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# TMED2 Potentiates Cellular IFN Responses to DNA Viruses by Reinforcing MITA Dimerization and Facilitating Its Trafficking

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## SUMMARY

**Mediator of IRF3 activation (MITA), also known as stimulator of interferon genes (STING), plays a vital role in the innate immune responses to cytosolic dsDNA. The trafficking of MITA from the ER to perinuclear vesicles is necessary for its activation of the downstream molecules, which lead to the production of interferons and pro-inflammatory cytokines. However, the exact mechanism of MITA activation remains elusive. Here, we report that transmembrane emp24 protein transport domain containing 2 (TMED2) potentiates DNA virus-induced MITA signaling. The suppression or deletion of TMED2 markedly impairs the production of type I IFNs upon HSV-1 infection. TMED2-deficient cells harbor greater HSV-1 load than the control cells. Mechanistically, TMED2 associates with MITA only upon viral stimulation, and this process potentiates MITA activation by reinforcing its dimerization and facilitating its trafficking. These findings suggest an essential role of TMED2 in cellular IFN responses to DNA viruses.**

## INTRODUCTION

The cyclic GMP-AMP synthase (cGAS)-mediator of IRF3 activation (MITA) signaling pathway is a vital process for sensing aberrant cytosolic DNA and initiating the production of type I interferon (IFN) and pro-inflammatory cytokines (Barber, 2014, 2015; Chen et al., 2016b). As cytosolic DNA can originate from invading pathogens, such as viruses or certain bacteria, or can be self-DNA that escapes from the mitochondria or nucleus, the cGAS-MITA pathway plays important roles in not only antimicrobial immune responses but also tumorigenesis and diseases related to mitochondrial or nuclear damage (Ahn et al., 2014; Rongvaux et al., 2014; West et al., 2015; White et al., 2014).

As a central cytosolic DNA sensor, cGAS detects aberrant cytosolic DNA, and catalyzes the generation of cyclic dinucleotide cGMP-AMP (cGAMP) (Sun et al., 2013; Wu et al., 2013). In turn, cGAMP functions as a secondary messenger to activate the downstream adaptor MITA. MITA, also known as stimulator of interferon genes (STING) and endoplasmic reticulum (ER) IFN stimulator (ERIS), is a transmembrane protein that is predominantly localized to the ER (Ishikawa and Barber, 2008; Ishikawa et al., 2009; Ran et al., 2014; Sun et al., 2009; Zhong et al., 2008, 2009; Zhou et al., 2014); upon association with cGAMP, MITA translocates from the ER through the Golgi apparatus to perinuclear microsomal compartments (Dobbs et al., 2015; Fu et al., 2017; Ishikawa and Barber, 2008; Ishikawa et al., 2009; Luo et al., 2016). Because treating cells with brefeldin A, an inhibitor that specifically disrupts protein transport from the ER to the Golgi, abrogates cGAS-MITA signaling in cells, MITA trafficking from the ER to the proper organelles is considered critical for its function (Konno et al., 2013). During trafficking, MITA recruits TANK-binding kinase 1 (TBK1), which in turn phosphorylates MITA. In perinuclear microsomal compartments, phosphorylated MITA recruits interferon regulatory factor 3 (IRF3), which leads to the phosphorylation of IRF3 by TBK1. Activated IRF3 further undergoes dimerization and translocates to the nucleus. As a result, the transcription of type I IFNs and other pro-inflammatory cytokines is triggered (Chen et al., 2016a; Lei et al., 2010; Saitoh et al., 2009; Tanaka and Chen, 2012). Despite numerous reports on the post-transcriptional regulation of cGAS and MITA, the detailed molecular events in the cGAS-MITA pathway remain unclear. Importantly, how activated MITA in the ER is picked up and designated for perinuclear microsomal compartments remain issues to be addressed.

