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Original Article

IFIT5 potentiates anti-viral response through enhancing innate immune signaling pathways

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Humans have a distinct combination of IFIT (IFN-induced protein with tetratricopeptide repeats) family orthologs, including IFIT1 (ISG56), IFIT2 (ISG54), IFIT3 (ISG60), and IFIT5 (ISG58). The function of IFIT1/IFIT2/IFIT3 has been intensively investigated. However, little is known about the role of IFIT5 in any cellular processes. In this study, we reported that both the mRNA and protein levels of IFIT5 are up-regulated in response to RNA virus infection or polyinosinic–cytidylic acid stimulation. Ectopic expression of IFIT5 could synergize IRF3- and NF-κB-mediated gene expression, whereas knockdown of IFIT5 impairs the transcription of these genes. Consistently, anti-viral responses of host cells are significantly increased or decreased in the presence or absence of IFIT5. Mechanistically, IFIT5 co-localizes partly with mitochondria and interacts with RIG-I and MAVS. Our study identified that IFIT5 is an important enhancer in innate immune response.

Keywords IFIT5; IRF3; NF-κB; innate immune response

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Introduction

In response to microbial infection, host cells defend themselves by initiating several innate immune signaling pathways, including IRF3 (IFN response factors 3) [1–4], nuclear factor-κB (NF-κB) [5, 6], and AP-1 [7, 8] signaling, which ultimately result in the induction of a series of chemokines, cytokines, and interferons [9]. These innate immune signaling pathways including the unique receptors and adaptors have been identified in recent years [7].

Upon RNA virus infection, virus-derived double-stranded RNA (dsRNA) is recognized by endosomal receptor TLR3 (Toll-like receptor 3) or cytoplasmic RNA sensors RLRs (RIG-like receptors), including RIG-I and MDA-5 [10–12]. In the cytosol, RIG-I and MDA-5 transduce the signal through MAVS (also known as Cardif, VISA, and IPS-1) [6, 13–15], a mitochondrial outer membrane protein with the C-terminal transmembrane domain [6]. Then MAVS recruits the downstream kinases TBK1/IKKε and IKKα/β/γ complex, respectively, leading to the activation of IRF3/NF-κB and the subsequent induction of type I interferon to counteract viral infections. In the endosome, dsRNA or its mimetic poly(I:C) (polyinosinic–cytidylic acid) can be recognized by TLR3 [16, 17], which recruits the adapter protein TRIF. Consequently, TRIF recruits TRAF3 and activates TBK1/IKKε and IKKα/β/γ complexes, thus phosphorylates IRF3, NF-κB, and AP-1 to induce the transcription of interferons [1, 3].

The IFIT1 family members, comprised of IFIT1/ISG56, IFIT2/ISG54, IFIT3/ISG60, and IFIT5/ISG58, are clustered on human chromosome 10 and conserved from mammals to fish [18]. However, there is no ortholog of human IFIT5 in mice [19–22]. A variety of stimuli, e.g. virus infection, interferons, dsRNA, or lipopolysaccharides, can induce the transcription of IFIT1 family members [23, 24]. The IFIT1 family proteins contain multiple helix–turn–helix motifs critical for a wide range of functions, including virus replication, translation initiation, dsRNA signaling, cell proliferation, and migration [18, 25, 26].

Previously, it was reported that IFIT1/2 plays diverse roles in anti-viral signal transduction either as a negative feedback modulator [26] or as a positive regulator [27]. IFIT3 has been shown to potentiate the anti-viral signaling by bridging MAVS and TBK1 [28]. Nevertheless, the function of IFIT5 still remains unknown.

In the present study, we reported that IFIT5 was significantly induced both at the mRNA and protein levels upon Sendai virus (SeV), poly(I:C), or IFN-β stimulation. IRF3- or NF-κB-mediated gene expression was promoted by the ectopic expression of IFIT5. In contrast, knockdown of IFIT5

diminished the expression of these genes. Finally, host anti-viral responses were markedly enhanced or impaired in the presence or absence of IFIT5. Our study showed that IFIT5 partly co-localizes with the mitochondria and associates with both RIG-I and MAVS, suggesting the adaptor function of IFIT5. Thus, our results characterized IFIT5 as an important modulator in the anti-viral innate immune response.

