new strategy for host cells to control excessive innate immune response after viral infection.

RESULTS

Identification of RNF5 as a MITA-Interacting Protein

Previous studies have shown that MITA plays a critical role in virus-induced IRF3 activation and type I IFN induction (Ishikawa and Barber, 2008; Zhong et al., 2008). To investigate how MITA is regulated in virus-induced signaling events, we attempted to identify MITA-interacting proteins by yeast two-hybrid screens. Using full-length MITA as bait, we screened ~2×10^6 independent clones from a combination of human 293 and B cell cDNA libraries and obtained 35 β-gal positive clones. Sequencing analysis and blast searches of the GenBank databases indicated that two of these clones encoded full-length RNF5 or its C-terminal region (aa 21~180).

RNF5 is an E3 ubiquitin ligase that has been implicated in cell motility, protein quality control in the ER, cancer, and degenerative myopathy (Bromberg et al., 2007; Didier et al., 2003; Younger et al., 2006). It has been shown that RNF5 contains a transmembrane domain at its C terminus and is localized to the ER (Younger et al., 2006). Because MITA has been shown to localize at the mitochondria and ER (Ishikawa and Barber, 2008; Zhong et al., 2008), we investigated whether MITA physically interacts with RNF5 in mammalian cells. In transient transfection and coimmunoprecipitation experiments, RNF5 interacted with MITA. Deletion of the C-terminal transmembrane domain of RNF5 abolished its interaction with MITA, whereas the C-terminal transmembrane domain containing fragment interacted with MITA (Figures 1A and 1B). These results suggest that the C-terminal transmembrane domain of RNF5 is required for its interaction with MITA.

Previously, studies suggest that MITA is a multiple transmembrane domain-containing protein and the third transmembrane domain of MITA (aa 111–150) is important for its mitochondrial localization (Zhong et al., 2008). Deletion mutagenesis indicated that this domain of MITA was also required for its interaction with RNF5 (Figures 1A and 1C). These results suggest that MITA and RNF5 interact through their respective transmembrane domains that are also required for their membrane localization.

We next determined whether MITA interacts with RNF5 in untransfected cells and the effects of viral infection on the interaction. Endogenous coimmunoprecipitation experiments indicated that MITA did not interact with RNF5 without stimulation but was associated with RNF5 after viral infection (Figure 1D). These results suggest that MITA is associated with RNF5 in a viral-infection-dependent manner.

We also performed coimmunoprecipitation experiments to examine whether RNF5 interacts with other molecules involved in virus-induced type I IFN signaling. As shown in Figure S1 (available online), RNF5 interacted with RIG-I, VISA, TRADD, and TRAF3 but not FADD, RIP, TRAF6, TBK1, and IRF3. Interestingly, MITA is found to be associated with TRAF3 and VISA but not RIP or TRAF6 (Zhong et al., 2008), and such a result is consistent with a physical interaction between MITA and RNF5.

Overexpression of RNF5 Inhibits SeV-Induced Activation of ISRE, NF-κB, and the IFN-β Promoter

Because RNF5 interacted with MITA in a viral-infection-dependent manner, we determined whether RNF5 is involved in virus-induced type I IFN signaling. Reporter assays indicated that

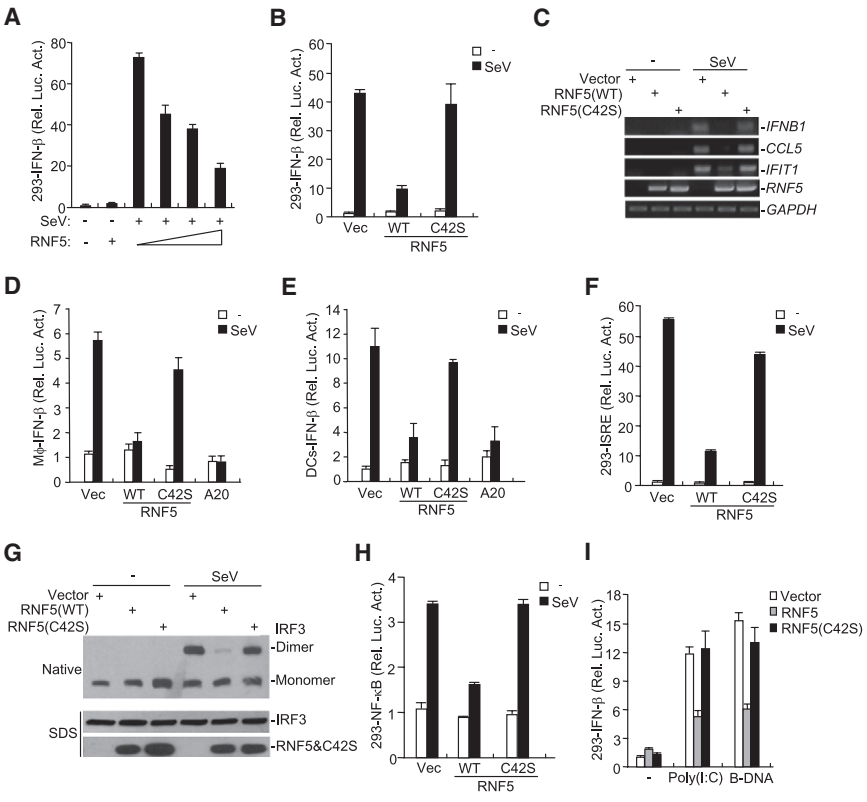

Figure 2. RNF5 Inhibits SeV-Triggered Signaling

(A) RNF5 inhibits SeV-induced activation of the IFN-β promoter in a dose-dependent manner. 293 cells (1×10^5) were transfected with an IFN-β promoter reporter (0.1 μg) and an increased amount of RNF5 expression plasmid. Twenty hours after transfection, cells were infected with SeV or left untreated. Luciferase assays were performed 12 hr after infection.

(B) Effects of RNF5 and RNF5 (C42S) on virus-induced IFN-β promoter activation. 293 cells (1×10^5) were transfected with an IFN-β promoter reporter (0.1 μg) and the indicated expression (0.1 μg each) plasmids. Twenty hours after transfection, cells were infected with SeV or left untreated. Luciferase assays were performed 12 hr after infection.

(C) RNF5 inhibits SeV-induced expression of endogenous *IFNB1*, *CCL5*, and *IFIT1*. 293 cells (2×10^5) were transfected with the indicated expression plasmids (1 μg each) for 20 hr. The cells were left untreated or infected with SeV for 10 hr before RT-PCR for the indicated genes was performed.

