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IFN-Induced TPR Protein IFIT3 Potentiates Antiviral Signaling by Bridging MAVS and TBK1

Xin-Yi Liu,¹ Wei Chen,¹ Bo Wei, Yu-Fei Shan, and Chen Wang

Intracellular RNA viruses are sensed by receptors retinoic acid-inducible gene I/MDA5, which trigger formation of the mitochondrial antiviral signaling (MAVS) complex on mitochondria. Consequently, this leads to the activation of TNFR-associated factor family member-associated NF- κ B activator-binding kinase 1 (TBK1) and phosphorylation of IFN regulatory factor 3 (IRF3). It remains to be elucidated how MAVS activates TBK1/IRF3. In this study, we report that IFN-induced protein with tetratricopeptide repeats 3 (IFIT3) is significantly induced upon RNA virus infection. Ectopic expression or knockdown of IFIT3 could, respectively, enhance or impair IRF3-mediated gene expression. Mechanistically, the tetratricopeptide repeat motif (E164/E165) of IFIT3 interacts with the N terminus (K38) of TBK1, thus bridging TBK1 to MAVS on the mitochondrion. Disruption of this interaction markedly attenuates the activation of TBK1 and IRF3. Furthermore, host antiviral responses are significantly boosted or crippled in the presence or absence of IFIT3. Collectively, our study characterizes IFIT3 as an important modulator in innate immunity, revealing a new function of the IFIT family proteins (IFN-induced protein with tetratricopeptide repeats). *The Journal of Immunology*, 2011, 187: 2559–2568.

Retinoic acid-inducible gene I and MDA5 have recently been characterized as ubiquitous sensors for detecting cytosolic RNA viruses (1). They trigger the formation of the mitochondrial antiviral signaling (MAVS) complex on the mitochondrial outer membrane, which includes adaptor proteins MAVS (also called IFN-promoter stimulator-1/virus-induced signaling adaptor/Cardif), TNFR-associated factor (TRAF) 2/3/5/6, NF- κ B essential modulator, TNFR-associated factor family member-associated NF- κ B activator, and TRADD (2–10). This ultimately leads to the activation of TNFR-associated factor family member-associated NF- κ B activator-binding kinase 1 (TBK1), which then phosphorylates IFN regulatory factor 3 (IRF3) on a series of Ser/Thr residues at its C terminus (11–13). In turn, IRF3

dimerizes, translocates into nucleus, and recruits p300/CBP, resulting in the early production of IFN- β and subsequent establishment of antiviral state (14–16). A dozen proteins have been reported to regulate this signaling pathway (17). In addition, MAVS complex could activate I κ B kinase (IKK) complex and NF- κ B (4).

MAVS per se localizes on the outer membrane of the mitochondria via its C-terminal transmembrane domain (4). Another study suggested that MAVS could possibly localize on the peroxisome (18). Notably, Hsp90 interacts constitutively with TBK1 and IRF3. There is no direct interaction between MAVS and the Hsp90/TBK1/IRF3 protein complex, because all of them reside in the cytosol (19–22). It remains to characterize how MAVS relays the activation signal to this protein complex.

The IFN-induced protein with tetratricopeptide repeats (IFIT) family of genes is clustered on human chromosome 10, including IFIT1, IFIT2, IFIT3/4, and IFIT5. All of them are robustly induced by IFNs, viral infection, and LPSs (23). Orthologs of the IFIT family are evolutionarily conserved from amphibians to mammals. Notably, no ortholog of human IFIT5 is present in mouse. It was reported that IFIT3 is widely expressed in neurons, kidney cells, T and B cells, plasmacytoid dendritic cells, and myeloid dendritic cells (24, 25).

IFIT family proteins are characterized by the unique helix-turn-helix motifs called tetratricopeptide repeats (TPRs), which mediates a variety of protein–protein interactions (26). The TPR motif is critical for a multitude of cellular and viral regulations, such as protein transport, translational initiation, cell migration and proliferation, antiviral signaling, and virus replication (23, 26–29). Recently, IFIT1/2 was characterized as a negative-feedback regulator of the retinoic acid-inducible gene I (RIG-I) antiviral signal transduction (28). In addition, IFIT1/2 preferentially recognized viral mRNA lacking 2'-O methylation. This selectively restricted the propagation of the mutants of poxvirus and coronavirus (such as West Nile virus) that lacked 2'-O methyltransferases activity (29). Notably, little is known about the function of IFIT3 in any cellular processes.

Translocase of outer membrane 70 (Tom70) is a mitochondrion protein transport receptor on the mitochondrial outer membrane.

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Abbreviations used in this article: BMDM, bone marrow-derived macrophages; CARD, caspase activation and recruitment domain; IAV, influenza A virus; IFIT, IFN-induced protein with tetratricopeptide repeats; IFIT3-C, C-terminal 210 aa (281–490 aa) of IFN-induced protein with tetratricopeptide repeats 3; IFIT3-N, N-terminal 280 aa (1–280 aa) of IFN-induced protein with tetratricopeptide repeats 3; IKK, I κ B kinase; IRF3, IFN regulatory factor 3; ISG, IFN-stimulated gene; MAVS, mitochondrial antiviral signaling; NC, nonspecific control; NDV-GFP, Newcastle disease virus-GFP; P-M ϕ , peritoneal macrophage; poly(I:C), polyinosinic-polycytidylic acid; Q-PCR, quantitative PCR; rIFIT3, RNA interference-resistant IFN-induced protein with tetratricopeptide repeats 3 construct; RIG-I, retinoic acid-inducible gene I; RNAi, RNA interference; SeV, Sendai virus; siRNA, small interfering RNA; TBK1, TNFR-associated factor family member-associated NF- κ B activator-binding kinase 1; Tom70, translocase of outer membrane 70; TPR, tetratricopeptide repeat; TRAF, TNFR-associated factor; VSV, vesicular stomatitis virus.

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Interestingly, Tom70 contains multiple TPR motifs (27). Our recent study revealed that a particular TPR motif of Tom70 could interact directly with Hsp90, thus recruiting the Hsp90/TBK1/IRF3 protein complex onto mitochondrion (22, 30). Given that IFIT proteins are inducible by viral infection, and they all contain TPR motif, we wondered if they could modulate the RIG-I antiviral signaling. In this study, we identified IFIT3 as an important adaptor bridging TBK1 to MAVS on the mitochondrion. IFIT3 is significantly induced by RNA viral infection or polyinosinic-polycytidylic acid [poly(I:C)] transfection. Ectopic expression or knockdown of IFIT3 markedly potentiates or attenuates IRF3-mediated gene expression, respectively. Interestingly, IFIT3 colocalizes partly with the mitochondrion and interacts specifically with MAVS. Mechanistically, the TPR motif (E164/E165) of IFIT3 interacts with the N terminus (K38) of TBK1. Disruption of this interaction sharply impairs the activation of TBK1 and IRF3. Taken together, our study characterizes IFIT3 as an important modulator in innate immunity, revealing a new function of the IFIT family proteins.

