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MIP-T3 Is a Negative Regulator of Innate Type I IFN Response

Ming-Him James Ng,* Ting-Hin Ho,* Kin-Hang Kok,* Kam-Leung Siu,* Jun Li,† and Dong-Yan Jin*  

TNF receptor-associated factor (TRAF) 3 is an important adaptor that transmits upstream activation signals to protein kinases that phosphorylate transcription factors to induce the production of type I IFNs, the important effectors in innate antiviral immune response. MIP-T3 interacts specifically with TRAF3, but its function in innate IFN response remains unclear. In this study, we demonstrated a negative regulatory role of MIP-T3 in type I IFN production. Overexpression of MIP-T3 inhibited RIG-1-, MDA5-, VISA-, TBK1-, and IKKε-induced transcriptional activity mediated by IFN-stimulated response elements and IFN-β promoter. MIP-T3 interacted with TRAF3 and perturbed in a dose-dependent manner the formation of functional complexes of TRAF3 with VISA, TBK1, IKKε, and IFN regulatory factor 3. Consistent with this finding, retinoic acid-inducible gene I- and TBK1-induced phosphorylation of IFN regulatory factor 3 was significantly diminished when MIP-T3 was overexpressed. Depletion of MIP-T3 facilitated Sendai virus-induced activation of IFN production and attenuated the replication of vesicular stomatitis virus. In addition, MIP-T3 was found to be dissociated from TRAF3 during the course of Sendai virus infection. Our findings suggest that MIP-T3 functions as a negative regulator of innate IFN response by preventing TRAF3 from forming protein complexes with critical downstream transducers and effectors.  

MIP-T3 binds to TRAF3 to impede its formation of functional complexes with downstream transducers and effectors including VISA, TBK1, IKKe, and IRF3. Finally, the function of MIP-T3 was characterized in the context of viral infection. Our findings might have implications in viral pathogenesis and the design of new antivirals.

**FIGURE 1.** MIP-T3 inhibits RIG-I/MDA5- and TBK1/IKKe-induced activation of ISRE and IFN-β promoter activity. Constant amounts of ISRE-Luc (A) or IFN-β–Luc (B) reporter plasmid were cotransfected with a fixed amount of activator plasmid and increasing amounts of Flag–MIP-T3 plasmid into HEK293 cells. The total amount of plasmids transfected was normalized with corresponding empty vectors. pRL-CMV was used as an internal control for transfection efficiency. Expression of MIP-T3 and other stimulatory proteins was verified by Western blotting (see inset in A for an example of Western blot analysis of MIP-T3 expression. *MIP-T3; #1, \( p = 0.010598 \) by Student t test; #2, \( p = 0.01745 \) by Student t test; #3, \( p = 0.018214 \) by Student t test; #4, \( p = 0.000028 \) by Student t test. C, Effect of MIP-T3 expression on activation of human T cell leukemia virus I long terminal repeats by viral trans-activator Tax. D, Effect of MIP-T3 on tunicamycin-induced activation of GRP78 promoter. Tunicamycin was added 12 h prior to cell harvest. E, Effect of MIP-T3 on RIG-I–induced activation of NF-κB. F, Effect of MIP-T3 on poly-IC–induced activation of TLR3. HEK293 cells were transfected with ISRE-Luc, TLR3 expression plasmid, and increasing amounts of expression plasmid for NS1-V5His or Flag-MIP-T3. Forty-eight hours after transfection, cells were stimulated with 1 μg/ml poly-IC for another 8 h. No ISRE-Luc activity was detected in the absence of TLR3 (data not shown). Results are representative of three independent experiments. Error bars indicate SD.
Materials and Methods