Materials and Methods

Plasmids

Human full-length IFIT5, TBK1, RIG-I, Hsp90, Tom70, IFIT3, and TRAF6 cDNAs were obtained by using polymerase chain reaction (PCR) from human thymus plasmid cDNA library (Clontech, Palo Alto, USA) and then subcloned into indicated vectors. pEGFP-mito for labeling mitochondria was a gift from Dr Jiansheng Kang (Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai, China). Flag-MAVS was kindly provided by Professor Hong-Bing Shu (College of Life Sciences, Wuhan University, Wuhan, China). All these constructs were confirmed by automatic DNA sequencing (Life Technologies, Shanghai, China).

Reagents

Anti-hemagglutinin (HA) antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, USA). Anti-Flag and anti-β-actin antibodies were obtained from Sigma-Aldrich (St Louis, USA). Anti-IFIT5 antibody was from Proteintech (Chicago, USA). Cy3-conjugated anti-mouse antibody was from Jackson ImmunoResearch (West Grove, USA). Poly(I:C) (GE Healthcare, Bucks, UK) was diluted in endotoxin-free sterile H_2O (Invitrogen, Carlsbad, USA) and used at 10 μg/ml. Hu-IFN-β (recombinant protein) was ordered from PBL Biomedical Laboratories (Leiden, Netherlands) and used at 100 μg/ml. Flag-M2-conjugated beads were purchased from Sigma-Aldrich. Non-specific control (NC) and IFIT5 siRNA were chemically synthesized by GenePharma (Shanghai, China). The IFIT5 siRNA sequences were as follows: 5-1, GGCAACAGCUUGAAUUUCUdTdT; 5-2, GCUGAUUUCAUCUGCUAUAdTdT; 5-3, CUCCAAUGUAACUCUUAAAdTdT. The control siRNA sequence was UUCUCCGAACGUGUCACGUdTdT. IFIT3 siRNA sequences were described previously [28].

Cell culture and transfection

Human embryonic kidney (HEK) 293T cells and 293 cells were cultured using Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, USA), penicillin (50 U/ml), and streptomycin (50 μg/ml) (Invitrogen). All transfections were performed with LipofectAMINE 2000 reagent (Invitrogen) according to manufacturer's instructions.

Real-time reverse transcription-PCR

Total RNA was isolated with TRIzol reagent (Invitrogen) according to manufacturer's instructions. Reverse transcription of purified RNA was performed by using oligo(dT) primer. The quantification of gene transcripts was performed by real-time PCR by using SYBR green I dye (Invitrogen). All values were normalized to the level of β-actin mRNA. The primers used were listed as follows: human IFIT5, 5′-TAAAAAAGGCCTTGGAGGTG-3′ (sense) and 5′-CCAGGTCTGTGTAGGCAAAT-3′ (antisense); human IFN-β, 5′-ATGAAGATCTCTGCAGCTGCC-3′ (sense) and 5′-TAGGCAAAGCAGCAGGGAGTG-3′ (antisense); human ISG56, 5′-CACAGTGATGCTAGTGGTAC-3′ (sense) and 5′-CTTAAGCGTGTCTACAGTCTG-3′ (antisense); human RANTES, 5′-GAAGGGCTCATGACCACAGT-3′ (sense) and 5′-GGATGCAGGGATGATGTTCT-3′ (antisense); human IL-8, 5′-AGGTGCAGTTTTGCCAAGGA-3′ (sense) and 5′-TTTCTGTGTTGGCGCAGTGT-3′ (antisense); human TNFα, 5′-ATGTGCTCCTCACCCACACC-3′ (sense) and 5′-CCCTTCTCCAGCTGGAAGAC-3′ (antisense); human IκBα, 5′-CTGAGCTCCGAGACTTTCGAGG-3′ (sense) and 5′-CACGTGTGGCCATTGTAGTTGG-3′ (antisense); human β-actin, 5′-AAAGACCTGTACGCCAACAC-3′ (sense) and 5′-GTCATACTCCTGCTTGCTGAT-3′ (antisense); NDV F gene, 5′-TACAACAGGACATTGACCAC-3′ (sense) and 5′-ATCTTCCCAACTGCCACTGC-3′ (antisense). Melt curve analysis was performed to check primer specificity