Materials and Methods

Plasmids

Human full-length IFIT3, IFIT5, MAVS, TBK1, IKK ϵ , RIG-I, TRAF3, TRAF2, TRAF6, TRADD, capsase activation and recruitment domain (CARD) 9, Hsp90, and Tom70 cDNA were cloned from human thymus plasmid cDNA library (Clontech) using standard PCR techniques and then subcloned into indicated vectors. All point mutants were generated by using a Quickchange XL (Stratagene). IFIT3-N is composed of the N-terminal 280 aa (1–280 aa) of IFIT3, whereas IFIT3-C represents the C-terminal 210 aa (281–490 aa) of IFIT3. IFIT3-2A has changed two aa, E164/E165, to AA at the second TPR motif, whereas TBK1 (K38A) has constructed by substitution of the N-terminal K38 with A. The truncation mutant TBK1 (111–729) is deprived of the N-terminal 110 aa. All IFIT3 small interfering RNA (siRNA)-resistant forms were generated by introducing silent mutations in the IFIT3 siRNA 3-2 target sequence (972-GGGACTAAACCCATAAAT-990). All constructs were confirmed by sequencing. IFIT1 and IFIT2 were kindly provided by Dr. Hongbing Shu (Wuhan University); pEGFP-mito for labeling mitochondria was provided by Dr. Jiansheng Kang (Shanghai Institutes for Biological Sciences).

siRNA

All siRNAs (GenePharma) were transfected using Lipofectamine 2000 as described (Invitrogen). HEK293 or HEK293T cells were plated in 12-well plates in antibiotic-free DMEM. At ~50% confluence, 40–80 pmol siRNA was transfected into cells. To determine efficiency of protein knockdown, at 48 h posttransfection, cells were lysed in RIPA buffer and immunoblotted with the indicated Abs. The siRNA sequences were as follows: nonspecific control (NC), 5'-UUCUCCGAAGGUGUCACGU-3'; IFIT3-1 siRNA human, 5'-GGCAAGCUGAAGAGUUAU-3'; IFIT3-1 siRNA mouse, 5'-GGCAAGCUGAAGAUUUAAG-3'; IFIT3-2 siRNA human, 5'-GGGACUGAAUCCUCUGAAU-3'; IFIT3-2 siRNA mouse, 5'-GAGGC-GAAGUCCUUUGAAC-3'; and IFIT3-3 siRNA human and mouse, 5'-GGUAUCUUAAGGAUUAU-3'.

Abs

Mouse polyclonal Ab against human MAVS recombinant protein was generated in this laboratory. This Ab was purified by affinity column conjugated with recombinant MAVS protein, and its specificity was confirmed. Abs obtained from commercial sources were as follows: anti-IFIT3 (Proteintech); anti-Flag, anti- β -actin, anti- α -tubulin (Sigma-Aldrich); anti-HA, anti-PARP (Santa Cruz Biotechnology); anti-TBK1 (Millipore); and anti-phospho-IRF3 (Epitomics).

Cell culture

HEK293T and HEK293 cells were cultured using DMEM plus 10% FBS supplemented with 1% penicillin-streptomycin (Gibco-BRL). Transient transfection was performed with Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions.

Manipulation of primary cells

Bone marrow-derived macrophages (BMDM) were obtained as described (31) with minor modifications. BMDM were harvested after ~6–10 d and

cultured in 12-well plates for siRNA transfection using Lipofectamine 2000 (Invitrogen) before culturing further. For peritoneal macrophages, 3 d after injection of 2 ml 4.0% (w/v) thioglycollate medium (Sigma-Aldrich), peritoneal cells were isolated from the peritoneal cavities of mice by peritoneal lavage with PBS. Macrophages were collected after washing twice with PBS and resuspended in DMEM containing 10% FBS in 12-well plates for further RNA interference (RNAi) experiments.

Luciferase reporter assays

Luciferase reporter assays were performed as described previously (30).

Quantitative PCR

Total cellular RNA was isolated with TRIzol (Invitrogen) according to the manufacturer's instructions. Reverse transcription of purified RNA was performed using oligo(dT) primer. The quantification of gene transcripts was performed by quantitative PCR (Q-PCR) using SYBR Green I dye (Invitrogen). All values were normalized to the level of β -actin mRNA. The primers used were listed as follows. Primers for human were: β -actin, sense (5'-AAAGACCTGTACGCCAACAC-3'), antisense (5'-GTCATACCTCTGCTTGCTGAT-3'); IFN- β , sense (5'-ATTGCTCAAGGACAGG-ATG-3'), antisense (5'-GGCCTTCAGGTAATGCAGAA-3'); IFN-stimulated gene (ISG) 56, sense (5'-GCCATTTTCTTTGCTTCCCCTA-3'), antisense (5'-TGCCCTTTTGTAGCCTCCTTG-3'); RANTES, sense (5'-TACAC-CAGTGGCAAGTGCTC-3'), antisense (5'-ACACACTTGGCGGTTCTT-TC-3'); IL-8, sense (5'-AGGTGCAGTTTTGCCAAGGA-3'), antisense (5'-TTTCTGTGTGGCGCAGTGT-3'); TNF- α , sense (5'-ATGTGCTC-CTCACCCACACC-3'), antisense (5'-CCCTTCTCCAGCTGGAAGAC-3'); and I κ B α , sense (5'-CTGAGCTCCGAGACTTTCGAGG-3'), antisense (5'-CACGTGTGGCCATTGTAGTTGG-3'). Primers for mouse were: β -actin, sense (5'-AGACCTCTATGCCAACACAG-3'), antisense (5'-TCGTACT-CCTGCTTGCTGAT-3'); IFN- β , sense (5'-AGATCAACCTCACCTACA-GG-3'), antisense (5'-TCAGAAACACTGTCTGCTGG-3'); RANTES, sense (5'-ATGAAGATCTCTGCAGCTGC-3'), antisense (5'-GTGACAA-ACACGACTGCAAG-3'); and IL-6, sense (5'-GAGAGGAGACTTCACAGAGG-3'), antisense (5'-GTACTCCAGAAGACCAGAGG-3').

Immunoblot analysis and immunoprecipitation assay

For immunoblotting, the immunoprecipitates or whole-cell lysates were resolved by SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Millipore). Immunoblotting was probed with indicated Abs. The proteins were visualized by using the Supersignal West Pico chemiluminescence ECL kit (Pierce). For immunoprecipitation, cells were collected ~24–48 h after transfection and then lysed in Nonidet P-40 buffer supplemented with a complete protease inhibitor mixture (Roche). The whole cell lysates were used for immunoprecipitation with indicated Abs. Generally, 5–10 μ l conjugated Flag beads (Sigma-Aldrich) was added to 500 μ l cell lysate and then incubated for 2–6 h at 4°C. Immunoprecipitates were extensively washed with lysis buffer and eluted with SDS loading buffer by boiling for 5 min.

Confocal imaging

HEK293T cells were plated on coverslips in 12-well plates and transfected with the indicated plasmids. Twenty-four hours posttransfection, coverslips with the 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 BSA (2%) and then incubated with a primary Ab in the staining buffer lacking Triton X-100 for 1 h. After washing three times in the staining buffer lacking Triton X-100, cells were incubated with a secondary Ab for 1 h and then with DAPI for 10 min. The coverslips were then washed extensively and fixed on slides. Imaging of the cells was carried out using Leica laser scanning confocal microscope (Leica Microsystems).

Nuclear extraction

Nuclear extraction of HEK293 cells was performed as described previously (30).

Rescue experiments

HEK293 cells were transfected with IFIT3 or control siRNA for 24 h and then transfected again with the indicated siRNA-resistant IFIT3 or control plasmid, followed by Sendai virus (SeV) infection the next day.

Measurement of IFN- β production

HEK293 cells were transfected with the indicated plasmids or siRNAs, and then cell culture supernatants were collected at 8 h after virus infection and

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

Virus manipulation

Viral infection was performed when 80% cell confluence was reached. Then, culture media was replaced by serum-free DMEM, and SeV, vesicular stomatitis virus (VSV), influenza A virus (IAV), or Newcastle disease virus (NDV)-GFP was added to the media at a multiplicity of infection of 0.2–1 according to specific experiments. After 1 h, the medium was removed, and the cells were fed with DMEM containing 10% FBS (32, 33). Human H1N1 IAV strain IAV-PR8/34 was kindly provided by Dr. Ze Chen (Wuhan Institute of Virology, Chinese Academy of Sciences).