(D and E) RNF5 inhibits SeV-induced activation of IFN-β promoter in human primary macrophages (Mϕ) and dendritic cells (DCs).

(D) Monocyte-derived Mϕ (5×10^5) and (E) DCs (1×10^6) were nucleofected with the indicated plasmids (1.5 μg each). Twenty-four hours after transfection, cells were left untreated or infected with SeV for 16 hr before reporter assays were performed.

(F) RNF5 inhibits SeV-induced ISRE activation. Experiments were performed similarly as in (B) except that an ISRE reporter was used.

(G) RNF5 inhibits SeV-induced IRF3 dimerization. 293 cells (2×10^5) were transfected with the indicated plasmids. Twenty hours after transfection, cells were infected with SeV or left uninfected for 6 hr. Cell lysates were separated by native (upper panel) or SDS (bottom two panels) PAGE and analyzed by immunoblotting with the indicated antibodies.

(H) RNF5 inhibits SeV-induced NF-κB activation. Experiments were performed similarly as in (B) except that an NF-κB reporter was used.

(I) RNF5 inhibits cytoplasmic dsRNA- and B-DNA-induced activation of the IFN-β promoter. 293 cells (1×10^5) were transfected with an IFN-β promoter reporter (0.1 μg) and the indicated expression plasmids (0.1 μg each). Twenty hours after transfection, cells were further transfected with poly(I:C) (1 μg) or B-DNA (1 μg) for 12 hr before luciferase assays were performed.

For (C) and (G), experiments were performed for three times with similar results. For (A) and (B), (D)–(F), and (H) and (I), graphs show mean ± SD, n = 3.

RNF5 inhibited SeV-induced activation of the IFN-β promoter in a dose-dependent manner in 293 cells (Figure 2A). A catalytic inactive mutant of RNF5, RNF5(C42S) (Younger et al., 2006), in which the Cys42 in the ring-finger domain is mutated to serine, lost its ability to inhibit SeV-induced activation of the IFN-β promoter in both 293 (Figure 2B) and HeLa (Figure S2) cells. Consistently, wild-type but not the C42S mutant RNF5 markedly inhibited SeV-induced expression of endogenous *IFNB1* and the downstream genes *CCL5* and *IFIT1* in 293 cells (Figure 2C).

We further examined whether RNF5 regulates virus-induced IFN-β expression in primary human macrophages and dendritic cells (DCs). We isolated $CD14^+$ monocytes and generated monocyte-derived macrophages and DCs according to standard procedures. Reporter assays indicated that wild-type RNF5 but not RNF(C42S) markedly inhibited SeV-induced activation of IFN-β promoter both in macrophages and DCs. In these experiments, A20, which has previously shown to negatively regulate virus-induced signaling (Lin et al., 2006b; Saitoh et al., 2005; Wang et al., 2004), also inhibited SeV-induced activation of IFN-β promoter (Figures 2D and 2E). These results suggest that RNF5 inhibits virus-triggered signaling in primary human immune cells.

Transcription induction of the *IFNB1* gene requires cooperative assembly of an enhanceosome containing both IRF3 and NF-κB (Maniatis et al., 1998). Because RNF5 inhibited virus-induced activation of the IFN-β promoter, we determined whether RNF5 inhibits virus-induced activation of IRF3 and NF-κB. The results indicated that wild-type RNF5 but not its C42S mutant markedly inhibited SeV-induced ISRE activation (Figure 2F), IRF3 dimerization (Figure 2G), and NF-κB activation (Figure 2H). These results suggest that RNF5 inhibits virus-induced activation of both IRF3 and NF-κB.

Previous studies demonstrate that MITA is also involved in cytoplasmic poly(I:C)- and B-DNA-induced type I IFN signaling (Ishikawa and Barber, 2008; Zhong et al., 2008). Consistent with a role for RNF5 in regulating MITA, wild-type RNF5 but not its C42S mutant inhibited poly(I:C)- and B-DNA-induced activation of the IFN-β promoter in reporter assays (Figure 2I). In similar experiments, RNF5 did not inhibit TNF-induced NF-κB activation (Figure S3), suggesting that RNF5 specifically

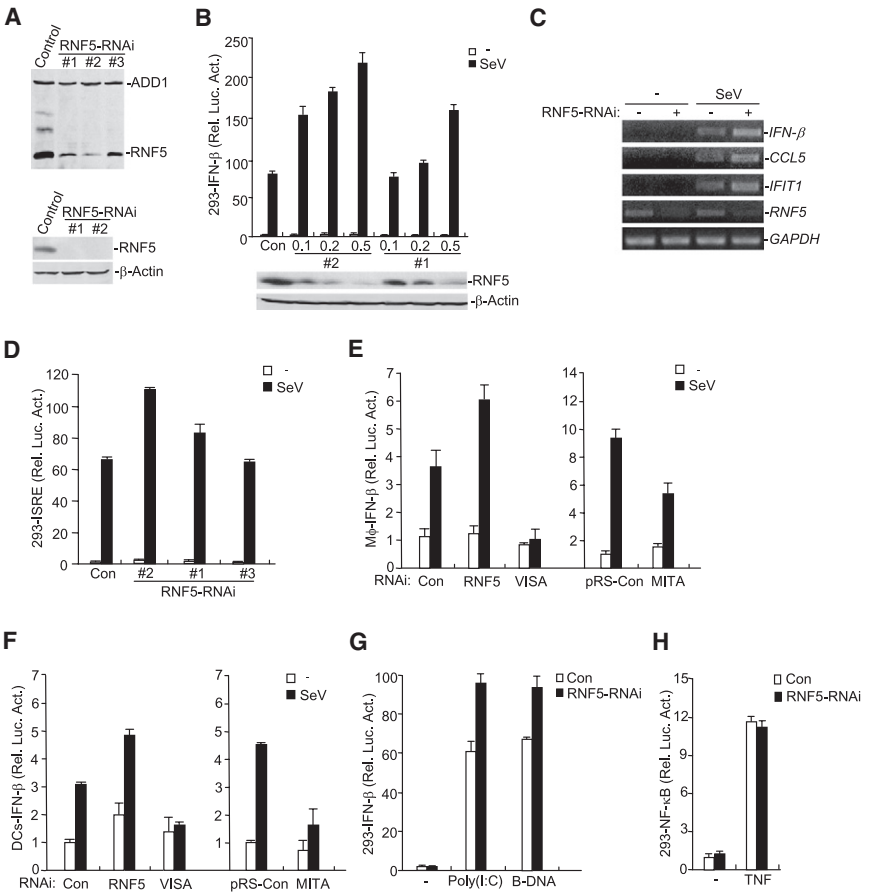

Figure 3. Effects of RNAi-Mediated Knockdown of RNF5 on SeV-Induced Signaling

(A) Effects of RNF5 RNAi plasmids on expression of RNF5. In the upper panel, 293 cells (2×10^5) were transfected with the indicated expression plasmids (0.1 μg each) and RNAi plasmids (1 μg). At 24 hr after transfection, cell lysates were analyzed by immunoblotting with anti-Flag. In the bottom panels, 293 cells (2×10^5) were transfected with control or RNF5 RNAi plasmids (1 μg each) for 24 hr. Cell lysates were analyzed by immunoblotting with the indicated antibodies.