Cell culture and reagents

HEK293, HEK293T, and Vero cells were obtained from the American Type Culture Collection. Primary mouse embryonic fibroblasts (MEFs) were generated from C57BL/6J background. Cells were maintained in DMEM with 10% FBS (Life Technologies) and antibiotics. Cells were grown at 37°C in a humidified atmosphere with 5% CO₂. Transfection of plasmid DNA was performed using Genejuice (Novagen) as described previously (15, 26). Tunicamycin was purchased from Calbiochem. Recombinant IκB was bought from Santa Cruz. Recombinant IRF3 fused to glutathione S transferase (GST) was obtained from Abnova. Sendai virus (Cantell strain) was obtained from the American Type Culture Collection. Primary mouse embryonic fibroblasts (MEFs) were provided by Dr. Takashi Fujita (Kyoto University, Kyoto, Japan) (31). Expression vector for human T cell leukemia virus type I oncoprotein was provided by Dr. Genhong Cheng (National Institute of Arthritis and Musculoskeletal and Skin Diseases, Bethesda, MD) (30). pIFNβ-Luc (p125-Luc) and Flag-RIG-I plasmids were provided by Dr. Liusheng He and Dr. Peter Lipsky (University of Rochester, Rochester, NY) (37). Expression plasmids for MIP-T3, VISA, TANK, IKKε, TRAF3 were gifts from Dr. Jacques Perrault (San Diego State University, San Diego, CA) and Dr. Brian Lichty (McMaster University, ON, Canada) (27, 28). Viral infection of HEK293 cells and MEFs was performed as described (29).

Plasmids
cDNA clones for MIP-T3, VISA, TANK, IKKε were purchased from imaGenes (Berlin, Germany). Flag-tagged MIP-T3 was created by cloning MIP-T3 cDNA into pCMV-Tag2B (Stratagene) using PCR amplification. It was further subcloned into pcDNA-3.1+ (Invitrogen) as described previously (15, 26). Tunicamycin was used to coexpress in HEK293T cells. Cell lysates were probed with anti-Flag (a-Flag) and the precipitates were probed with anti-V5 (a-V5). Immunoprecipitation (IP) was performed with α-Flag, and the precipitates were probed with α-Flag and α-V5, respectively.

Immunoprecipitation, Western blotting, reporter assay, and protein kinase assay

Coinmunoprecipitation, Western blotting, dual luciferase assay, and protein kinase assay were performed as described previously (15, 39). Mouse monoclonal anti-Flag Ab M2 (F3165) and rabbit polyclonal anti-Flag Abs (F7425) were purchased from Sigma. Mouse monoclonal anti-V5 Ab (46-0705) was purchased from Invitrogen. Mouse monoclonal anti-Myc Ab (sc-40), rabbit polyclonal anti-IRF3 (sc-9082) and anti-MIP-T3 (C20) Abs were purchased from Santa Cruz Biotechnology. Rabbit monoclonal anti-phospho-IRF3 (S396) Ab 4D4G (4741) was obtained from Cell Signaling. For TRAF3 ubiquitination assay, 0.1% SDS was added to the immunoprecipitation buffer and wash buffer.

Primers, small interfering RNAs, and quantitative PCR

Sequences of human-specific MIP-T3 primers are 5’-TCACACAATT CTGCAATATGA AG-3’ (MT3-497F) and 5’-TCACTTTCTC TCAGT-CAAAT TG-3’ (MT3-691R). Primers for amplification of GAPDH and procedures for semiquantitative RT-PCR have been described elsewhere (40, 41).

Quantitative real-time PCR was performed using Power SYBR Green Master Mix (Applied Biosystems). Quantitative PCR (qPCR) primers for mouse MIP-T3 transcript are 5’-TGAACGCTGA CATTTCCTGTG-3’ (forward) and 5’-ATCACCTCCG GCTTATCTTG-3’ (reverse). Primers for mouse IFN-β are 5’-CAGCTCCCAAG AAGGACGAA C-3’ (forward) and 5’-GGCAGTGTAA CTTCTTCTGA T-3’ (reverse). Primers for mouse GAPDH are 5’-AGGGTCGTTT GAACGGATT G-3’ (forward) and 5’-TGAGCACAT GTAGTGGAGG TCA-3’ (reverse). QPCR reaction was performed with a StepOne Real-Time PCR System (Applied Biosystems). Target mRNA expression was quantitated with the comparative Ct method. Relative expression level of target mRNA was calculated from 2^{-ΔΔCt}.