Statistics

Student *t* test was used for the comparison of two independent treatments. For all tests, a *p* value <0.05 was considered statistically significant.

Results

Identification of IFIT3 as a positive modulator of RIG-I signaling

Recently, we reported that Tom70, a mitochondrial outer membrane protein with multiple TPR motifs, is essential for the activation of the TBK1/IRF3 during RNA viral infection. So we wondered if other TPR-containing proteins play a regulatory role in the RIG-I signaling. Bioinformatics analysis uncovers a family of TPR-containing proteins, which are highly inducible by IFNs. A diagram of the IFIT family proteins is shown in Fig. 1A. Interestingly, IFIT1 and IFIT2 were implicated recently as the negative regulators of the antiviral innate immunity (28). It remains unknown whether other members of the IFIT family proteins regulate the RIG-I signaling.

To explore the possibility, we conducted the IFN- β -luciferase reporter assays. Apparently, ectopic expression of IFIT1 or IFIT2 marginally reduced the expression of IFN- β -luciferase reporter stimulated by SeV, whereas IFIT5 displayed some potentiating effect. Notably, ectopic expression of IFIT3 could markedly synergize the induction of IFN- β -luciferase reporter under the same condition (Fig. 1B). In addition, IFIT3 potentiated the expression of PRDIII-I- and κ B-luciferase reporters upon SeV infection (Fig. 1C). As a control, IFIT3 displayed no effect on the induction of κ B- or AP-1-luciferase reporter stimulated by TNF- α stimulation (Fig. 1D).

As expected, both the mRNA and protein of IFIT3 were robustly induced by SeV, poly(I:C), and IFN- β . However, IFIT3 could be detected constitutively in low abundance (Fig. 1E, 1F). Interestingly, we observed that a significant fraction of IFIT3 colocalized with mitochondrion, whereas TBK1 is exclusively localized in the cytoplasm (Fig. 1G), suggesting that IFIT3 is a potential regulator of MAVS antiviral signaling.

Exogenous expression of IFIT3 synergizes the activation of IRF3 and NF- κ B

To further corroborate the function of IFIT3, we measured the induction of endogenous mRNAs from IRF3-responsive genes (including IFN- β , ISG56, and RANTES) and NF- κ B-responsive genes (including IL-8, I κ B α , and TNF- α) via Q-PCR, after exogenously expressing IFIT3 and treating cells by the indicated stimuli. Consistently, IFIT3 markedly potentiated the expression of endogenous IRF3-responsive genes in response to SeV challenge (Fig. 2A). In addition, IFIT3 also promoted the induction of endogenous NF- κ B-responsive genes upon SeV infection (Fig. 2B). Furthermore, we observed the same potentiating effect of IFIT3 when stimulating cells with poly(I:C) (Fig. 2C, 2D). Collectively, these data suggest that IFIT3 synergizes the activation of IRF3 and NF- κ B during virus infection.

Knockdown of IFIT3 impairs the activation of IRF3 and NF- κ B

Alternatively, we took the knockdown approach to investigate the function of IFIT3 via Q-PCR. We designed multiple siRNAs for IFIT3 and then screened out two effective oligonucleotides that could reduce the endogenous protein levels of IFIT3 by >90% (namely 3-1 and 3-2) and use the siRNA 3-3 as a negative control (Fig. 3A, left and middle panels). Initially, we analyzed in HEK293 cells, the effect of IFIT3 knockdown on the induction of endogenous IRF3- and NF- κ B-responsive genes (including IFN- β , RANTES, and IL-8) stimulated, respectively, by SeV or poly(I:C). Consistently, knockdown of IFIT3 by siRNA 3-1 and 3-2 dramatically inhibited the transcription of both IRF3- and NF- κ B-responsive genes upon SeV or poly(I:C) stimulation, whereas the siRNA 3-3 had no such inhibitory effects (Fig. 3B, 3C).

To make it more physiologically relevant, we isolated BMDM and peritoneal macrophages (P-M ϕ) and, respectively, transfected these cells with indicated siRNAs, followed by SeV or IAV infection. Then, we examined whether IFIT3 regulated the expression of IRF3- and NF- κ B-induced genes (including IFN- β , RANTES, and IL-6) in these primary cells. Consistently, knockdown of IFIT3 markedly attenuated SeV- or IAV-induced expression of endogenous IRF3-responsive genes in both BMDM (Fig. 3D, Supplemental Fig. 1A) and P-M ϕ (Fig. 3E, Supplemental Fig. 1B). In addition, reduction of endogenous IFIT3 displayed the same effects toward NF- κ B-responsive genes (Fig. 3D, 3E, Supplemental Fig. 1).

To rule out potential off-target effects of the IFIT3 siRNAs, we performed the rescue experiments by using siRNA 3-2 in the knockdown procedure. An RNAi-resistant IFIT3 construct corresponding to siRNA 3-2 (rIFIT3) was generated, in which silent mutations were introduced into the sequence targeted by the same siRNA without changing the amino acid sequence of the protein (Fig. 3A, right panel). HEK293 cells were firstly transfected with control or IFIT3 siRNA 3-2, followed by transfection of control or rIFIT3 plasmid as indicated, respectively. Then the induction of IFN- β mRNA was measured by Q-PCR after SeV treatment. As shown in Fig. 3F, SeV-stimulated IFN- β mRNA induction was restored by introducing rIFIT3 into the IFIT3-knockdown cells. Collectively, these results indicate that IFIT3 is a positive regulator of IRF3 activation.

IFIT3 functions downstream of MAVS and upstream of TBK1

To explore the mechanism of IFIT3 action, we tried to delineate the topology of IFIT3 in RIG-I/MAVS/TBK1 signaling pathway. We observed that exogenous expression of RIG-I N-terminal tandem CARD or MAVS could induce the expression of IFN- β -luciferase reporter, and this induction was markedly potentiated by the ectopic expression of IFIT3 (Fig. 4A). Consistently, knockdown of IFIT3 impaired the expression of IFN- β reporter under the same condition (Fig. 4B). Apparently, neither ectopic expression nor knockdown of IFIT3 had any effects on the induction of IFN- β reporter when stimulating with exogenous TBK1 (Fig. 4A, 4B).

To further substantiate the above observations, we examined the phosphorylation status and nuclear translocation of IRF3 during SeV infection when ectopically expressing IFIT3. As shown in Fig. 4C, IFIT3 increased the IRF3 phosphorylation induced by SeV. Consistently, introduction of IFIT3 into HEK293 cells markedly enhanced the nuclear translocation of phosphorylated IRF3 (Fig. 4D). Taken together, these data indicate that IFIT3 functions downstream of MAVS and upstream of TBK1.