(B) Effects of RNF5 RNAi on SeV-induced activation of the IFN-β promoter in 293 cells. 293 cells (1×10^5) were transfected with the indicated amount of RNAi plasmids. An empty vector was added to ensure that each transfection received the same amount of total DNA. Twenty-four hours after transfection, cells were left uninfected or infected with SeV for 10 hr before luciferase assays were performed. Immunoblot analysis was carried out so that the expression level of RNF5 and β-actin (lower panels) could be examined.

(C) Effects of RNF5 RNAi on SeV-induced expression of downstream genes. 293 cells (2×10^5) were transfected with a control or RNF5 RNAi plasmid (#2) (1 μg). Twenty-four hours after transfection, cells were left uninfected or infected with SeV for 10 hr before RT-PCR was performed.

(D) Effects of RNF5 RNAi on SeV-induced ISRE activation in 293 cells. 293 cells (1×10^5) were transfected with a control or the indicated RNF5 RNAi plasmids (0.5 μg). Twenty-four hours after transfection, cells were left untreated or infected with SeV for 10 hr before reporter assays were performed.

(E and F) Effects of RNF5 RNAi on SeV-induced activation of the IFN-β promoter in human primary Mϕ and DCs. Monocyte-derived Mϕ (5×10^5, shown in [E]) and DCs (1×10^6, shown in [F]) were nucleofected with the indicated plasmids (1.5 μg each). Twenty-four hours after transfection, cells were left untreated or infected with SeV for 16 hr before reporter assays were performed.

(G) Effects of RNF5 RNAi on cytoplasmic dsRNA- and B-DNA-induced activation of the IFN-β promoter in 293 cells. 293 cells (1×10^5) were transfected with a control or RNF5 RNAi plasmid (#2) (0.5 μg). Twenty-four hours after transfection, cells were further transfected with poly(I:C) (1 μg) or B-DNA (1 μg) or left uninfected for 10 hr before luciferase assays were performed.

(H) Effects of RNF5 RNAi on TNF-induced NF-κB activation in 293 cells. Reporter assays were performed similarly as in (D) except that TNF (50 ng/ml) was used. All experiments were repeated for at least three times with similar results. The bar graphs show mean ± SD, n = 3.

inhibits signaling leading to induction of IFN-β and that this inhibitory function requires its E3 ubiquitin ligase activity.

Knockdown of RNF5 Potentiates SeV-Induced Activation of the IFN-β Promoter

Because overexpression of RNF5 inhibited virus-induced activation of IRF3, NF-κB, and the IFN-β promoter, we next determined whether endogenous RNF5 regulates virus-induced signaling in untransfected cells. To do this, we constructed three human RNF5 RNAi plasmids. Two of these RNAi plasmids could markedly inhibit the expression of transfected and endogenous RNF5 in 293 cells as suggested by immunoblot analysis (Figure 3A). In reporter assays, these two RNF5 RNAi plasmids potentiated SeV-induced activation of the IFN-β promoter in a dose-dependent manner in 293 cells (Figure 3B). Knockdown of RNF5 also potentiated SeV-induced transcription of endogenous *IFNB1*, *CCL5*, and *IFIT1* genes (Figure 3C) as well as activation of ISRE (Figure 3D). We also determined whether endogenous RNF5 is involved in regulation of virus-induced signaling other cell types. We found that RNF5 RNAi potentiated SeV-induced IFN-β promoter activation in HeLa cells (Figure S4). More importantly, knockdown of VISA and MITA markedly inhibited SeV-induced IFN-β promoter activation, whereas knockdown of RNF5 potentiated SeV-induced IFN-β promoter activation in human primary macrophages (Figure 3E) and DCs (Figure 3F). These observations suggest that the regulatory function of RNF5 on virus-induced type I IFN signaling is not cell specific. Similarly, we found that knockdown of RNF5 potentiated cytoplasmic dsRNA- and B-DNA-induced activation of the IFN-β promoter (Figure 3G) but not TNF-induced NF-κB activation (Figure 3H). These results suggest that RNF5 is a specific physiological inhibitor of virus-triggered IRF3 activation and IFN-β induction in various cell types.

RNF5 Regulates Virus-Induced Signaling at the MITA Level

Because RNF5 was associated with MITA after viral infection and acted as an inhibitor of virus-triggered signaling, we