The transmembrane emp24 domain/p24 (TMED) family is a group of proteins that participate in vesicle transport in the cytoplasm. The vertebrate TMED family contains 10 members that can be classified into four subfamilies based on sequence homology (Dominguez et al., 1998; Sohn et al., 1996; Strating and Martens, 2009). The deficiency of several TMED members in yeast leads to the failure of secretory vesicle formation, and TMED knockout in mice is embryonic lethal (Denzel et al., 2000; Jerome-Majewska et al., 2010; Marzioch et al., 1999). It has been reported that TMEDs link

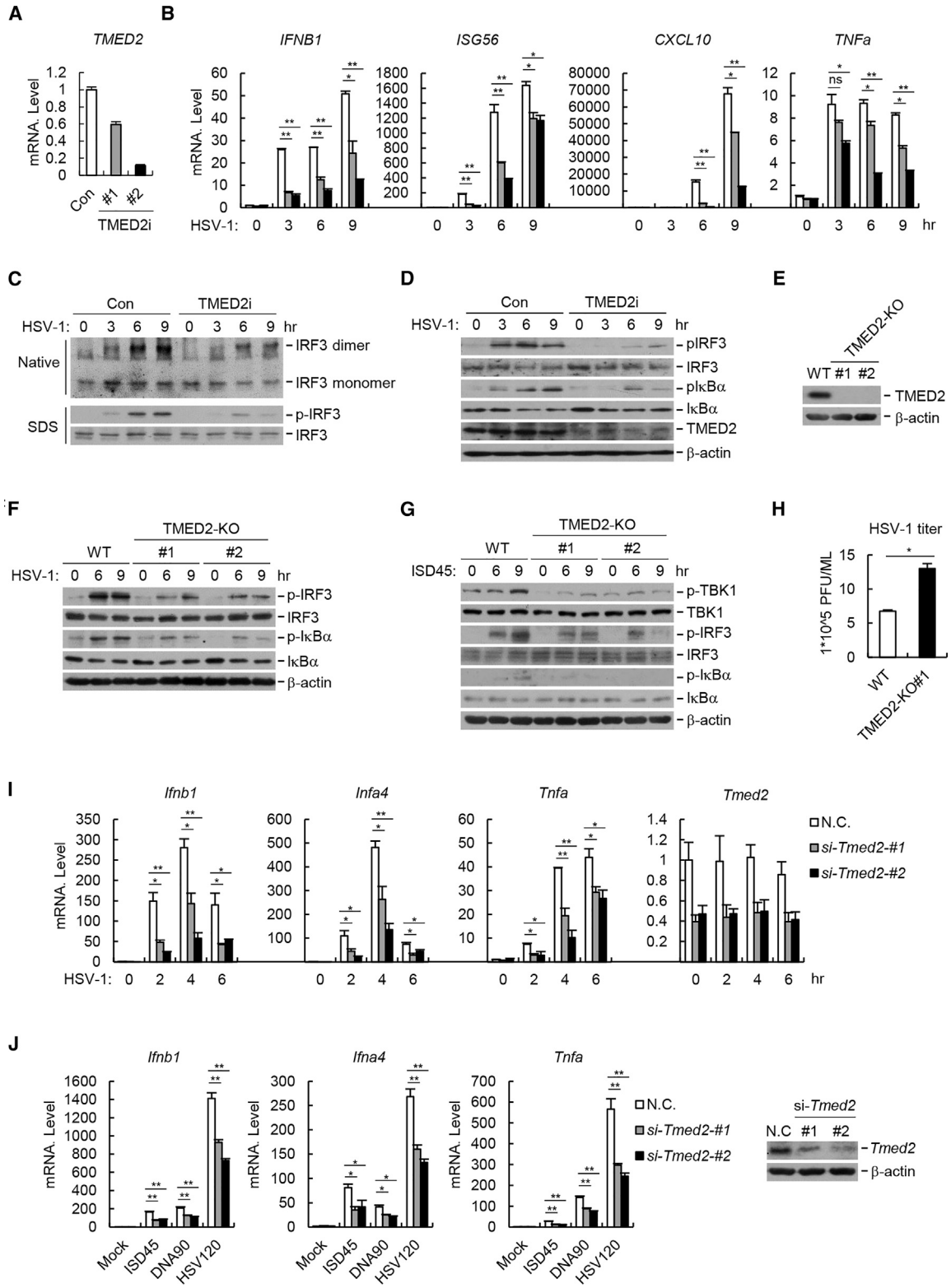



**Figure 1. TMED2 Is Important for Exogenous Cytosolic DNA-Induced Innate Immune Responses**

(A) *TMED2* mRNA expression in stable TMED2 knockdown THP-1 cells was analyzed by qPCR.

(B) qPCR analysis of the indicated gene mRNA levels in stable TMED2 knockdown THP-1 cells infected with HSV-1 for the indicated time.