IFIT3 is a new component of MAVS complex

As observed above, IFIT3 colocalized partly with the mitochondrion (Fig. 1G). We wondered whether IFIT3 is an integral com-

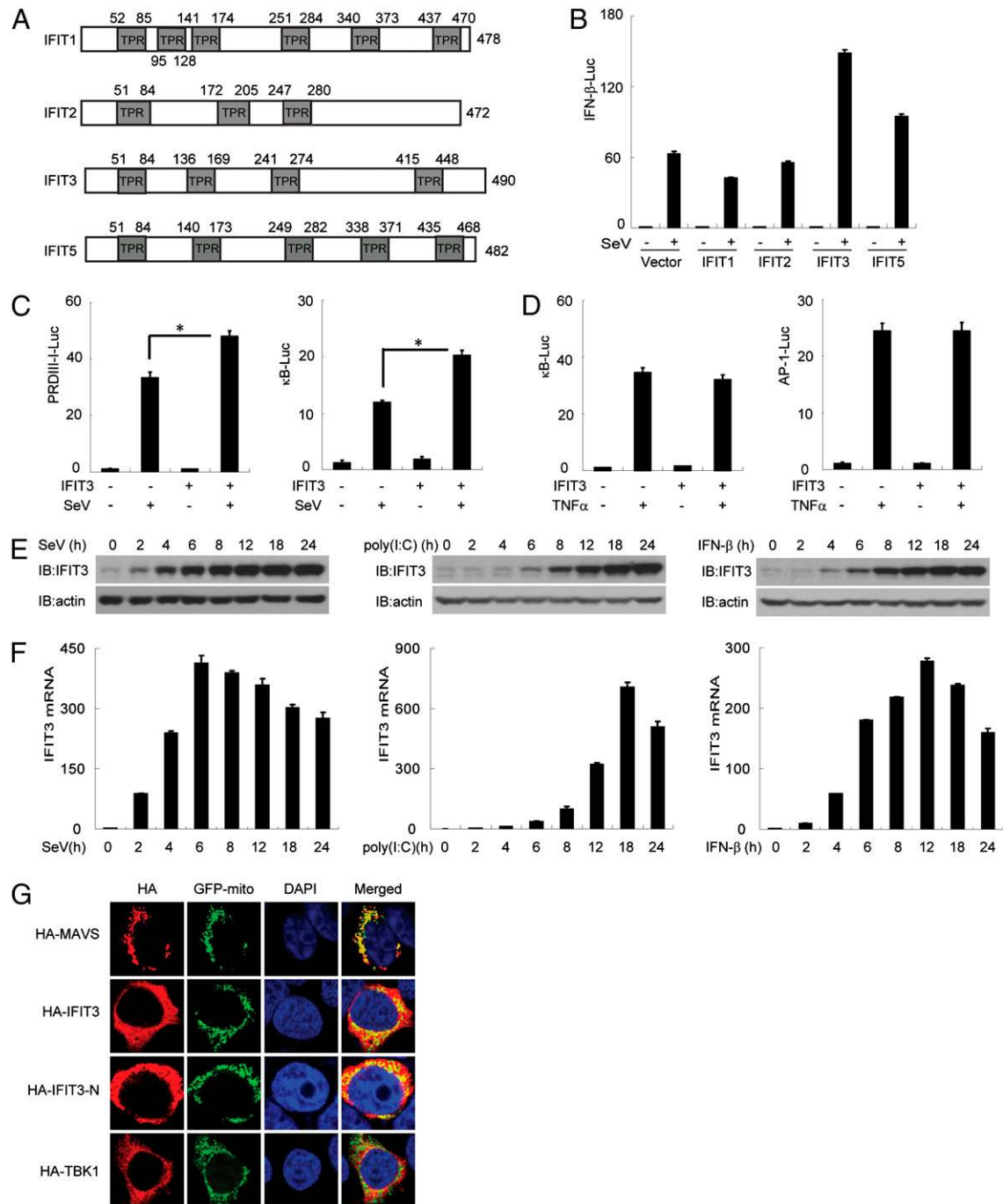


FIGURE 1. Identification of IFIT3 as a new regulator of RIG-I signaling. *A*, The domain structures of IFIT family proteins. *B*, HEK293 cells were transfected with IFN- β -luc reporter plasmid, together with pTK-Renilla reporter and empty vector or indicated IFIT constructs. Twenty-four hours after transfection, cells were stimulated with SeV for 8 h before luciferase reporter assays were performed. *C*, HEK293 cells were transfected individually with PRDIII-I- or κ B-luc reporter plasmid, together with pTK-Renilla reporter and empty vector or IFIT3 construct. Twenty-four hours after transfection, cells were stimulated with SeV for 8 h before luciferase reporter assays were performed. *D*, HEK293 cells were transfected individually with κ B- or AP-1-luc reporter plasmid, together with pTK-Renilla reporter and empty vector or IFIT3 construct. Twenty-four hours after transfection, cells were stimulated with TNF- α for 8 h before luciferase reporter assays were performed. *E*, HEK293 cells were stimulated with SeV, poly(I:C), and IFN- β , respectively, for the indicated time periods, and the cell lysates were immunoblotted with anti-IFIT3 and anti- β -actin Abs. *F*, HEK293 cells were stimulated as in *E*, and the inductions of IFIT3 mRNA were measured by Q-PCR. *G*, HA-IFIT3, HA-IFIT3-N, HA-MAVS, or HA-TBK1 were transfected into HEK293T cells together with enhanced GFP-mito plasmid; the cells were then stained with anti-HA Ab and imaged by confocal microscopy. The images were captured with a $\times 40$ oil objective. * $p < 0.05$.

ponent of the MAVS signalosome. To address the hypothesis, HA-IFIT3 was cotransfected into HEK293T cells with Flag-MAVS, TRAF2, TRAF3, TRAF6, TBK1, IKKe, RIG-I, Tom70, Hsp90, CARD9, or TRADD, respectively. Cell lysates were immunoprecipitated with conjugated anti-Flag beads. It was revealed that

IFIT3 could interact strongly with MAVS and TBK1 (Fig. 5A, left panel), whereas the IFIT1 cannot bind to either MAVS or TBK1 (Supplemental Fig. 2C) (28). In addition, IFIT3 could coimmunoprecipitate marginally with TRAF6 and RIG-I. Notably, IFIT3 did not interact with Hsp90 or Tom70 (Fig. 5A, left panel).