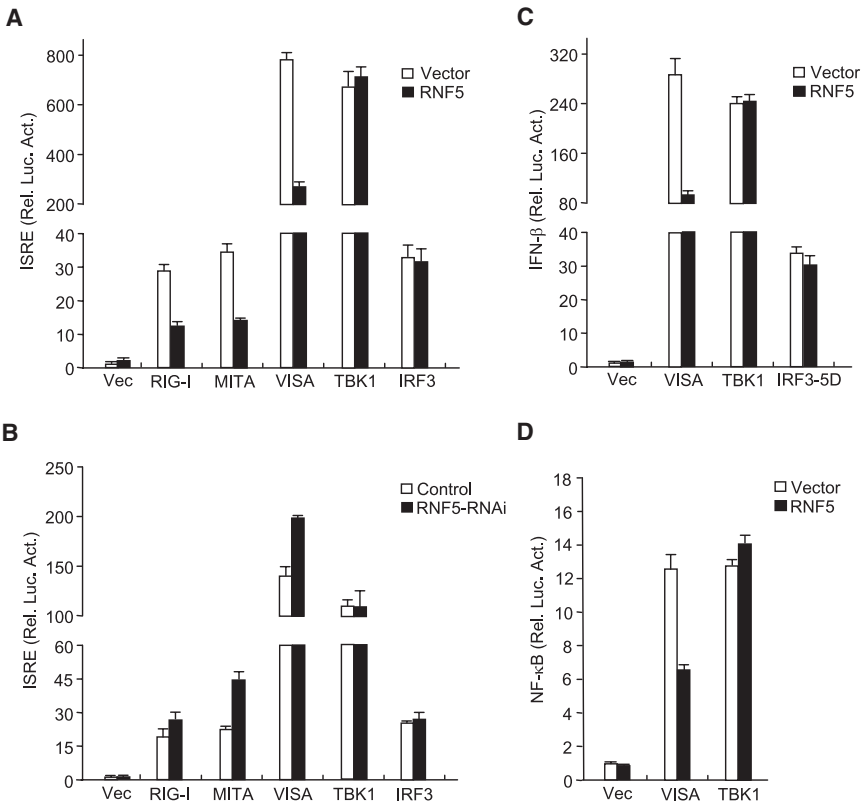

Figure 4. RNF5 Regulates Virus-Induced Signaling at the MITA Level

(A) RNF5 inhibits RIG-I-, VISA-, and MITA- but not TBK1- and IRF3-mediated ISRE activation. 293 cells (1×10^5) were transfected with the indicatedl plasmids (0.2 μg each) together with an ISRE reporter plasmid (0.1 μg). Reporter assays were performed 20 hr after transfection.

(B) Knockdown of RNF5 enhances RIG-I-, VISA-, and MITA- but not TBK1- and IRF3-mediated ISRE activation. 293 cells (1×10^5) were transfected with a control or RNF5-RNAi plasmid (#2) (0.5 μg). Twelve hours later, cells were selected with puromycin (1 μg/ml) for 24 hr and further transfected with the indicated plasmids (0.1 μg each) for 20 hr before reporter assays were performed.

(C) RNF5 inhibits VISA- but not TBK1-mediated activation of the IFN-β promoter. The experiments were similarly performed as in (A).

(D) RNF5 inhibits VISA- not TBK1-mediated NF-κB activation. The experiments were similarly performed as in (A).

Graphs show mean ± SD, n = 3.

determined whether RNF5 functions through MITA. In reporter assays, RNF5 inhibited activation induced by MITA as well as by its upstream components RIG-I and VISA, but RNF5 did not inhibit ISRE activation induced by its downstream components TBK1 and IRF3 (Figure 4A). Conversely, knockdown of RNF5 had opposite effects (Figure 4B). Similarly, RNF5 inhibited VISA- but not TBK1-induced activation of NF-κB (Figure 4C) and the IFN-β promoter (Figure 4D). These results suggest that RNF5 negatively regulates virus-triggered signaling at the MITA level.

RNF5 Targets MITA for Ubiquitination and Degradation

Because RNF5 is an E3 ubiquitin ligase and its enzymatic activity is critical for inhibition of virus-induced signaling, we determined whether RNF5 could ubiquitinate MITA. As shown in Figures 5A and 5B, wild-type RNF5 but not RNF5(C42S) caused ubiquitination and downregulation of MITA. Because MITA-RNF5 interaction was viral infection dependent in untransfected cells, we determined whether viral infection caused ubiquitination of endogenous MITA. The results indicated that endogenous MITA was ubiquitinated and downregulated after SeV infection (Figure 5C). Moreover, SeV-induced MITA ubiquitination and downregulation were reversed by RNAi-mediated knockdown of RNF5 (Figure 5C), whereas the proteasome inhibitor MG132 could reverse SeV-induced downregulation of endogenous MITA as well as RNF5-mediated downregulation of overexpressed MITA (Figure S5). These results suggest that RNF5 targets MITA for ubiquitination and degradation after viral infection through a proteasome-dependent pathway.

Because proteasomes normally recognize and degrade proteins modified with K48-linked polyubiquitin chains, we determined whether RNF5 catalyzes K48- or K63-linked ubiquitination of MITA. We made expression plasmids for ubiquitin mutants retaining only one single lysine residue, K48 (ubiquitin-K48) or K63 (ubiquitin-K63). As shown in Figure 5D, immunoprecipitation and immunoblot analysis indicated that RNF5 catalyzed MITA ubiquitination with wild-type ubiquitin and ubiquitin-K48 but not with ubiquitin-K63, whereas TRAF6 caused IRF7 ubiquitination with wild-type ubiquitin and ubiquitin-K63 but not ubiquitin-K48; such a finding is consistent with a previous report that TRAF6 catalyzes K63-linked ubiquitination of IRF7 (Ning et al., 2008). Consistently, RNF5-catalyzed ubiquitination of MITA was detected with an antiubiquitin but not with an anti-K63-linked polyubiquitin antibody, whereas TRAF6-mediated ubiquitination of IRF7 was detected with both antibodies (Figure 5E). Taken together, these data suggest that RNF5 catalyzes K48-linked ubiquitination of MITA, which is recognized and degraded by the proteasome system.

During our coimmunoprecipitation experiments, we routinely observed that RNF5 caused shifts of MITA(1-160) to higher molecular bands (Figure 1C), suggesting the possibility that the ubiquitination site of MITA is located in this region. Sequence analysis identified three lysine residues in this region, which are K20, K137, and K150. We mutated these lysine residues into arginines individually and found that mutation of K150 to arginine abolished RNF5-mediated MITA ubiquitination (Figure 5F). Consistently, RNF5 caused degradation of wild-type but not K150R mutant MITA (Figures 5F and Figure S6). In reporter assays, MITA(K150R) could induce ISRE activation to a higher level than its wild-type counterpart. In addition, MITA(K150R)-induced ISRE activation was markedly more resistant to the inhibitory effect of RNF5 (Figure 5G). These results