Sequences for siGFP are 5’-GCAACUGUCG CCGAGAGGC at-3’ (sense) and 5’-CAUAUCUGAG CUGACGUCCG CTG-3’ (antisense). Sequences for si3A and si3B against human MIP-T3 are 5’-GAGGAGAGC GCUGAAUAAt r-3’ (sense for si3A), 5’-UAAUUUACUG CGCUUCCUCt r-3’ (antisense for si3A), 5’-GCAUGACGCU GAAAGAAAt r-3’ (sense for si3B), and 5’-UAAUUUACUG CGCUUCCUCt r-3’ (antisense for si3B). Sequences for si3C and si3D against mouse MIP-T3 are 5’-GAGGAGAGC GCUGAAUAAt r-3’ (sense for si3C), 5’-GCAUGACGCU GAAAGAAAt r-3’ (antisense for si3C). Sequences for si3E against mouse MIP-T3 were 5’-GUCUCUCUCU UAUAUUAUCG CGCUUCCUCt r-3’ (antisense for si3E).

Materials and Methods

Cell culture and reagents

HEK293, HEK293T, and Vero cells were obtained from the American Type Culture Collection. Primary mouse embryonic fibroblasts (MEFs) were generated from E13 embryos of wild-type mouse in C57BL/6J background. Cells were maintained in DMEM with 10% FBS (Life Technologies) and antibiotics. Cells were grown at 37°C in a humidified atmosphere with 5% CO₂. Transfection of plasmid DNA was performed using Genejuice (Novagen) as described previously (15, 26). Tunicamycin was purchased from Calbiochem. Recombinant IκB was bought from Santa Cruz. Recombinant IRF3 fused to glutathione S transferase (GST) was obtained from Abnova. Sendai virus (Cantell strain) was obtained from the American Type Culture Collection. Recombinant vesicular stomatitis virus (VSV)-GFP was a gift from Dr. Jacques Perrault (San Diego State University, San Diego, CA) and Dr. Brian Lichty (McMaster University, ON, Canada) (27, 28). Viral infection of HEK293 cells and MEFs was performed as described (29).

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AGAAUGUCA GCCUCAUCU r-3' (antisense for si3C), 5'-GUGCAGAGCACAAGAAT r-3' (sense for si3D), and 5'-UUCUGUUGUGCUCUCAUCU r-3' (antisense for si3D). Transfection of small interfering RNA (siRNA) into HEK293 cells was performed as described (40) using Lipofectamine 2000 according to the manufacturer’s protocol. Transfection of siRNA into MEF cells was performed using X-treme-Gene siRNA transfection reagents (Roche).

**ELISA**

ELISA was performed using a mouse IFN-β ELISA kit (PBL Biomedical Laboratories) and the manufacturer’s protocol.

**Plaque formation assay**

Vero cells were seeded in 12-well plates. When the cells grew to 100% confluence, they were infected with serial 10-fold dilutions of the virus in DMEM culture medium for with routine rocking. One hour postinfection, infection medium was removed and infected Vero cells were then overlaid with 1:1 DMEM culture medium containing 1% agarose. At 30 h postinfection, cells were fixed with 3.7% paraformaldehyde and stained with 0.05% crystal violet.

**Results**

MIP-T3 inhibits RIG-I/MDA5- and TBK1/IKKε-induced activation of IFN-β promoter

To test whether and how expression of MIP-T3 might affect type I IFN production, we used luciferase reporter constructs that are driven by canonical IFN-stimulated response elements (ISRE) or IFN-β promoter. These ISREs are known to be recognized by IRF3 (42, 43). As expected, luciferase activity from both ISRE-Luc (Fig. 1A) and IFNβ-Luc (Fig. 1B) reporter constructs was remarkably stimulated by RIG-I, MDA5, VISA, TBK1, IKKε, and IRF3, which are key activators of type I IFN production (44). When MIP-T3 was expressed, a significant and dose-dependent suppression of ISRE-Luc (Fig. 1A) and IFN-β-Luc (Fig. 1B) reporter activity induced by RIG-I, MDA5, VISA, TBK1, IKKε, or IRF3 was observed in HEK293 cells. For example, when the dose of MIP-T3 plasmid introduced into cells increased from 50 to 150 ng (see inset in Fig. 1B for a representative Western blot of MIP-T3), a more than 2-fold reduction of IKKε-induced ISRE-Luc and IFN-β-promoter-driven luciferase reporter expression was seen (Fig. 1A, B, lower third panel). In the control experiments, MIP-T3 did not affect the basal transcriptional activity of ISRE and IFN-β promoter (Fig. 1A, 1B, upper left panel).