Immunoblotting

Cells were washed with phosphate-buffered saline (PBS) and lysed at 4°C in lysis buffer (pH 7.0, containing 1% Nonidet P-40, 20 mM Tris, pH 7.5, 20 mM β-glycerol phosphate, 10 mM NaF, 0.5 mM Na_3VO_4, 150 mM NaCl, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 0.2 mM ethylenediaminetetraacetic acid plus a protease inhibitor cocktail). Fifteen minutes later, lysates were centrifuged at 4°C, 15,000 *g* for 15 min. The supernatant was analyzed by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to a polyvinylidene difluoride membrane (Millipore, Billerica, USA). After blocking in 5% non-fat dry milk and incubation overnight with indicated primary antibodies, the membrane was then washed with Tris-buffered saline–Tween-20 and incubated for 1 h with horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibodies (Jackson ImmunoResearch Laboratories). Indicated proteins were visualized by using Supersignal West Pico chemiluminescence ECL kit (Pierce, Rockford, USA).

Immunoprecipitation

HEK293T cells were harvested 36 h after transfection and then lysed in Nonidet P-40 buffer supplemented with a complete protease inhibitor mixture (Roche, Basel, Switzerland).

For each assay, 10 μl of conjugated Flag beads (Sigma-Aldrich) was added to 500 μl of whole cell lysates and then incubated for 6 h at 4°C. Immunoprecipitates were extensively washed with lysis buffer and eluted with SDS loading buffer by boiling for 5 min.

Virus manipulation

Viral infection was performed when 80% cell confluence was reached. Then, culture media were replaced by serum-free DMEM, and SeV or Newcastle disease virus (NDV)-green fluorescent protein (GFP) was added to the media at a multiplicity of infection (MOI) of 0.2–1. One hour later, the medium was removed and the cells were fed with DMEM containing 10% FBS.

Enzyme-linked immunosorbent assay

IFN-β production was analyzed by ELISA kits (PBL Biomedical Laboratories) according to manufacturer's instructions.

Confocal imaging

HEK293T cells were plated on coverslips in 12-well plates and co-transfected with GFP-mito and HA-IFIT5 plasmids. Twenty-four hours after transfection, coverslips with cells were washed once with PBS and fixed in 3.7% formaldehyde in PBS for 15 min. Cells were permeabilized and blocked for 30 min at room temperature in a staining buffer containing Triton X-100 (0.1%) and bovine serum albumin (2%) and then incubated with anti-HA antibody in staining buffer lacking Triton X-100 for 1 h. After washing three times in staining buffer lacking Triton X-100, cells were incubated with Cy3-conjugated anti-mouse antibody (1 : 500) for 1 h and then with 4′,6-diamino-2-phenylindole for 10 min. The coverslips were then washed extensively and fixed on slides. Imaging of cells was carried out by using a Leica laser scanning confocal microscope (Wetzlar, Germany).

Statistical analysis

Data were expressed as mean ± SD from at least three independent experiments.

Results

IFIT5 can be induced by virus, poly(I:C) or IFN-β

Recently, we have reported that IFIT3 serves as an essential adaptor to bridge TBK1 onto mitochondrial MAVS [28]. Interestingly, IFIT1 and IFIT2 are implicated to have diverse functions in anti-viral signal transduction [26, 27]; however, it remains unknown whether IFIT5 regulates the anti-viral signaling. In the present study, IFN-β luciferase reporter assays were carried out. Results showed that ectopic expression of IFIT5 could markedly synergize the induction of IFN-β-luciferase reporter stimulated by SeV (data not shown), suggesting that IFIT5 was a potential regulator of anti-viral signaling. Western blot assay was performed to detect the induction of IFIT5 upon SeV, poly(I:C), or IFN-β stimulation. As expected, the protein expression of IFIT5 was markedly induced 24 h after the stimulation [**Fig. 1(A)**]. Unlike other IFIT1 family members, however, IFIT5 could be detected constitutively in high abundance [**Fig. 1(A)**]. Real-time reverse transcription-PCR (RT-PCR) analysis showed that the transcriptional level of IFIT5 reached the peak at different time points in response to specific treatments [**Fig. 1(B)**].