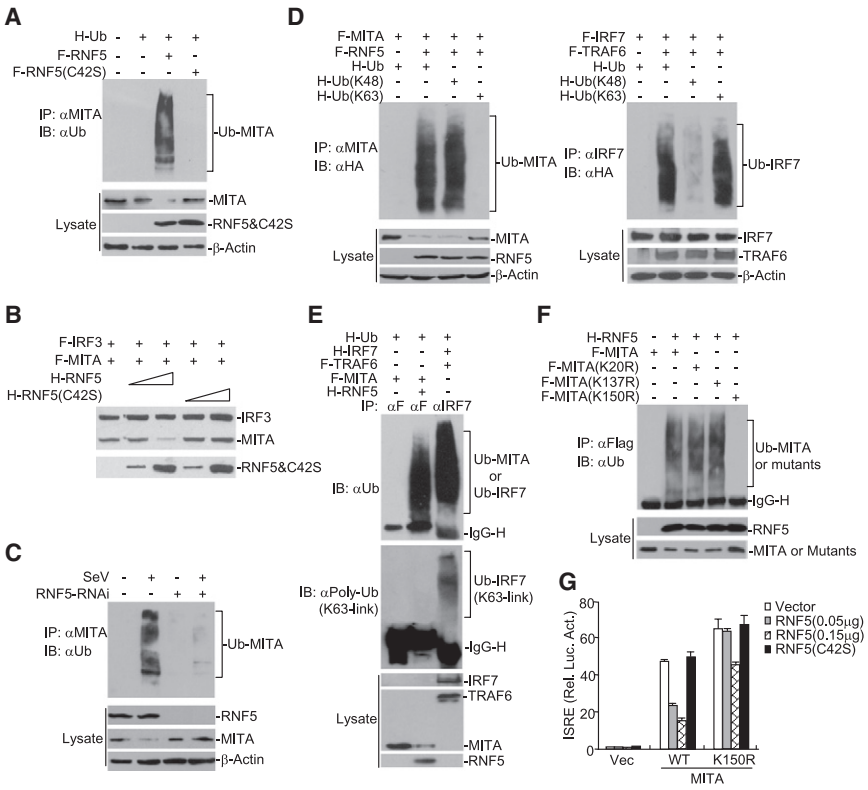

Figure 5. RNF5 Targets MITA for Ubiquitination and Degradation

(A) Overexpression of wild-type but not mutant RNF5 promotes ubiquitination of MITA. 293 cells (1×10^7) were transfected with the indicated plasmids. Twenty hours after transfection, cell lysates were immunoprecipitated with anti-MITA. The immunoprecipitates were denatured and reimmunoprecipitated with anti-MITA and then analyzed by immunoblotting with antiubiquitin (upper panel). The expression levels of the proteins were examined by immunoblotting with the indicated antibodies (lower panels).

(B) Overexpression of RNF5 caused downregulation of MITA. 293 cells (2×10^5) were transfected with the indicated plasmids. Twenty-four hours after transfection, cells were lysed for immunoblot analysis with anti-Flag (upper panel) or anti-HA (lower panel).

(C) Effects of RNF5 RNAi on SeV-induced ubiquitination of endogenous MITA. 293 cells (5×10^7) were transfected with a control or the RNF5 RNAi plasmid (#2). Twenty-four hours after transfection, cells were infected with SeV or left untreated for 6 hr. The cell lysates were immunoprecipitated with anti-MITA. The immunoprecipitates were denatured and reimmunoprecipitated with anti-MITA and then analyzed by immunoblotting with antiubiquitin (upper panel). The expression of the related proteins was examined by immunoblotting with the indicated antibodies (lower panels).

(D) RNF5 catalyzes K48-linked ubiquitination of MITA, whereas TRAF6 catalyzes K63-linked ubiquitination of IRF7. 293 cells (2×10^6) were transfected with the indicated plasmids. Twenty hours after transfection, immunoprecipitation, reimmunoprecipitation and immunoblot analysis were performed with the indicated antibodies (upper panels). The expression of the proteins was examined by immunoblotting with the indicated antibodies (lower panels).

(E) RNF5-mediated ubiquitination of MITA is not detected by a K63-linkage specific ubiquitin antibody. 293 cells (2×10^6) were transfected with the indicated plasmids. Twenty hours after transfection, immunoprecipitation, reimmunoprecipitation and immunoblot analysis were performed with the indicated antibodies (upper two panels). The expression of the transfected proteins was examined by immunoblotting with the indicated antibodies (lower panels).

(F) Mapping of RNF5-targeted ubiquitination sites of MITA. 293 cells (2×10^6) were transfected with the indicated plasmids. Twenty-four hours after transfection, the cell lysates were immunoprecipitated with anti-Flag. The immunoprecipitates were denatured and reimmunoprecipitated with anti-Flag and then analyzed by immunoblotting with antiubiquitin (upper panel). The expression of the tranfected proteins was examined by immunoblotting with the indicated antibodies (lower panels).

(G) Effects of RNF5 on wild-type and K150R mutant MITA-mediated ISRE activation. 293 cells (1×10^5) were transfected with the indicated plasmids. Luciferase assays were performed 24 hr after transfection. Graphs show mean ± SD, n = 3.

For (A)–(F), experiments were repeated for three times with similar results.

suggest that RNF5 targets MITA at K150 for ubiquitination and this modification is important for RNF5-mediated negative regulation of virus-induced signaling.

RNF5 Interacts with and Ubiquitinates MITA at the Mitochondria

Having established that RNF5 negatively regulates virus-triggered signaling by targeting MITA for ubiquitination and degradation, we next examined whether viral infection affects expression of MITA and RNF5 in a temporal manner. We found that the total amount of cellular MITA was noticeably reduced at 6 hr after SeV infection and then restored at 9 hr after SeV infection. In contrast, the amount of cellular RNF5 was not noticeably changed before and after viral infection (Figure 6A). The reduction of MITA at 6 hr after viral infection was caused by RNF5 because RNAi-mediated knockdown of this protein abolished the effect (Figure 6A). These results further support our conclusion that virus-induced MITA ubiquitination and degradation is dependent on RNF5.