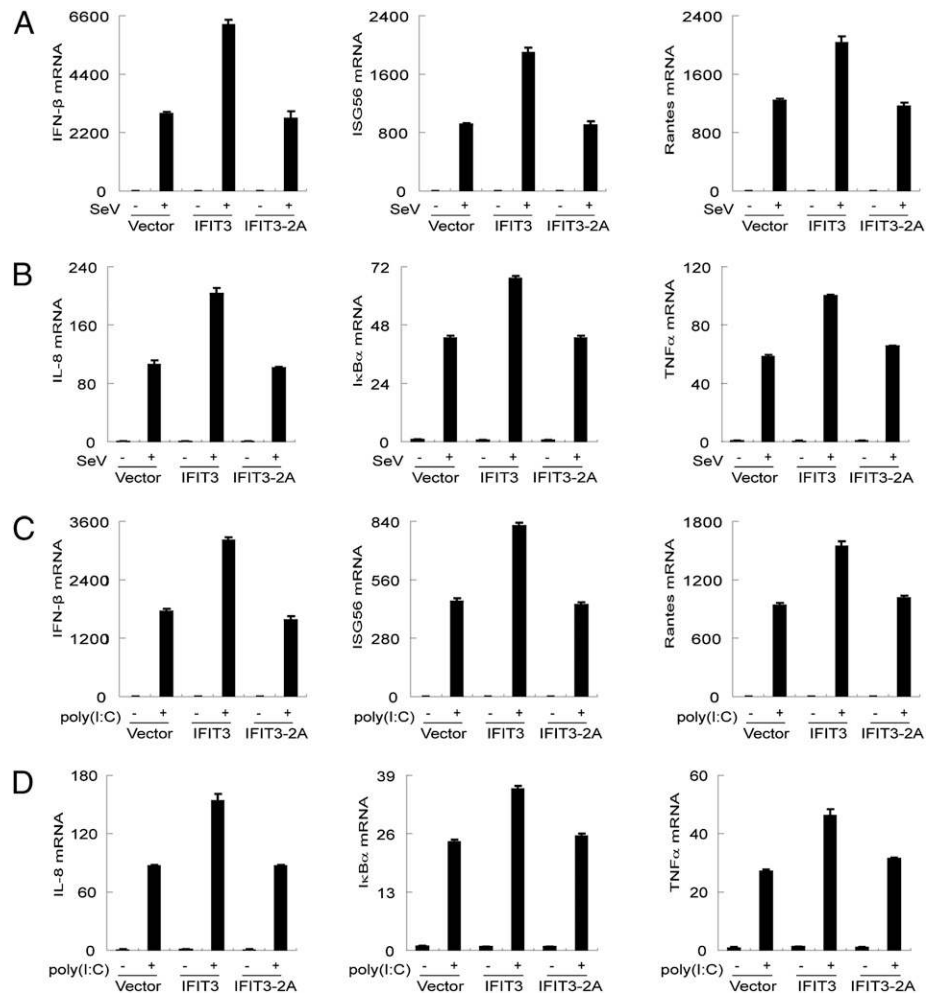


FIGURE 2. IFIT3 synergizes IRF3 activation during SeV or poly(I:C) stimulation. HEK293 cells were transfected with indicated plasmids and then challenged by SeV infection. Induction of IFN- β , ISG56, RANTES (A), IL-8, I κ B α , and TNF- α (B) mRNA was measured by Q-PCR. C and D, HEK293 cells were transfected with indicated plasmids and then challenged by poly(I:C) transfection. Induction of IFN- β , ISG56, RANTES (C), IL-8, I κ B α , and TNF- α (D) mRNA was measured by Q-PCR. Data are presented as means \pm SD ($n = 3$). 2A, the point mutant of IFIT3.

Furthermore, HA-IFIT3 could, to a lesser extent, be coimmunoprecipitated by Flag-tagged TRAF2, TRAF3, IKK ϵ , and TRADD, but not by CARD9 (Fig. 5A, right panel). These data indicate that IFIT3 is a novel component in the MAVS signalosome.

To map the domain of IFIT3 critical for its interaction with MAVS, we generated two plasmids encoding IFIT3 truncates. The IFIT3-N is composed of the N-terminal 1–280 aa, whereas IFIT3-C contains the C-terminal 281–490 aa (Fig. 5B). It was observed that IFIT3-N could, as well as wild-type IFIT3, interact with MAVS, TBK1, TRAF6, and RIG-I (Fig. 5C), suggesting that the N terminus of IFIT3 is critical for the MAVS signalosome. For unknown reason, the IFIT3-C did not express in all of the cells we tested. Notably, the IFIT3-N per se could potentiate the SeV-induced of IFN- β and IL-8 expression (Fig. 5D). Interestingly, the IFIT3-N also partly colocalizes with the mitochondria (Fig. 1G), indicating that the C terminus of IFIT3 is dispensable for either antiviral function or mitochondrial localization. These data indicate that the N terminus of IFIT3 mediates its interaction with both MAVS and TBK1, and it is sufficient to modulate the MAVS antiviral signaling.

IFIT3 is a novel adaptor bridging TBK1 to MAVS

We went on to align the protein sequences of IFIT3-N across the species and have identified several conserved amino acids in the TPR motifs (Fig. 5B, 5E). Consequently, a series of point mutants of IFIT3 were generated, and their binding capacity to both MAVS and TBK1 was tested via coimmunoprecipitation assays. Interestingly, IFIT3-2A, with two amino acids mutated in the

second TPR motif (Fig. 5B, 5E), failed to interact with TBK1 (Fig. 5F, left panel). However, IFIT3-2A could strongly interact with MAVS (Fig. 5F, left panel). In addition, we constructed the truncation mutant and point mutants of TBK1 (Supplemental Fig. 2A). The truncation mutant TBK1 (111–729), which is deprived of the N-terminal 110 aa, could not bind to the wild-type IFIT3 (Supplemental Fig. 2B). Furthermore, TBK1 (K38A) could not interact with the wild-type IFIT3 (Fig. 5F, right panel), indicating that the N-terminal of TBK1 mediates this interaction.

To further corroborate the physiological relevance of this interaction, it was observed that IFIT3-2A could not potentiate the expression of endogenous IRF3- and NF- κ B-responsive genes, stimulated, respectively, by SeV infection or poly(I:C) transfection (Fig. 2). In addition, we generated the corresponding RNAi-resistant construct of IFIT3-2A, namely rIFIT3-2A. Consistently, this construct was not able to rescue the SeV-induced expression of IFN- β , after the endogenous IFIT3 was knocked down (Fig. 3F).

To explore whether IFIT3 served as a bridge between MAVS and TBK1, we coexpressed Flag-MAVS and Myc-TBK1 in the presence or absence of IFIT3. Coimmunoprecipitation assay revealed that MAVS could barely associate with TBK1 in the absence of IFIT3. Notably, the association between MAVS and TBK1 was apparently enhanced when ectopically expressing IFIT3, whereas ectopic expression of IFIT3-2A displayed no such effect (Fig. 5G). Consistently, knockdown of endogenous IFIT3 attenuated the interaction between ectopically expressed MAVS and TBK1 (Fig. 5H). In addition, endogenous IFIT3 could coimmunoprecipitate