Ectopic expression of IFIT5 enhances IRF3- and NF-κB-mediated gene expression

To further investigate the function of IFIT5, we determined the induction of endogenous mRNA expression of IRF3-responsive genes (including IFN-β, ISG56, and Rantes) and NF-κB-responsive genes (including IL-8, IκBα, and TNFα) by real-time RT-PCR, after stimulating cells by SeV infection in the presence of exogenously expressed IFIT5. IFIT3, as a control, has been reported to enhance the expression of these genes [28]. **Figure 2(A)** showed that IFIT5 and IFIT3 significantly enhanced the expression of endogenous IRF3-responsive genes in responsive to SeV challenge. However, neither IFIT5 nor IFIT3 alone induced any expression of these genes [**Fig. 2(A)**]. Furthermore, IFIT5 and IFIT3 also potentiated the induction of endogenous NF-κB-responsive genes upon SeV infection [**Fig. 2(B)**]. In addition, we observed the same potentiating effect of IFIT5 when stimulating cells with poly(I:C) (data not shown). Taken together, these data suggest that IFIT5 potentiated the function of IRF3 and NF-κB during virus infection, which was similar with IFIT3.

Knockdown of IFIT5 attenuates IRF3- and NF-κB-mediated gene expression

Figure 2(C) showed that the effective siRNA oligonucleotides named 5-1 and 5-2 were screened out and could reduce endogenous IFIT5 protein level by ~85%. As a negative control, the non-effective siRNA 5-3 was used. We analyzed the effect of IFIT5 knockdown on the induction of endogenous IRF3- and NF-κB-responsive genes (including IFN-β, Rantes, and IL-8) in HEK293 cells stimulated by SeV. Consistently, the knockdown of IFIT5 by siRNA 5-1 and 5-2 dramatically inhibited the transcription of both IRF3- and NF-κB-responsive genes upon SeV stimulation, whereas the siRNA 5-3 had no such inhibitory effects [**Fig. 2(D)**]. These results strongly suggested that IFIT5 was a positive regulator of IRF3 and NF-κB signaling.

IFIT5 is required for anti-viral response

Although a variety of cytokines and chemokines are produced by host cells, type I IFNs are the principal cytokines involved

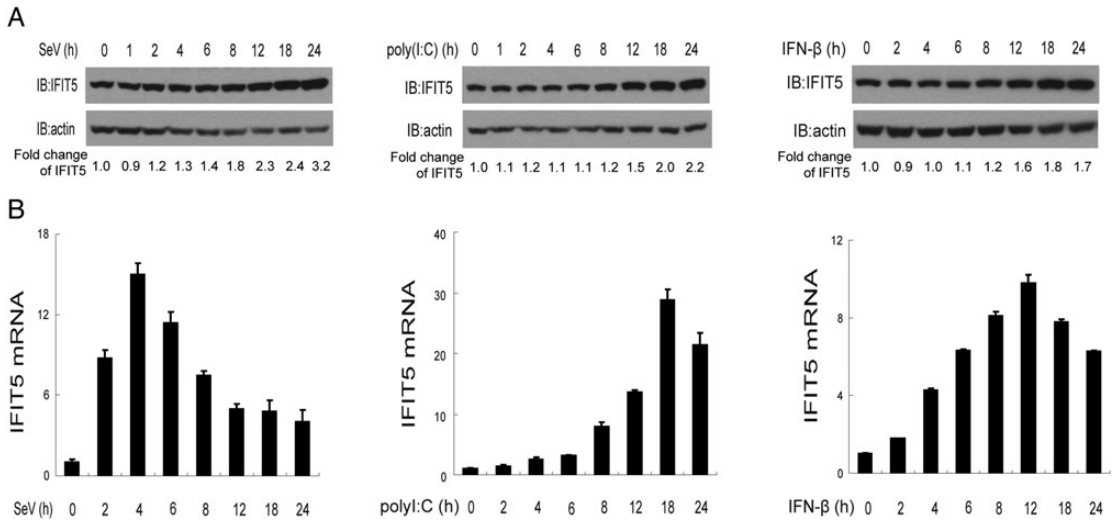

Figure 1 IFIT5 could be induced by SeV, poly(I:C), or IFN-β stimulation (A) HEK293 cells were challenged by SeV, poly(I:C), and IFN-β, respectively, for the indicated time periods, and the cell lysates were immunoblotted with anti-IFIT5 and anti-β-actin antibodies. The induction of IFIT5 was quantitatively analyzed using the Image J software to show the fold change. (B) HEK293 cells were treated as described in (A), and the mRNA level of IFIT5 were measured by real-time PCR.