In addition, to exclude the possibility of a general inhibition effect induced by overexpression of MIP-T3, we showed that MIP-T3 did not inhibit Tax-induced activation of a reporter driven by human T cell leukemia virus type I long terminal repeats (Fig. 1C). Tax is a retroviral transactivator that activates transcription from viral and cellular promoters (34, 45, 46). In addition, MIP-T3 had no effect on tunicamycin-induced activation of GRP78 promoter (Fig. 1D). GRP78 resides in endoplasmic reticulum is a protein chaperone activated by endoplasmic reticulum stressors such as tunicamycin that inhibits glycosylation (36, 47). Thus, MIP-T3

**FIGURE 4.** MIP-T3 blocks TRAF3 complex formation. The indicated proteins were coexpressed in HEK293T cells. Immunoprecipitation (IP) and Western blotting (WB) were performed with the indicated Abs. The formation of protein complexes TRAF3–TBK1 (A), TRAF3–IKKε (B), TRAF3–IRF3 (C), TRAF3–VISA (D), TRAF3–TANK (E), and TBK1–IRF3 (F) was examined.
is not a general repressor of cellular transcription or translation. Because both IRF3 and NF-κB are activated in innate IFN response (31, 48, 49), we asked whether MIP-T3 might also exert an inhibitory effect on NF-κB signaling. We found that MIP-T3 had no influence on RIG-I-induced NF-κB activation (Fig. 1E), suggesting that MIP-T3 acts specifically on TRAF3, but not on NF-κB. However, a strong inhibitory effect of MIP-T3 was observed when we activated TLR3 with polyinosinic-polycytidylic acid (poly-IC) to boost ISRE activity (Fig. 1F). The inhibition by MIP-T3 was even more dramatic than that mediated by influenza A virus NS1 protein, a known viral antagonist of IFNs (38). Our results indicate that MIP-T3 specifically suppresses type I IFN production induced by various activators including RIG-I, MDA5, VISA, TLR3, TBK1, IKKe, and IRF3.

**Interaction of MIP-T3 with TRAF3 and influence of MIP-T3 on TRAF3 ubiquitination**

Above we showed the inhibition of RIG-I, MDA5, TBK1, and IKKe activity by MIP-T3 (Fig. 1). To investigate the cause, we asked whether MIP-T3 might physically associate with these activators in addition to TRAF3. We coexpressed MIP-T3 and RIG-I, TANK, TRAF3, TBK1, and IKKe in HEK293T cells and performed coimmunoprecipitation assay. The target proteins were efficiently expressed and successfully precipitated (Fig. 2). MIP-T3 was found only in the TRAF3-containing precipitate (Fig. 2, lane 3), but not in all other precipitates. Thus, MIP-T3 binds specifically to TRAF3, but not to RIG-I, TANK, TBK1, or IKKe. In other words, the suppressive effect of MIP-T3 on RIG-I, TBK1, or IKKe was unlikely due to direct interaction.

TRAF3 is a ubiquitin ligase, and several negative regulators of TRAF3 have been shown to modulate K63- or K48-linked polyubiquitination of TRAF3 (8, 10, 12). In view of this, we next investigated whether the interaction of MIP-T3 with TRAF3 might also influence TRAF3 ubiquitination. We coexpressed HA-tagged ubiquitin and Flag-tagged TRAF3 in HEK293T cells and then probed the anti-Flag immunoprecipitate with anti-Flag and anti-HA (Fig. 3). The ladder of ubiquitinated TRAF3 proteins in the precipitate was evident (Fig. 3, lane 5). However, the ubiquitination signal of TRAF3 was only slightly reduced upon expression of MIP-T3 (Fig. 3, lane 6). Therefore, MIP-T3 did not appear to affect TRAF3 ubiquitination dramatically.