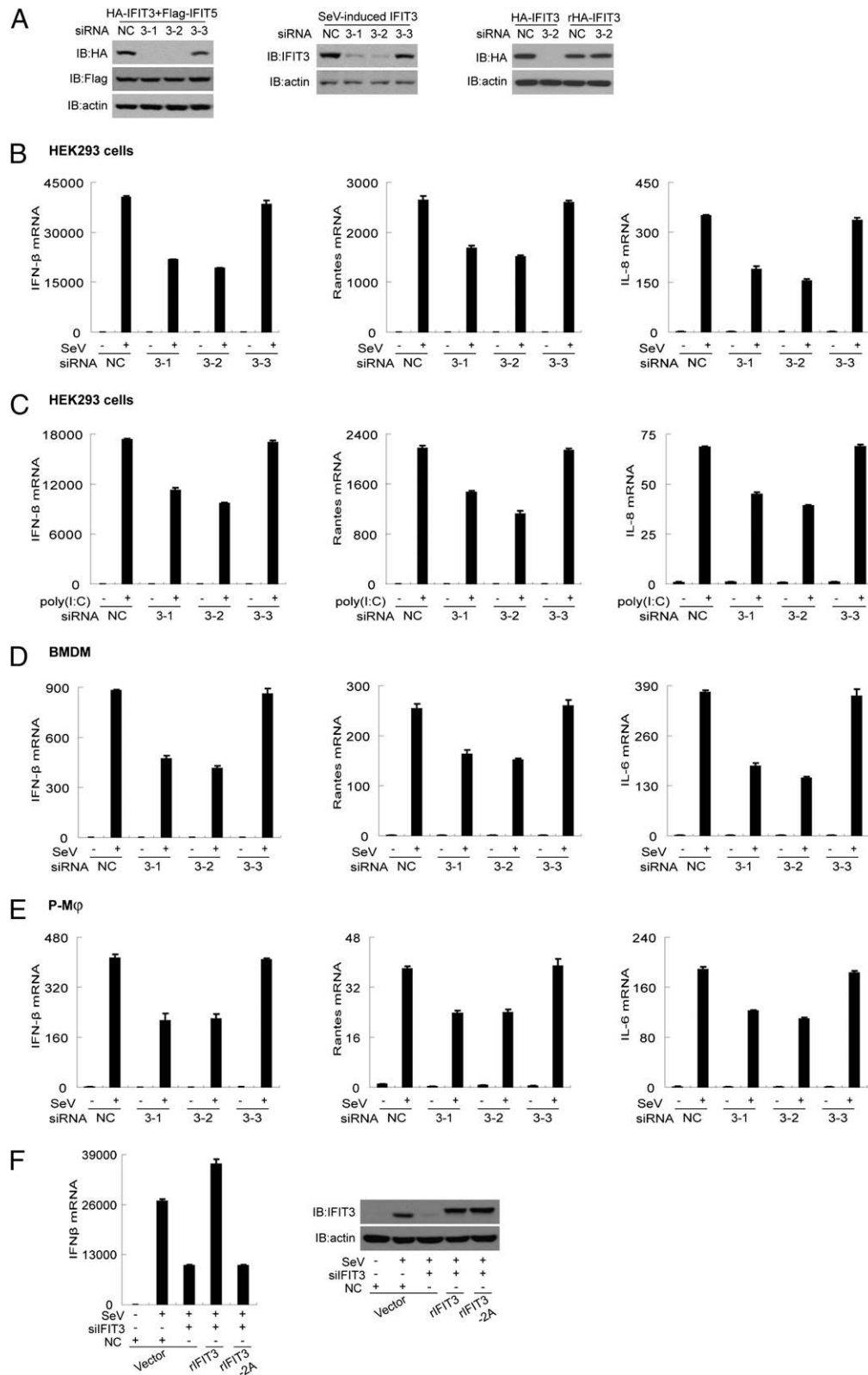


FIGURE 3. Knockdown of IFIT3 attenuates IRF3 activation upon challenging by SeV or poly(I:C). *A*, HEK293 cells were transfected with HA-IFIT3 and Flag-IFIT5 plasmids and then treated with NC or IFIT3 siRNA, respectively. Cell lysates were immunoblotted with anti-HA and anti-Flag Abs (*left panel*). HEK293 cells were transfected with NC or IFIT3 siRNA and then infected with SeV. Cell lysates were immunoblotted with anti-IFIT3 Ab (*middle panel*). HEK293 cells were transfected with HA-IFIT3 or siRNA-resistant IFIT3 plasmid and then treated with NC or IFIT3 siRNA, respectively. Cell lysates were immunoblotted with anti-HA Ab (*right panel*). HEK293 cells were transfected with indicated siRNAs and then challenged by SeV infection (*B*) or poly(I:C) transfection (*C*). Induction of IFN- β , RANTES, and IL-8 mRNA was measured by Q-PCR. BMDM (*D*) or P-M ϕ (*E*) were transfected with indicated siRNAs and then stimulated by SeV. Induction of IFN- β , RANTES, and IL-6 mRNA was measured by Q-PCR. *F*, HEK293 cells were transfected with indicated siRNAs for 24 h. Then, siRNA-resistant IFIT3 or IFIT3-2A was transfected into the knockdown cells. After SeV infection, induction of IFN- β mRNA was measured by Q-PCR. Data in *B–F* are presented as means \pm SD ($n = 3$).

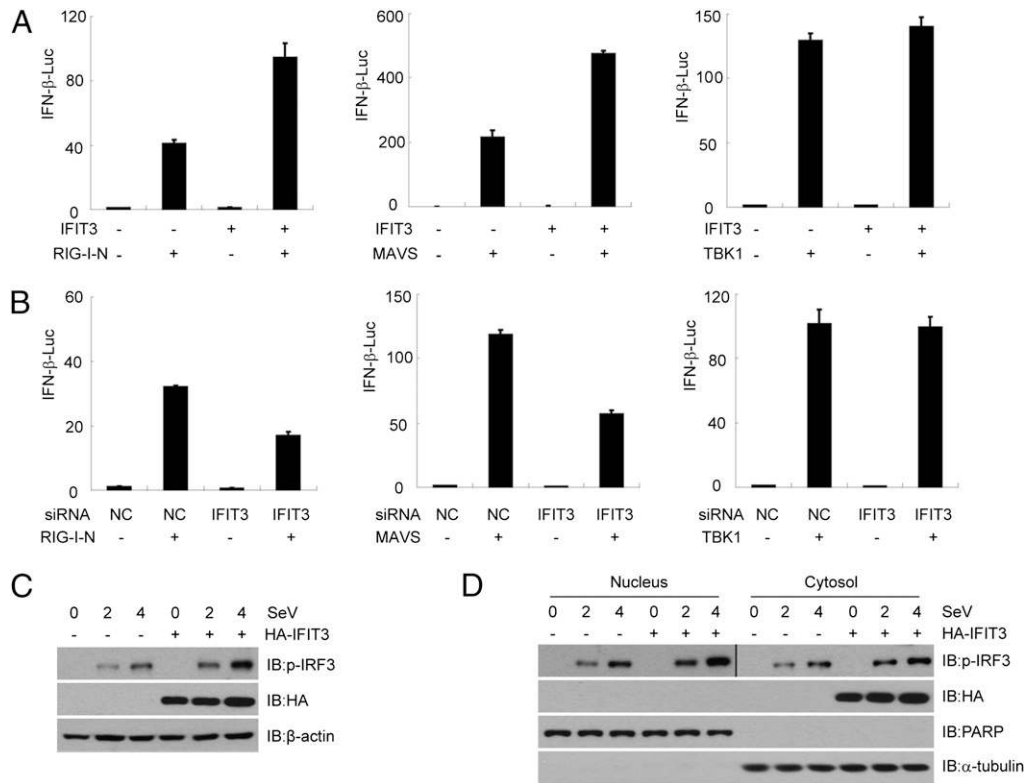


FIGURE 4. IFIT3 functions downstream of MAVS and upstream of TBK1. *A*, IFN- β -luc and pTK-Renilla reporters were transfected into HEK293T cells together with empty vector or IFIT3 plasmid and then challenged by RIG-I N-terminal tandem CARD, MAVS, or TBK1 construct. *B*, HEK293T cells were transfected and stimulated as in *A*, except that siRNA 3-2 was transfected in lieu of plasmid. *C*, HEK293 cells were transfected with HA-IFIT3 or control plasmid and then stimulated with SeV for indicated time. Cell lysates were immunoblotted with anti-phospho-IRF3 and anti-HA Abs. *D*, HEK293 cells were transfected and stimulated as in *C* and then fractionated into cytosolic and nuclear subfractions. The amount of phosphorylated IRF3 in the nucleus was analyzed by immunoblot.

both endogenous MAVS and TBK1, and these interactions were significantly enhanced after SeV stimulation (Fig. 5*J*). Taken together, these data established that IFIT3 serves as an essential adaptor bridging TBK1 to MAVS on the mitochondrion.