in anti-viral response. IFN-β is one important member of type I IFNs [29]. To further explore whether IFIT5 regulates the production of IFN-β, IFIT5 plasmids were transfected into HEK293 cells followed by SeV treatment. Enzyme-linked immunosorbent assay (ELISA) showed that the ectopic expression of IFIT5, like that of IFIT3, significantly promoted IFN-β protein release [**Fig. 3(A)**]. Consistently, the knockdown of endogenous IFIT5 or IFIT3 drastically attenuated the production of IFN-β [**Fig. 3(B)**].

Since IFN-β could protect cells from infection, we assessed whether IFIT5 modulates virus replication by challenging cells with NDV-GFP. HEK293 cells were directly treated with NDV-GFP and the replication of NDV was visualized by using a fluorescence microscope. As shown in **Fig. 3(C)** (left panel), the exogenous expression of IFIT5 or IFIT3 markedly suppressed the NDV-GFP replication in HEK293 cells. Consistently, IFIT5 knockdown by siRNA 5-1 or 5-2 significantly augmented the levels of NDV-GFP-positive cells [**Fig. 3(D)**, left panel]. As a negative control, the transfection of siRNA 5-3 rarely had effects on the virus replication [**Fig. 3(D)**, left panel]. Alternatively, NDV-GFP replication was detected by using real-time RT-PCR with NDV F gene specific primers and consistent results were obtained [**Fig. 3(C,D)**, right panel]. These data indicated that IFIT5 played an important role in virus restriction.

IFIT5 co-localizes with mitochondrion and interacts with RIG-I and MAVS

Previously, we have reported that IFIT3 co-localizes with mitochondrion and potentiates anti-viral signaling through bridging TBK1 to MAVS on mitochondrion [28]. To explore the mechanism of IFIT5 action, initially we investigated the localization of IFIT5 by confocal imaging. A partial fraction of IFIT5 co-localized with mitochondrion [**Fig. 4(A)**], which was similar with that of IFIT3, suggesting that IFIT5 might function by interacting with MAVS.

To explore whether IFIT5 is an integral component of MAVS signalosome, HA-IFIT5 was co-transfected into HEK293T cells with Flag-MAVS, TRAF6, TBK1, RIG-I, Hsp90, or Tom70, respectively. Cell lysates were immunoprecipitated with anti-Flag-conjugated beads. It was shown that IFIT5 could interact strongly with MAVS, RIG-I, and TRAF6 [**Fig. 4(B)**]. In addition, IFIT5 associated marginally with TBK1. These data indicated that IFIT5 was a novel component in the MAVS signalosome.

Discussion

IFIT5, also known as ISG58, belongs to IFIT1 family genes. To our knowledge, the function of IFIT5 in viral restriction is poorly elucidated. Here, the current study revealed that IFIT5 potentiates anti-viral responses through enhancing innate immune signaling pathways.

In the past decade, mitochondrial anti-viral immunity has been established with the identification of RIG-I/MDA5 and MAVS [13]. A large amount of investigation has since accumulated and provides a detailed understanding of intracellular anti-viral signaling pathway. However, little is known about the mechanism how RIG-I transduces the upstream signaling to the downstream adaptor MAVS. Both RIG-I and MAVS have caspase recruitment domains (CARD), which is implicated to mediate protein–protein interactions [6]. However, there is no evidence until now that supports the direct interaction between the two