**MIP-T3 prevents TRAF3 from complex formation with VISA, TBK1, IKKe, and IRF3**

We demonstrated that MIP-T3 could mitigate type I IFN production (Fig. 1), but was unable to counteract TRAF3 ubiquitination (Fig. 3). However, MIP-T3 specifically interacts with TRAF3, but not with other downstream transducers or effectors in IFN production (Fig. 2), which are normally associated with TRAF3 (16). These TRAF3-associated transducers and effectors include RIG-I, MDA5, VISA (50–53), TBK1, IKKe, IRF3 (4, 5), and TANK (33). The formation of protein complexes comprising TRAF3 and these downstream proteins is essential for the activation of IFN production (4, 5). Because MIP-T3 was not in the complexes that contain RIG-I, TANK, TBK1, and IKKe (Fig. 2), we asked whether MIP-T3 and these downstream proteins might be mutually exclusive in forming a complex with TRAF3. To address this question, we extensively examined the formation of TRAF3 complexes with different downstream transducers and effectors in the presence and in the absence of MIP-T3.

We found that MIP-T3 inhibited the formation of TRAF3–VISA, TRAF3–TBK1, TRAF3–IKKe, and TRAF3–IRF3 complexes (Fig. 4, A–D); neither did MIP-T3 have any influence on the interaction between RIG-I and TRAF3 or between MDA5 and TRAF3 (data not shown). Particularly, when V5–MIP-T3 was expressed,

**FIGURE 5.** MIP-T3 inhibits TRAF3–IKKe complex formation in a dose-dependent manner. Progressively increasing amounts of MIP-T3 plasmid were cotransfected with TRAF3 and IKKe constructs into HEK293T cells. Immunoprecipitation (IP) and Western blotting (WB) experiments were performed as in Fig. 4B.

**FIGURE 6.** MIP-T3 inhibits RIG-I–induced and TBK1–induced IRF3 phosphorylation in cultured cells without affecting TBK1 kinase activity. A, IRF3 phosphorylation in cultured cells. Indicated proteins were expressed in HEK293T cells. Phosphorylated IRF3 (pIRF3) was detected by guest on April 28, 2017 http://www.jimmunol.org/ Downloaded from
FIGURE 7. siRNA-mediated depletion of MIP-T3 potentiated viral activation of IFN-β promoter. A, Two independent MIP-T3–targeting siRNAs (si3A and si3B) at a concentration of 50 nM were used to deplete endogenous MIP-T3 in HEK293 cells. siGFP was used as a negative control. At the 24th hour after knockdown, reporter plasmids p125-Luc and pRL-CMV were cotransfected into the cells. Twenty-four hours later, transfected cells were infected with Sendai virus (80 hemagglutinating U/ml). Cells were harvested 0 and 24 h postinfection. Results are representative of three independent experiments. Error bars represent SD. *p = 0.024 by Student t test, #p = 0.041 by Student t test. h.p.i., hours postinfection.

B and C, Verification of MIP-T3 knockdown by siRNAs. RT-PCR was performed to amplify MIP-T3 and GAPDH transcripts. Western blot analysis was performed using anti–MIP-T3 and anti–β-tubulin Abs. D and E, RNAi depletion of MIP-T3 in primary MEFs augmented the production of IFN-β transcript. MIP-T3 knockdown in MEF cells before and after Sendai virus infection was verified. MEFs were transfected with siGFP and siRNAs targeting mouse MIP-T3 (si3C and si3D) 48 h before Sendai virus infection. Expression level of MIP-T3 mRNA was normalized with that of GAPDH transcript. Induction of IFN-β transcript in MEFs infected with Sendai virus was also analyzed. Error bars represent SD. *p = 0.028 by Student t test, #p = 0.015 by Student t test. F, Depletion of MIP-T3 augmented IFN-β production. Infection and transfection of MEFs were performed as in D and E. Production of IFN-β was analyzed by ELISA. Error bars denote SD. (Figure legend continues)
V5-TRAF3 was not detected in the Flag-TBK1 immunoprecipitate obtained with anti-Flag Ab, whereas abundant V5-TRAF3 was found to be associated with the Flag–TBK1–containing protein complex in cells that did not express V5–MIP-T3 (Fig. 4A, lane 2 compared with lane 1). Likewise, when V5–MIP-T3 was overexpressed, V5-TRAF3 was absent from the Flag–IκKε or Myc–IRF3 immune complex (Fig. 4B, 4C, lane 2 compared with lane 1), and V5-VISA was almost undetectable in the Flag–TRAF3 precipitate (Fig. 4D, lane 2 compared with lane 1). In sharp contrast, MIP-T3 appeared to have no influence on TANK–TRAF3 or IRF3–TBK1 interaction (Fig. 4E, 4F). As such, comparable amounts of V5-TRAF3 were recovered from Flag–TANK–containing immune complex in cells either not expressing or expressing V5–MIP-T3 (Fig. 4E), whereas similar levels of endogenous IRF3 were found to be associated with TBK1-Flag immunoprecipitate in V5–MIP-T3-expressing or V5–MIP-T3–nonexpressing cells (Fig. 4F). That is to say, MIP-T3 did not prevent TRAF3 from interacting with all its partners promiscuously, nor did it impede all downstream events. Instead, MIP-T3 specifically perturbs the formation of particular TRAF3 complexes.