It was reported that RNF5 is localized to the ER or endosome (Younger et al., 2006; Zhang et al., 2005). Whether RNF5 is also localized to the mitochondria was not examined in the previous studies (Younger et al., 2006). Cell fractionation and confocal microscopy experiments indicated that RNF5 was identified both in the mitochondrion- and the ER-containing membrane fractions in unstimulated cells (Figures 6B and 6C). Consistent with previous reports (Jin et al., 2008; Zhong et al., 2008), MITA was mostly found in the mitochondria in unstimulated cells and barely detectable in the ER (Figure 6B). Confocal microscopy experiments also indicated that a fraction of RNF5 colocalized with MITA at the mitochondria (Figure 6C). Interestingly, infection of cells with SeV for 6 hr caused upregulation of RNF5 and simultaneous downregulation of MITA in the mitochondria (Figure 6B). Noticeably, SeV also caused the upregulation of MITA in the ER at 6 hr after infection (Figure 6B). We next determined whether virus-induced ubiquitination of MITA by RNF5 occurs in the mitochondria or the ER. We isolated the mitochondria- and ER-containing membrane fractions and

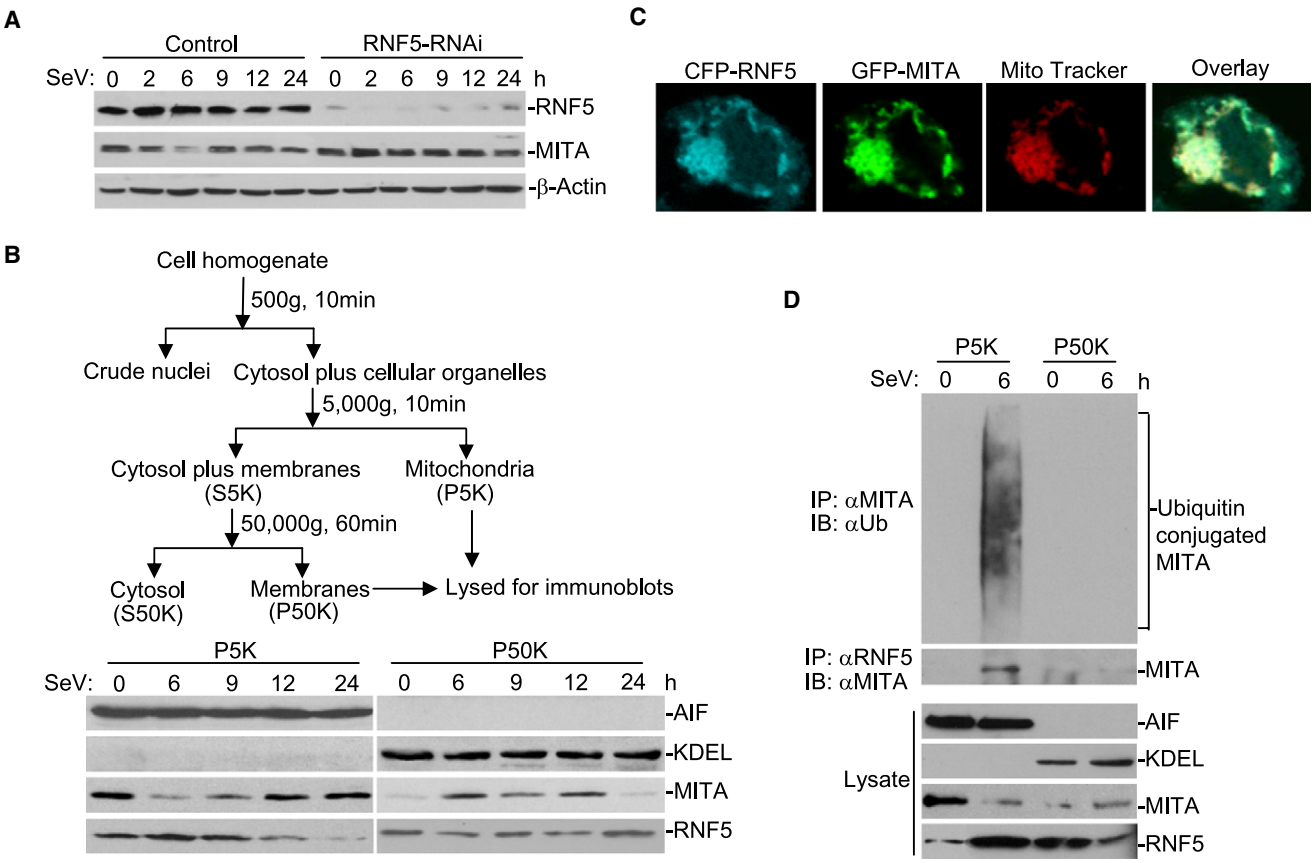

Figure 6. RNF5 Interacts with and Ubiquitinates MITA at the Mitochondria

(A) Effects of SeV infection on expression of MITA and RNF5. 293 cells (2×10^6) were transfected with a control or the RNF5-RNAi plasmid (#2). Twenty-four hours later, cells were infected with SeV for the indicated time points followed by immunoblot analysis with the indicated antibodies.

(B) Effects of SeV infection on subcellular distribution of MITA and RNF5. 293 cells (1×10^7) were left untreated or infected with SeV for the indicated time points and fractionated as shown in the diagram. The cellular fractions were analyzed by immunoblotting with the indicated antibodies.

(C) RNF5 and MITA colocalize to the mitochondria. 293 cells were transfected with CFP-RNF5 and YFP-MITA. Twenty hours after transfection, cells were stained with the Mito Tracker Red (1 mM) for 30 min. The cells were fixed with 4% paraformaldehyde and subjected for confocal microscopy.

(D) RNF5 interacts with and ubiquitinates MITA at the mitochondria. 293 cells (1×10^8) were infected with SeV or left uninfected for 6 hr. Cell lysates were fractionated as in (B). The fractions were lysed and the lysates were immunoprecipitated with anti-MITA; the immunoprecipitates were denatured and reimmunoprecipitated with anti-MITA and analyzed by immunoblotting with antiubiquitin (upper panel). Alternatively, the lysates were immunoprecipitated with anti-RNF5 and the immunoprecipitates were analyzed by immunoblotting with anti-MITA (middle panel). A fraction of lysate was taken for immunoblot analysis to detect the expression levels of the indicated proteins (bottom four panels).

All experiments were repeated for three times with similar results.

performed coimmunoprecipitation analysis. As shown in Figure 6D, RNF5 interacted with and ubiquitinated MITA in the mitochondrial but not the ER fraction at 6 hr after SeV infection. These results suggest that RNF5 targets MITA for ubiquitination and degradation in the mitochondria but not the ER after viral infection.

RNF5 Inhibits MITA-Mediated Cellular Antiviral Response

Because RNF5 targeted MITA for ubiquitination and degradation, and acted as an inhibitor for virus-induced activation of IRF3, NF-κB, and the IFN-β promoter, we investigated whether RNF5 regulates MITA-mediated cellular antiviral innate immunity. In plaque assays, overexpression of wild-type but not the C42S mutant RNF5 abolished MITA- and cytoplasmic poly(I:C)-mediated inhibition of VSV production in both 293 and HeLa cells (Figure 7A). Conversely, knockdown of RNF5 enhanced the inhibitory effects on viral replication mediated by MITA or triggered by cytoplasmic poly(I:C) (Figure 7B). These results suggest that RNF5 is a suppressor of MITA-mediated cellular antiviral response.