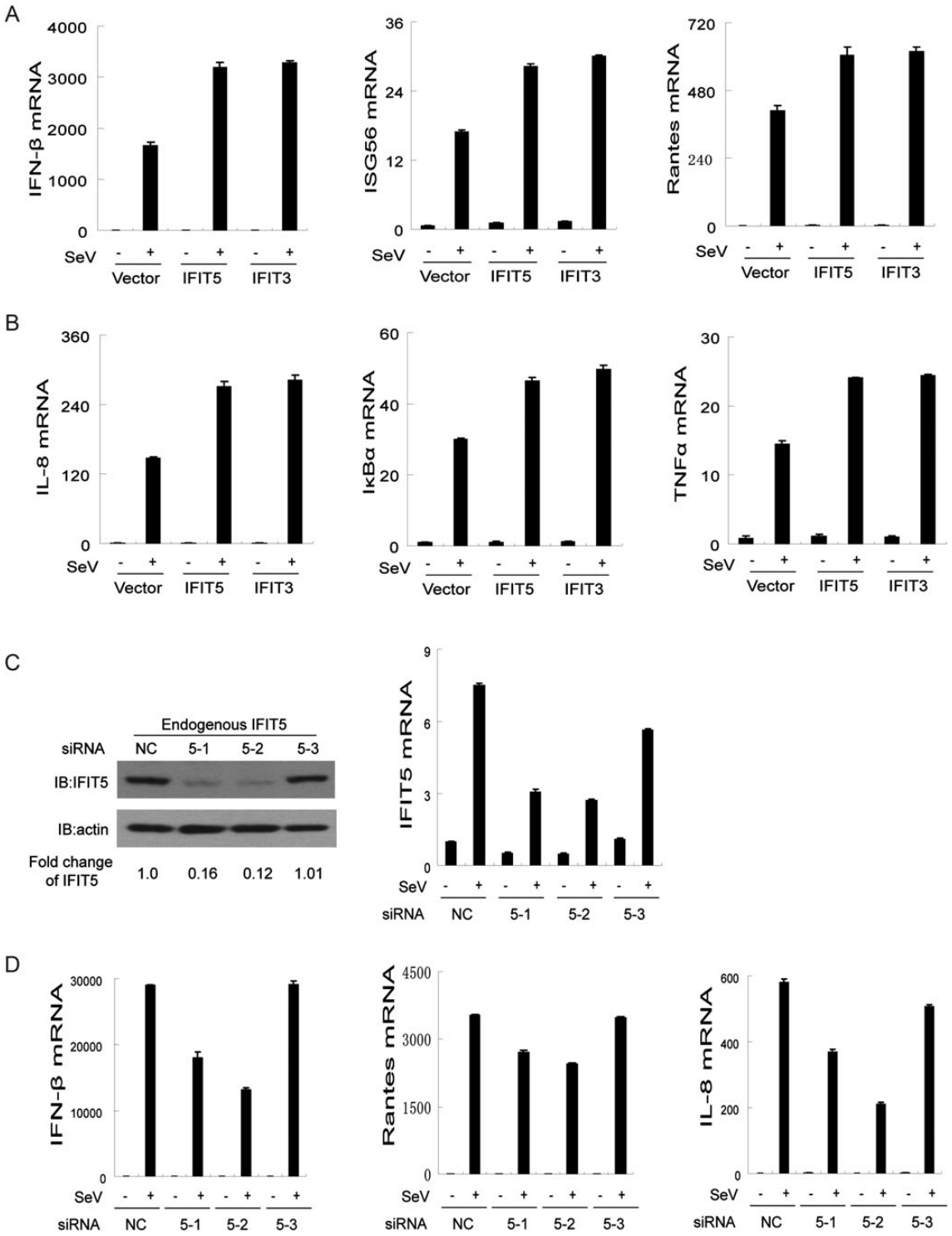

Figure 2 IFIT5 regulates the induction of IRF3- and NF-κB-responsive genes during SeV stimulation HEK293 cells were transfected with indicated plasmids, and then treated with or without SeV for 8 h. Induction of IFN-β/ISG56/Rantes (A) and IL-8/IκBα/TNFα (B) mRNA was measured by real-time PCR. (C) HEK293 cells were transfected with NC or IFIT5 siRNA and then treated with SeV. Cell lysates were immunoblotted with anti-IFIT5 or anti-β-actin antibodies. Fold change of IFIT5 protein level was quantitatively analyzed using the Image J software (left panel). HEK293 cells were transfected with indicated siRNAs and then challenged by SeV infection. IFIT5 mRNA level was detected by real-time PCR (right panel). (D) HEK293 cells were transfected with NC or IFIT5 siRNA (5-1, 5-2, and 5-3), and then challenged by SeV infection. Induction of IFN-β, Rantes, and IL-8 mRNA was measured by real-time PCR.