Using TRAF3-IκKε as an example, we showed that the progressively elevated expression of MIP-T3 in HEK293T cells correlated with a gradual diminution of IκKε-associated TRAF3 (Fig. 5, lanes 2–4). Likewise, dose-dependent inhibition of TRAF3–VISA, TRAF3–TBK1, and TRAF3–IRF3 by MIP-T3 was also observed when similar experiments were performed (data not shown). Hence, one functional consequence of the interaction between MIP-T3 and TRAF3 is the perturbation of complex formation between TRAF3 and downstream proteins including VISA, TBK1, IκKε, and IRF3.

**MIP-T3 inhibits phosphorylation of IRF3 in cultured cells without affecting TBK1 kinase activity in vitro**

Above we showed that MIP-T3 inhibits type I IFN production by inhibiting TRAF3 complex formation (Fig. 4). IRF3 is one major transcription factor regulating IFN-β transcription. Phosphorylation of IRF3 is a critical event in IFN production (54). Phosphorylated IRF3 would dimerize and translocate into the nucleus to activate the IFN-β promoter (55). To shed light on the mechanism by which MIP-T3 inhibits IFN production, we next explored whether MIP-T3 might affect RIG-I–induced and TBK1–induced phosphorylation of IRF3. Phosphorylated IRF3 in cultured cells overexpressing RIG-I or TBK1 was readily detected with a phosphospecific Ab (Fig. 6A, lanes 1 and 3). Upon overexpression of MIP-T3, the level of phospho-IRF3 induced by RIG-I or TBK1 was significantly reduced to undetectable level (Fig. 6A, lanes 2 and 4). Thus, MIP-T3 plausibly perturbs phosphorylation and activation of IRF3 in cultured cells.

To investigate whether the activity of kinases that phosphorylate IRF3 is also inhibited by MIP-T3, in vitro kinase assay was performed using TBK1 immunoprecipitated from HEK293T cells expressing increasing levels of MIP-T3 (Fig. 6B). Because TBK1 recovered from MIP-T3–expressing cells could still phosphorylate recombinant IκBα and GST-IRF3 to a similar extent as the enzyme prepared from cells that did not express MIP-T3 (Fig. 6B, lanes 1–3), the kinase activity of TBK1 is not inhibited by MIP-T3. Similar results were also obtained with another IRF3 kinase IκKε (data not shown). Thus, the perturbation of IRF3 phosphor-

*FIGURE 8.* MIP-T3 is dissociated from TRAF3 during the course of Sendai virus infection. HEK293T cells were transfected with Flag–TRAF3 and V5–MIP-T3. Forty-eight hours after transfection, cells were challenged with Sendai virus (80 hemagglutinating U/ml) for 0, 2, and 4 h. Infected cells were harvested, lysed, and immunoprecipitated with anti-Flag Ab. Composition of the immunoprecipitated TRAF3 complexes was analyzed by Western blotting.