IFIT3 enhances MAVS-mediated antiviral responses

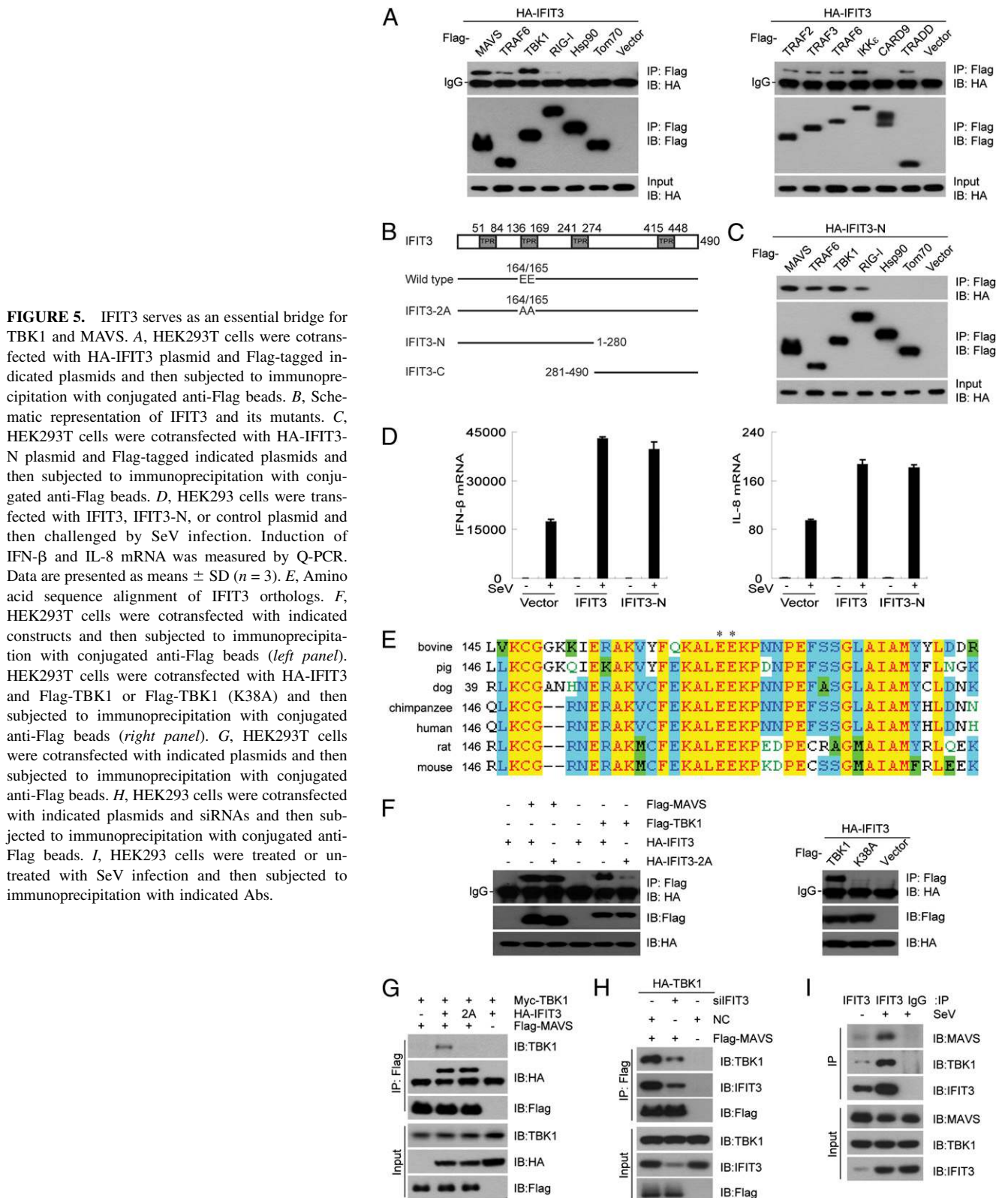
Finally, we investigated the physiological importance of IFIT3 in innate immunity. It is well known that the induction of IFN- β is a hallmark of host antiviral responses. We transfected IFIT3 into HEK293 cells, followed by SeV infection. The supernatants were checked by ELISA. As expected, IFIT3 significantly promoted IFN- β protein production (Fig. 6*A*). In contrast, knockdown of endogenous IFIT3 by the siRNA 3-1 and 3-2 drastically impaired the IFN- β protein production, whereas the siRNA 3-3 had no effect (Fig. 6*B*).

Because IFN- β could protect cells from viral infection, we wondered if IFIT3 played a role in virus restriction. So HEK293 cells were transfected with the indicated siRNAs and then treated with VSV for 16 h, and then the titers of the VSV in the supernatants were analyzed by standard plaque assay. As shown in Fig. 6*C*, IFIT3 knockdown resulted in a significant increase in the VSV titer. We also infected cells directly with the Newcastle disease virus-GFP (NDV-GFP) and visualize the replication of the NDV via fluorescence microscope. Consistently, exogenous expression of IFIT3 markedly suppressed the NDV-GFP replication in HEK293 cells (Fig. 6*D*). In contrast, knockdown of IFIT3 augmented the levels of NDV-GFP-positive cells (Fig. 6*E*). These data indicate that IFIT3 enhances the host antiviral responses upon virus infection (Fig. 7).

Discussion

Little was known, until a decade ago, about the mechanism of how host cells detect invading viruses and restrict their replication and proliferation. A large body of investigations has since accumulated, and this provides a detailed understanding of the molecular basis of the pattern recognition receptor-mediated recognition of RNA viruses (34). One major breakthrough is the realization that RIG-I-like helicase receptors and TLRs detect the RNA viruses in different cellular compartments, respectively, initiating the mitochondrial and endosomal antiviral signaling pathways (17, 35). A major converging point of these pathways is the protein complex Hsp90/TBK1/IRF3, which could ultimately induce the robust expression of a series of cytokines and chemokines important for antiviral innate immune responses (30). The well-studied example is the rapid and robust induction of type I IFNs (in particular IFN- β), which in turn induces a wide array of ISGs (36). Among the ISGs, there is a subfamily named IFITs for which the functions are scarcely understood.

Recently, several proteins (Tom70, TRAF2/3/5/6, and TRADD) are characterized to perform functions in MAVS (5, 8, 9, 22, 37). Notably, it remains to elucidate how MAVS directly links to the critical protein kinase TBK1 and understand the detailed mechanism of signal transduction. Our recent report uncovers a novel shuttling mechanism for targeting the Hsp90/TBK1/IRF3 protein complex onto the mitochondrial outer membrane via the mitochondrial Tom70 receptor. However, MAVS per se does not interact directly with the Hsp90/TBK1/IRF3 complex. Although a few proteins have been demonstrated as the integral components of the MAVS signalosome, to our knowledge, no protein has been shown to specifically bridge TBK1 to MAVS. In particular,



knockdown of Tom70 could only partially attenuate the association between MAVS and TBK1. This led us to hypothesize that Tom70 serves to recruit Hsp90/TBK1/IRF3 onto the proximity of the mitochondrial outer membrane. Then, some unknown protein directly consolidates the association between MAVS and Hsp90/TBK1/IRF3, resulting in the TBK1 activation.

Domain analysis reveals that Tom70 resides on the mitochondrion via its N-terminal transmembrane domain. The rest of Tom70 is exposed in the cytosol and composed of a cluster of TPR motifs (22, 27). So we wondered if other TPR-containing proteins could play the regulatory role in the RIG-I signaling. Bioinformatics analysis of the microarray database uncovers the IFIT family of