CARD-containing proteins. In our study, we detected the binding of IFIT5 to both RIG-I and MAVS in co-immunoprecipitation assays when these proteins were co-overexpressed. Also, we observed that IFIT5 partially co-localized with mitochondria. So a tentative model on the action of IFIT5 is proposed, that IFIT5 serves as a critical

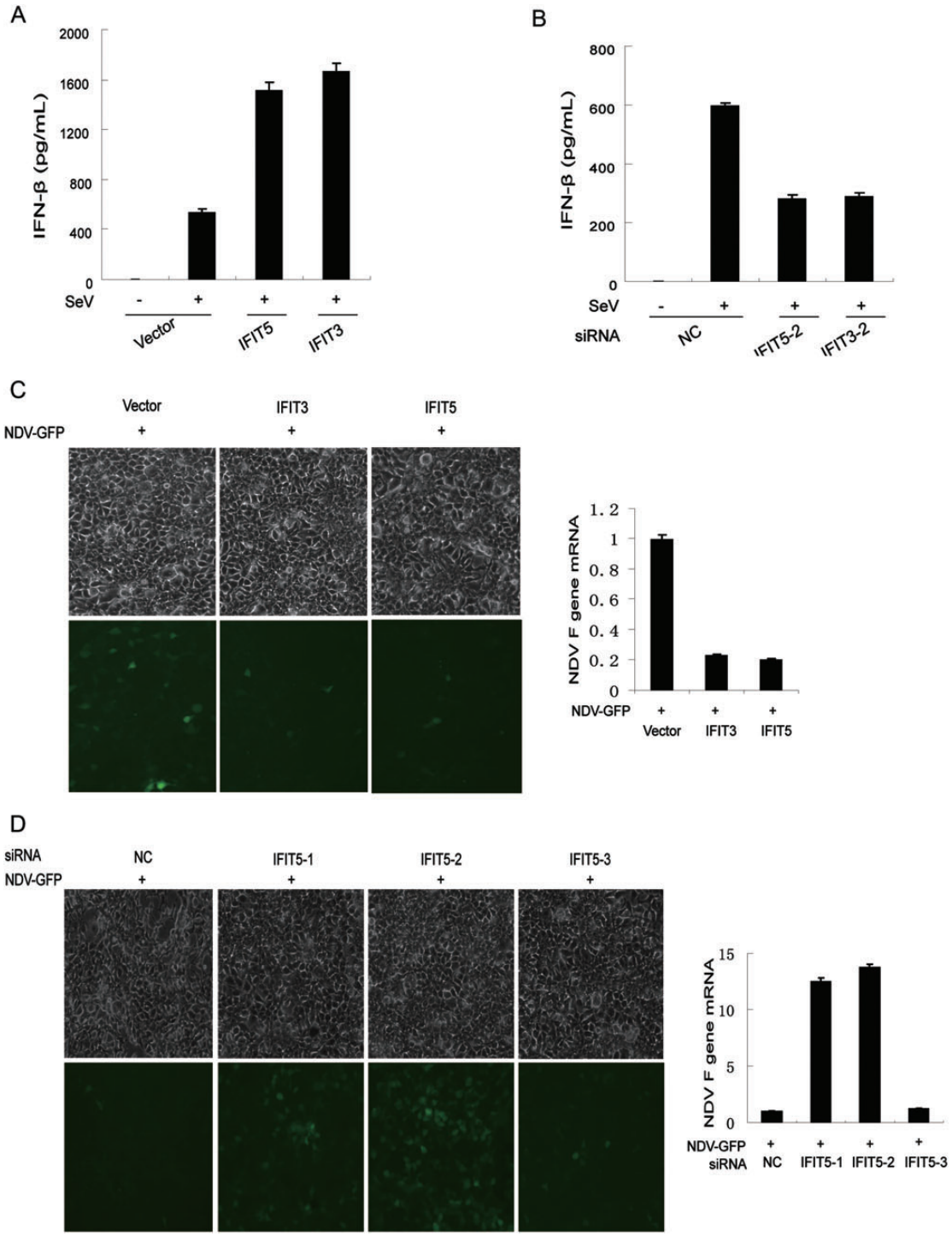

Figure 3 IFIT5 potentiates host anti-viral responses HEK293 cells were transfected with indicated plasmid (A) or siRNA (B). After SeV stimulation, IFN-β induction was determined by ELISA. HEK293 cells were transfected with indicated plasmid (C) or siRNA (D) followed by infection with NDV-GFP at an MOI of 1 (C) or 0.2 (D). And then the virus replication was visualized by fluorescence microscopy at 18 or 22 h post-infection (C and D, left panel). HEK293 cells were transfected and treated as described above, and virus replication was measured by real-time RT-PCR using NDV F gene specific primers (C and D, right panel).

adaptor bridging RIG-I to MAVS on the mitochondria. It is a great challenge to elucidate the molecular dynamics of the RIG-I/IFIT5/MAVS signaling complex in future investigations.