**Depletion of MIP-T3 potentiates viral induction of IFN-β production**

The above experiments were all conducted with overexpression assays (Figs. 1–6). To verify our findings in a loss-of-function experiment, we used MIP-T3–targeting siRNAs to deplete endogenous MIP-T3 in HEK293 and MEFs (Fig. 7). To control for nonspecific effects induced by siRNA in general, an siRNA against GFP (siGFP) was also used. In addition, to minimize the nonspecific effects caused by any particular siRNA targeting MIP-T3, two independent MIP-T3–depleting siRNAs targeting different regions of MIP-T3 were used in each experiment. siRNA-transfected cells were further challenged with Sendai virus. The activity of IFN-β–Luc reporter was significantly induced by Sendai virus (Fig. 7A, column 4 compared with column 1). Notably, this induction was further augmented in cells transfected with MIP-T3–targeting siRNAs (Fig. 7A, columns 5 and 6 compared with column 4). The augmentation observed was statistically significant ($p < 0.05$ by Student t test). The two independent MIP-T3–depleting siRNAs (si3B and si3A) behaved in a similar manner, lending support to the specificity of the effect. The effectiveness of MIP-T3 knockdown was verified by semiquantitative RT-PCR and Western blotting using MIP-T3 specific primers and Abs, respectively (Fig. 7B, 7C).

To gain further insight into the physiologic role of MIP-T3 in normal fibroblasts, we also depleted the expression of MIP-T3 in MEFs using two siRNAs, which specifically target mouse MIP-T3 transcript. Real-time qPCR analyses indicated that these siRNAs were highly effective in dampening the expression of MIP-T3.
mRNA (Fig. 7D). Concurrently, the levels of IFN-β mRNA and protein in MIP-T3-compromised cells were escalated (Fig. 7E, 7F), and the escalation was found to be statistically significant (p < 0.05 by Student t test). Plausibly, this overproduction of type I IFNs in infected cells would compromise viral replication. To investigate this, we silenced the expression of MIP-T3 in MEFs and measured the replication of VSV-GFP. VSV-GFP was used in this experiment because the titer of live virus could be measured readily by plaque formation assay in Vero cells. Indeed, RNA interference (RNAi) depletion of MIP-T3 caused a significant reduction of the number of VSV-GFP plaques, supporting the negative regulatory role of MIP-T3 in antiviral response (Fig. 7G). Consistently, our results demonstrate that compromising the expression of endogenous MIP-T3 in established and primary fibroblasts further potentiates viral induction of IFN production leading to the attenuation of viral replication.

**MIP-T3 is dissociated from TRAF3 during the course of Sendai virus infection**

The effect of MIP-T3 knockdown on viral induction of IFN was moderate (Fig. 7A, 7E, 7F). This finding led us to the hypothesis that MIP-T3 might be inactivated during the course of viral infection. To investigate this and to shed further light on the biologic relevance of MIP-T3 in the context of viral infection, we monitored the dynamic changes in the composition of the TRAF3 complex in Sendai virus-infected HEK293T cells. Interestingly, the amounts of MIP-T3 communoprecipitated with TRAF3 were gradually diminished during the course of Sendai virus infection (Fig. 8, lanes 2 and 3 compared with lane 1). Concurrently and in keeping with the essential role of TRAF3–IRF3 complex in viral activation of innate immunity, the relative amounts of both phosphorylated IRF3 and TRAF3-associated IRF3 increased with time (Fig. 8, lanes 2 and 3 compared with lane 1). Thus, the dissociation of MIP-T3 from TRAF3 accompanied by an enhanced association of IRF3 with TRAF3 and enhanced phosphorylation of IRF3 in virus-infected cells might play an important role in viral activation of IFN response.