DISCUSSION

Previous studies have demonstrated that MITA plays an essential role in virus-triggered induction of type I IFNs and innate antiviral immunity (Ishikawa and Barber, 2008; Zhong et al., 2008). However, how its activities are regulated is unknown. We performed yeast two-hybrid screens and identified RNF5 as a MITA-interacting protein. Although RNF5 interacted with MITA constitutively in overexpression system, their association was viral infection dependent in untransfected cells.

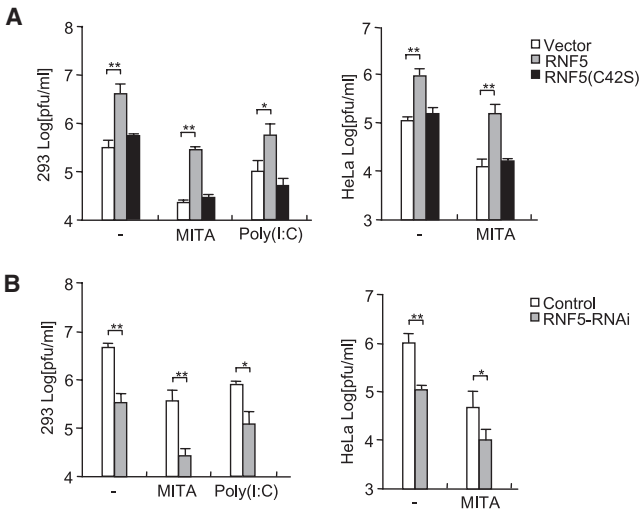

Figure 7. Roles of RNF5 in Cellular Antiviral Response

(A) Overexpression of wild-type but not the C42S mutant RNF5 increases VSV replication. 293 (left) or HeLa (right) cells (1×10^5) were transfected with the indicated expression plasmids (0.5 μg each). For 293 cells (left), 24 hr later, transfection cells were further transfected with poly(I:C) or left untransfected for 12 hr before cells were infected with VSV (MOI = 0.01). The supernatants were harvested 24 hr after infection for standard plaque assays. Graphs show mean ± SD, n = 3.

(B) Knockdown of RNF5 inhibits VSV replication. Plaque assays were performed as in (A) except that a control or RNF5 RNAi plasmid (#2) (1 μg) was transfected and VSV at MOI of 0.1 were used for infection. Graphs show mean ± SD, n = 3. (**$p < 0.01$; *$p < 0.05$).

Domain mapping experiments indicated that the transmembrane domains required for their respective membrane localization were also required for their interaction. Previous studies have demonstrated that RNF5 is an E3 ubiquitin ligase (Matsuda et al., 2001; Younger et al., 2006; Zhang et al., 2005). Correspondingly, overexpression of wild-type RNF5 but not its enzymatic inactive mutant caused ubiquitination and degradation of MITA. Site-directed mutagenesis indicated that RNF5 ubiquitinated MITA at K150. Moreover, viral infection caused ubiquitination and degradation of endogenous MITA by RNF5, as evidenced by the observation that RNAi-mediated knockdown of RNF5 diminished these effects. Further experiments indicate that RNF5 catalyzed K48-linked ubiquitination of MITA, and SeV-induced or RNF5-mediated downregulation of MITA was reversed by addition of the proteasome inhibitor MG132. These results suggest that RNF5 targets MITA for K48-linked ubiquitination and degradation by the proteasome pathway. Consistent with these observations, overexpression of wild-type but not enzymatic inactive mutant RNF5 inhibited SeV-induced activation of IRF3, NF-κB, and the IFN-β promoter as well as endogenous expression of *IFNB1*, whereas knockdown of RNF5 had the opposite effects in various types of cells including primary human macrophages and DCs. In plaque assays, overexpression of RNF5 inhibited MITA-mediated cellular antiviral response, whereas knockdown of RNF5 potentiated MITA-mediated inhibition of viral replication. These observations collectively suggest that RNF5 acts as a negative regulator of virus-induced type I IFN expression and cellular antiviral response by targeting MITA for ubiquitination and degradation.

Signaling triggered by viral infection occurs promptly. Previously, we demonstrate that MITA recruits TBK1 and IRF3 to the mitochondrion-bound VISA complex at 2 hr after viral infection (Zhong et al., 2008). However, the "activated" state of host cells needs to be timely terminated to avoid autoimmune diseases. Consistently, we found that MITA was ubiquitinated and degraded at 6 hr after viral infection. In this context, it is observed that IRF3 undergoes ubiquitination and degradation at 4–6 hr after viral infection (Bibeau-Poirier et al., 2006).

Although previous studies demonstrated that RNF5 is located at the ER (Younger et al., 2006), our cell fractionation experiments also identified similar amount of RNF5 in the mitochondria. In unstimulated cells, RNF5 did not interact with MITA, even though both proteins were present at the mitochondria. After viral infection, RNF5 interacted with MITA at the mitochondria, where it ubiquitinated and degraded MITA. It is possible that viral infection causes a dynamic recruitment of RNF5 to MITA, though the exact mechanism is unknown. Although both MITA and RNF5 were found in the ER after viral infection, the ER-associated MITA was not detected to be associated with and ubiquitinated by RNF5. Our results suggest that RNF5-mediated ubiquitination and degradation of MITA after viral infection occur at the mitochondria, and such a finding is consistent with previous observations that VISA is located at the mitochondria and required for virus-induced type I IFN expression (Seth et al., 2005). In this study, we found that after viral infection, the amount of RNF5 was noticeably decreased in the ER and increased in the mitochondria, whereas the amount of MITA had an opposite shift. The simplest explanation for these observations is that viral infection induces translocation of a fraction of RNF5 from the ER to the mitochondria, resulting in increased ubiquitination and degradation of MITA at the mitochondria after viral infection. In this context, it has been extensively shown that ~20% mitochondrial membrane is positioned against the ER and various molecules, including lipid, ions, cellular and viral proteins, are translocated from the ER to the mitochondria continually or under certain conditions (Franzini-Armstrong, 2007).