Our recent report uncovers an adaptor role of IFIT3 in bridging MAVS and TBK1. In the IFN-β luciferase reporter assays, we found that IFIT3 functions downstream of MAVS [28], while IFIT5 was shown to function upstream of MAVS

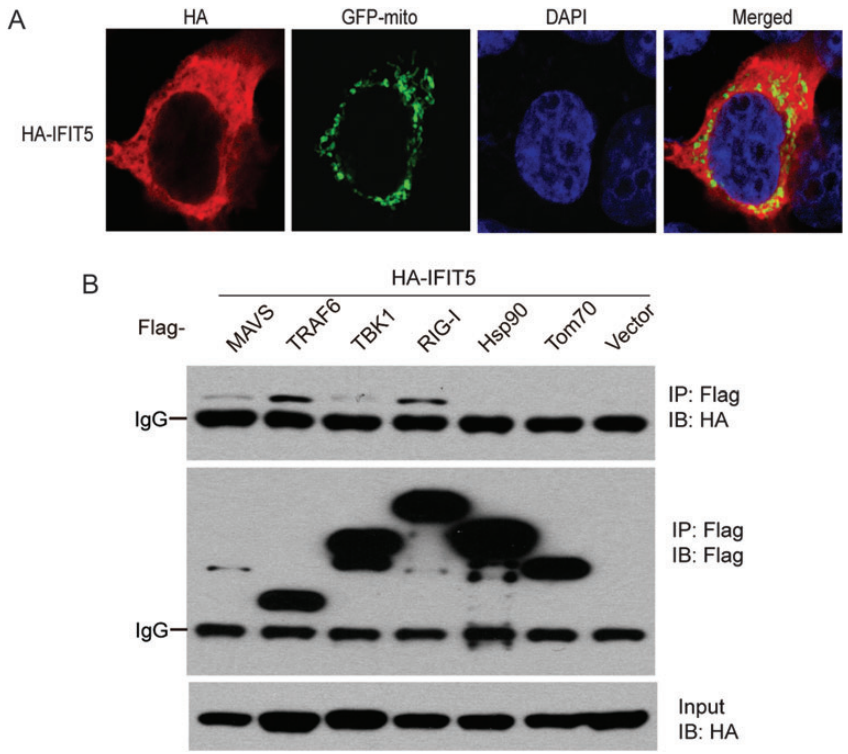

Figure 4 IFIT5 could associate with RIG-I and MAVS (A) HA-IFIT5 was transfected into HEK293T cells together with GFP-mito plasmid; the cells were then stained with anti-HA antibody and HA-IFIT5 was visualized with Cy3-conjugated secondary antibody. The nucleus was visualized by 4′,6-diamino-2-phenylindole staining. The images were captured with a 40× oil objective. (B) HEK293T cells were co-transfected with HA-IFIT5 plasmid and Flag-tagged indicated plasmids and then subjected to immunoprecipitation with anti-Flag-conjugated beads. Samples were resolved by SDS–PAGE and analyzed by western blot assay using indicated antibody.

(data not shown). These results suggest that IFIT3 and IFIT5 play non-redundant roles in the anti-viral signal transduction, which is further supported by the co-immunoprecipitation assays. Unlike IFIT3, IFIT5 associate strongly with the RNA sensor RIG-I, rather than the downstream kinase TBK1.

In conclusion, the current study reveals a novel and essential function of IFIT5 in host anti-viral responses.

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