**Discussion**

Type I IFNs are important components of innate immunity, which combats microbial infections at the front line (44, 56, 57). To ensure a rapid but transient response, the production of type I IFN is tightly regulated. The identification of MIP-T3 as a TRAF3 binding partner was made in 2000 (17). Although there is increasing evidence to indicate a critical role of TRAF3 in the regulation of innate type I IFN response (4, 5), how TRAF3 function is influenced by its binding proteins remains largely unknown. In this study, we provided several lines of evidence to support the conclusion that MIP-T3 functions as a negative regulator of type I IFN production. First, overexpression of MIP-T3 suppressed RIG-I/MDA5- and TBK1/IKKe-induced ISRE and IFN-β promoter activity (Fig. 1). Second, MIP-T3 interacted specifically with TRAF3 (Fig. 2) and prevented it from forming protein complexes with downstream transducer and effector proteins (Figs. 4, 5). Third, MIP-T3 inhibited RIG-I– and TBK1-induced IRF3 phosphorylation in cultured cells without affecting the IRF3 kinase activity (Fig. 6). Fourth, siRNA-mediated depletion of MIP-T3 augmented viral induction of IFN production and compromised viral replication in normal fibroblasts (Fig. 7). Finally, MIP-T3 was dissociated from TRAF3 during the course of Sendai virus infection (Fig. 8).

Negative regulators of type I IFN production have been well described (58). PIN1 and LGP2 have been suggested to downregulate type I IFN production at IRF and RIG-I–like receptor levels, respectively (59–61), although a positive regulatory role of laboratory of genetics and physiology 2 upstream of RIG-I and MDA5 has also been reported recently (62). Deubiquitinases DUBA (11) and OTUB1/2 (10) as well as ubiquitin ligases cIAP1/2 (12) and Triad3A (9) have been shown to be negative regulators of innate immunity by modulating degradative and nondegradative polyubiquitination of TRAF3. Other deubiquitinases and ubiquitin ligases such as A20, TAX1BP1 (63, 64), CYLD (65), and RNF125 (66) have also been thought to inhibit innate immunity by targeting other transducers and effectors such as RIG-I, TBK1, and IRF3. In addition, a newly identified inhibitor of IFN production named optineurin is also a ubiquitin-binding protein that likely fulfills its repressive role by modulating ubiquitination (67). However, the formation of functional TRAF3-containing signaling complexes has been shown to be targeted by various viral proteins (13–15). In this study, we characterized MIP-T3 to be a cellular inhibitor of type I IFN production that impedes TRAF3 complex formation (Fig. 4). MIP-T3 is unique among cellular inhibitors of innate immunity, because it appears to affect TRAF3 ubiquitination only slightly (Fig. 3) but is capable of preventing TRAF3 from engaging downstream transducers (Fig. 4). This finding indicates that both cellular and viral inhibitors of IFN production might function by the same mechanism of modulating the formation of TRAF3 complexes. It will be of great interest to understand whether MIP-T3 competes with selected TRAF3-interacting proteins by preoccupying the same binding domains in TRAF3 with higher affinity, speed, or stability.

Although the knockdown of MIP-T3 by RNAi further enhanced viral induction of IFNs (Fig. 7), the potentiating effect was not as dramatic as in the case of other known cellular negative regulators of IFN production such as PIN1 (59), DUBA (11), and mitofusin proteins (68–70). This finding might be explained by the incomplete knockdown of MIP-T3 as seen in Fig. 7B–D. Alternatively, for viral activation of IFN production, MIP-T3 might already be inactivated. Therefore, further knockdown of MIP-T3 might not have a substantial effect on top of viral induction. Consistent with this, we demonstrated the dissociation of MIP-T3 from TRAF3 during the course of Sendai virus infection (Fig. 8), which lent further support to the notion that MIP-T3 is biologically important in viral activation of IFN production.

MIP-T3 is a ciliary protein required for ciliogenesis and intraflagellar transport (18–21). Particularly, MIP-T3 has been suggested to be a new component of intraflagellar transport subcomplex B (71). Primary cilia are ubiquitous in mammalian cells, and they fulfill important functions in cell motility in addition to cell signaling and homeostasis (72, 73). In particular, primary cilia serve as a platform for integration of the hedgehog signaling pathway and might also regulate platelet-derived growth factor receptor-α and Wnt signaling (72, 74). Thus, it will not be too surprising if the signal transduction process leading to type I IFN production might also take place at the primary cilia. Of note, some viruses such as influenza A virus and severe acute respiratory syndrome coronavirus preferentially infected apical ciliated side of the respiratory and gastrointestinal epithelia (75, 76). It is therefore intriguing to elucidate whether MIP-T3 and MIP-T3–binding proteins concentrated in the cilia might facilitate viral infection by compromising innate immunity.

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Disclosures

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