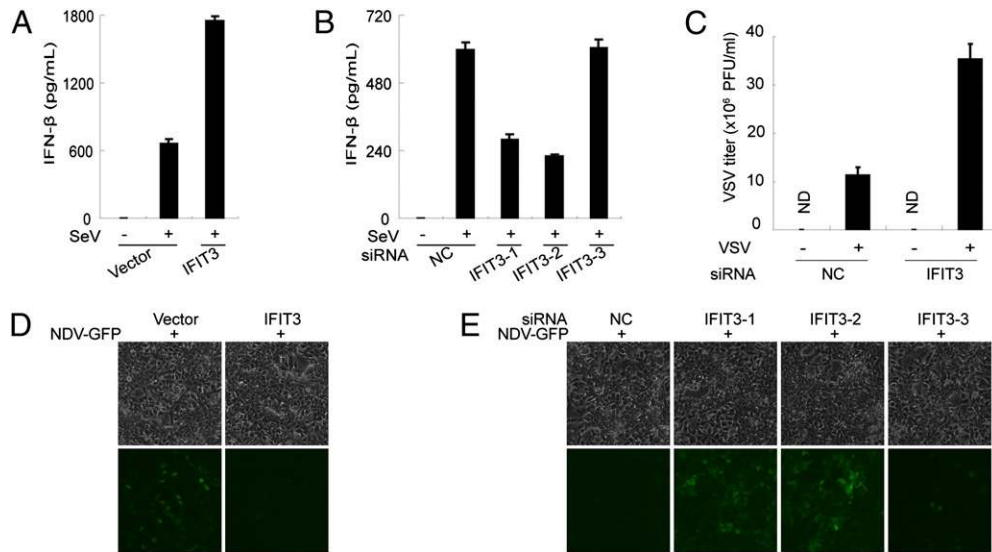


FIGURE 6. IFIT3 enhances MAVS-mediated host antiviral responses. HEK293 cells were transfected with indicated plasmid (A) or siRNA (B). After SeV infection, IFN- β production was determined by ELISA. Data are presented as means \pm SD ($n = 3$). C, HEK293 cells transfected with control or IFIT3 siRNA 3-2 were infected with VSV. The titers of VSV were determined by standard plaque assay. Data are presented as means \pm ASD ($n = 3$). NDV-GFP replication in HEK293 cells transfected with IFIT3 plasmid (D) or IFIT3 siRNA (E) was visualized by fluorescence microscopy. Data are representative of three independent experiments.

TPR-containing proteins, which are highly inducible by IFNs. The current study reveals that IFIT3 is an essential adaptor to bridge TBK1 to MAVS on mitochondrion.

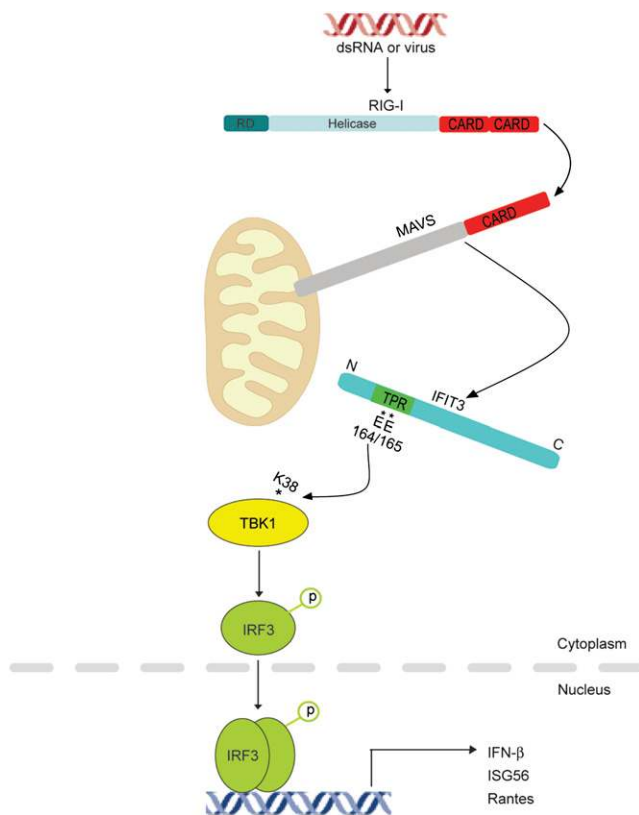


FIGURE 7. Schematic diagram of IFIT3 as a critical adaptor in innate immunity. Upon RNA virus infection, RIG-I binds to dsRNA, which causes RIG-I to undergo dramatic conformational change and results in the exposure of its N-terminal CARD domains. Then RIG-I was recruited to MAVS. IFIT3 serves as a new adaptor bridging TBK1 to MAVS on the mitochondrion. Consequently, this leads to the activation of TBK1 and phosphorylation of IRF3. Ultimately, phosphorylated IRF3 translocates into nucleus to promote antiviral gene transcription.

Several lines of evidence substantiate the novel function of IFIT3 for the MAVS signaling. Firstly, exogenous expression of IFIT3 potentiates the induction of IRF3- and NF- κ B-responsive genes upon SeV infection, but does not affect the TNF- α -induced NF- κ B activation. Secondly, knockdown of IFIT3 unequivocally results in significant reduction of IRF3- and NF- κ B-mediated gene expression, and this attenuation could be rescued by exogenously expressing a siRNA-resistant IFIT3. Thirdly, the phosphorylation and nuclear translocation of IRF3, the downstream events of TBK1 activation, are apparently impaired when knocking down endogenous IFIT3 during virus infection. Fourthly, IFIT3 interacts specifically, via its N-terminal domain, with both MAVS and TBK1. In the absence of IFIT3, the association between MAVS and TBK1 is disrupted. Fifthly, IFIT3-2A is unable to interact with TBK1; conversely, TBK1 K38A could not interact with IFIT3. Consistently, the corresponding RNAi-resistant mutants (rIFIT3-2A) failed to rescue the expression of IFN- β in IFIT3 knockdown cells. Ectopic expression of IFIT3-2A could not potentiate the induction of IRF3- and NF- κ B-responsive genes upon SeV infection. Sixthly, gain or loss of IFIT3 could, respectively, promote or impair IFN- β protein production upon SeV infection, thus boosting or crippling the host antiviral responses.

Intriguingly, MAVS was reported to also localize on the peroxisome and probably induced the rapid IFN-independent expression of defense factors that provide short-term protection. In contrast, mitochondrial MAVS activates an IFN-dependent signaling pathway with delayed kinetics, which amplifies and stabilizes the antiviral response (18). As observed in this study, the IFIT family members can be induced robustly by IFNs. However, the use of cell lines with appropriate genetic deficiencies of JAK-STAT pathway components demonstrated that viral and bacterial molecular patterns can directly (i.e., independently of IFN action) induce transcription of a subset of ISGs, including IFIT family genes (38, 39). So it is possible that IRF3 and NF- κ B activated by peroxisomal MAVS can induce the production of IFIT3 directly, which then promotes the interaction between TBK1 and mitochondrial MAVS, further amplifying the antiviral signaling. It will be interesting for future study to explore the mechanism of IFIT3

expression in the context of the interplay of the peroxisomal and mitochondrial MAVS.

Protein sequence alignment indicates that the N terminal region of IFIT3 is highly conserved among the vertebrates, whereas the C terminal domain is less conservative and displays little similarities (data not shown). This implies that the N-terminal region is critical for a conserved function. The implication is supported by the observation that the N-terminal of IFIT3 alone is sufficient to potentiate the MAVS signaling. Indeed, the IFIT3 proteins from both mouse and rat lack a portion of the C-terminal region. The knockdown of mouse IFIT3 confirmed its essential function for potentiating the expression of IFNs.

As reported previously, IFIT1 and IFIT2 exhibited some inhibitory effect toward RIG-I/MAVS signaling. The IFIT1/2 inhibitory effect was only marginal when we repeated the experiment. The same study proposed a mechanism in which IFIT1/2 interfered with the interaction between stimulator of IFN gene and MAVS or TBK1. Probably, there is uncovered protein that could offset the action of IFIT1/2. Another possibility is that IFIT1/2 could have more important functions in other aspects of the innate immunity.

In summary, the current study characterizes IFIT3 as an important adaptor to bridge TBK1 onto mitochondrial MAVS, revealing a new function of the IFIT family proteins in innate immunity (Fig. 7).

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Disclosures

The authors have no financial conflicts of interest.

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