The functional significance of virus-induced increase of MITA in the ER is unknown at this time. It has been reported by various groups that IRF3 was activated 4–6 hr after SeV infection (Bibeau-Poirier et al., 2006), which proceeds the increase of ER-associated MITA. In addition, previous studies have demonstrated that MITA is required for signaling by the RLR adaptor protein VISA, which has been demonstrated by several groups to be a mitochondrial protein (Ishikawa and Barber, 2008; Lin et al., 2006a; Seth et al., 2005; Zhong et al., 2008). Therefore, it is less possible that the increase of ER-associated MITA is more relevant to RLR signaling. Previous studies have demonstrated that MITA is also required for cytoplasmic poly(I:C)- and B-DNA-induced type I IFN signaling (Ishikawa and Barber, 2008; Zhong et al., 2008). Consistently, we found that RNF5 negatively regulated cytoplasmic poly(I:C)- and B-DNA-induced signaling. These observations further support our conclusion that RNF5 is a negative regulator of MITA-mediated signaling.

Available studies suggest that host cells adopt at least two distinct mechanisms to control excessive antiviral innate immune response. First, some inhibitory proteins, such as

DAK, SIKE and NLRX1, are physically associated with key components of virus-induced type I IFN signaling pathways to sequester them in inactive forms. Viral infection leads to release of these inhibitors and activation of the signaling components (Diao et al., 2007; Huang et al., 2005; Moore et al., 2008). The second mechanism involves the ubiquitin-proteosome system. Several members of the E3 ubiquitin ligase family, such as RNF125, Pin1, RBCK1, and Rho52, target the key components of the virus-induced type I IFN signaling pathways for degradation (Arimoto et al., 2007; Higgs et al., 2008; Saitoh et al., 2006; Zhang et al., 2008). The discovery that RNF5 targets MITA for ubiquitination and degradation in the mitochondria provides new insight into the mechanism responsible for control of excessive cellular antiviral response.

EXPERIMENTAL PROCEDURES

Reagents

TNF (R&D Systems), mouse monoclonal antibodies against Flag, HA and β-actin (Sigma), AIF, and KDEL (Santa Cruz Biotechnology), K63-linkage specific polyubiquitin chain (Biomol), and rabbit polyclonal antibodies against IRF3 and IRF7 (Santa Cruz Biotechnology) were purchased from the indicated manufacturers. SeV, VSV, and rabbit and mouse MITA antibodies were previously described (Zhong et al., 2008). Mouse anti-RNF5 antiserum were raised against recombinant human RNF5(1∼168).

Constructs

NF-κB, ISRE, and the IFN-β promoter luciferase reporter plasmids and mammalian expression plasmids for HA- or Flag-tagged MITA and its mutants, RIG-I, TBK1, RIP, TRAF6, TRAF3, and TRADD were previously described (Diao et al., 2007; Huang et al., 2005; Xu et al., 2005; Zhong et al., 2008). Mammalian expression plasmids for human HA-, Flag-, or CFP-tagged RNF5 and its truncated mutants were constructed by standard molecular biology techniques. The RNF5(C42S) was a generous gift from D. Cyr (University of North Carolina).

Yeast Two-Hybrid Screens, Transfection and Reporter Assays, RT-PCR, Coimmunoprecipitation, Immunoblot Analysis, Native PAGE and VSV Plaque Assay

These experiments were performed as described (Diao et al., 2007; Huang et al., 2005; Xu et al., 2005; Zhong et al., 2008).

Reimmunoprecipitation Experiments

First-round immunoprecipitation was performed as described (Diao et al., 2007; Huang et al., 2005; Xu et al., 2005; Zhong et al., 2008). The immunoprecipitates were re-extracted in lysis buffer containing 1% SDS and denatured by heating for 5 min. The supernatants were diluted with regular lysis buffer until the concentration of SDS was decreased to 0.1%. The diluted supernatants were reimmunoprecipitated with the indicated antibodies, and the immunoprecipitates were analyzed by immunoblotting with the ubiquitin antibody.

Generation and Transfection of Human Primary Macrophages and Dendritic Cells

Peripheral blood monocytes (PBMCs) were isolated from healthy human peripheral blood by density gradient separation with Ficoll-Paque (GE Healthcare) in accordance with the manufacturer's instructions. The isolated PBMCs were incubated with human CD14-specific antibody conjugated to paramagnetic microbeads (Miltenyi Biotec), and the $CD14^+$ monocytes were isolated on the LS columns (Miltenyi Biotec). The monocytes were suspended in RPMI 1640 supplemented with 10% heat-inactivated FBS (ExCell), 1% nonessential amino acids, and 1 mM sodium pyruvate. For generation of macrophages and DCs, the $CD14^+$ monocytes were treated with either recombinant human (rh) M-CSF (50 ng/ml) or rhGM-CSF (50 ng/ml) and rhIL-4 (100 ng/ml) (PeproTech) for 6 days. The media with the cytokines were changed every other day during the differentiation period. Differentiated macrophages and dendritic cells were harvested and transfected with the human macrophage or dendritic cell nucleofactor kit (Amaxa). Immediately after transfection, cells were resuspended in the cytokine-containing media. Twenty-four hours after transfection, cells were infected with SeV for 16 hr before reporter assays were performed.

RNAi

Double-strand oligonucleotides corresponding to the target sequences were cloned into the pSuper.Retro RNAi plasmid (Oligoengine). The following sequences were targeted for human RNF5 cDNA: RNF5-RNAi #1: 5′-gcgcgaccttcgaatgtaa-3′; #2: 5′-cggcaagagtgtccagtat-3′; #3: 5′-gggaagctgtggtcagtgt-3′.

Subcellular Fractionation

The cell fractionation experiments were performed as previously described (Zhong et al., 2008). In brief, 293 cells (1×10^7) infected with SeV or left uninfected for various time points were washed with PBS and lysed by douncing for 40 times in 2 ml homogenization buffer (10 mM Tris-HCl [pH 7.4], 2 mM MgCl2, 10 mM KCl, and 250 mM Sucrose). The homogenate was twice centrifuged at 500 g for 10 min, and the pellet (P5) was saved as crude nuclei. The supernatant (S5) was centrifuged at 5,000 g for 10 min for crude mitochondria (P5K) precipitation. The supernatant (S5K) was further centrifuged at 50,000 g for 60 min for S50K and P50K generation. The fractions of P5K and P50K were lysed in lysis buffer (20 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1 mM EDTA, and 1% NP-40, protease inhibitor cocktail) for 20 min; this was followed by immunoprecipitation or immunoblot analysis.

SUPPLEMENTAL DATA

Supplemental Data include six figures and can be found with this article online at http://www.immunity.com/supplemental/S1074-7613(09)00106-X.

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