*(legend continued on next page)*

glycosylphosphatidylinositol-anchored proteins to coat protein complex II (COP II), which mediates the anterograde trafficking of eukaryotic cargo proteins from the ER to the Golgi (Castillon et al., 2009; Takida et al., 2008). In addition, TMEDs have been reported to regulate innate immune signaling. TMED1 is required for optimal IL-33 signaling because it interacts with the IL-33 receptor ST2L (Connolly et al., 2013). TMED7 facilitates myeloid differentiation marker 88-dependent TLR4 signaling by promoting TLR4 translocation from the Golgi to the cell surface (Liaunardy-Jopeace et al., 2014). Furthermore, TMED7 inhibits myeloid differentiation marker 88-independent TLR4 signaling by disrupting signalosome in late endosomes (Doyle et al., 2012).

To search for new MITA signaling participants, we performed a library screening and identified TMED2 as a positive regulator of MITA. TMED2 deficiency impaired aberrant cytosolic DNA-induced cGAS-MITA signaling and reduced the production of type I IFNs and pro-inflammatory cytokines in response to DNA viruses. Mechanistically, TMED2 interacts with MITA upon HSV-1 infection, and reinforces MITA dimerization, translocation into the ER, trafficking to the Golgi, and the consequent TBK1 and IRF3 recruitment and phosphorylation. These findings expand our knowledge on the molecular mechanisms of MITA-IFN signaling.

## RESULTS

### TMED2 Is Important for Exogenous Cytosolic DNA-Induced Innate Immune Responses

To identify potential molecules involved in the cGAS-MITA pathway, we screened ~10,000 independent human cDNA expression plasmids for their ability to regulate the activation of interferon-stimulated response element (ISRE) by luciferase assays. These efforts led to the identification of human TMED2 as one of the candidates, as TMED2 overexpression potentiated the cGAS-MITA-mediated activation of ISRE (Figure S1A).

To confirm the role of TMED2 in antiviral innate immune responses, we constructed two RNAi plasmids for human TMED2 (TMED2i) and established THP-1 cell lines stably expressing TMED2i. The efficiencies of RNAi knockdown were determined by qRT-PCR analysis. As shown in Figure 1A, the TMED2i-#1 plasmid inhibited the TMED2 level to 60% of the control cells, whereas the TMED2i-#2 plasmid markedly inhibited the TMED2 level to 10%. Using these two stable cell lines, we quantified the transcription of *IFNB1*, *IFN-stimulated gene 56* (*ISG56*), and the pro-inflammatory cytokine genes *C-X-C motif chemokine ligand 10* (*CXCL10*) and *tumor necrosis factor α* (*TNFA*) upon Herpes simplex virus 1 (HSV-1, belonging to DNA virus) and Sendai virus (SeV, belonging to RNA virus) infection. Both RNAi cell lines showed significantly reduced transcription of *IFNB1*, *ISG56*, *CXCL10*, and *TNFA* in response to HSV-1 compared with the control cells (Figure 1B). The suppression degree correlated with the knockdown efficiency of the corresponding RNAi plasmid. However, the transcription of these cytokine genes induced by SeV was not markedly affected by TMED2 knockdown (Figure S1B). Moreover, TMED2 knockdown markedly suppressed the dimerization of IRF3 induced by HSV-1 but not SeV (Figures 1C and S1C). Consistently, the phosphorylation of IRF3 or IκBα upon HSV-1 but not SeV infection was impaired by TMED2 knockdown (Figures 1D and S1D), suggesting that TMED2 functions specifically in the DNA virus-induced cGAS-MITA pathway.

We further generated TMED2-deficient THP-1 cells using CRISPR-Cas9 technology. Two independent TMED2-deficient clones were obtained and confirmed (Figure 1E). TMED2-deficient cells were infected with HSV-1 or transfected with a nucleic acid mimic, a 45-bp interferon stimulatory dsDNA lacking CpG sequence (ISD45). The results showed that TMED2 deficiency impaired the phosphorylation of not only IRF3 and IκBα but also TBK1 (Figures 1F and 1G), suggesting that TMED2 acts upstream of TBK1. In plaque assays with HSV-1 infection, markedly higher HSV-1 titers were produced in TMED2-deficient THP-1 cells than the wild-type (WT) control cells (Figure 1H), further demonstrating that TMED2 is important for innate immune responses against aberrant cytosolic DNA.

We then determined the role of mouse homolog Tmed2 in mouse lung fibroblasts (MLFs). MLFs were transfected with two small interfering RNAs (siRNAs) against Tmed2 and then infected with HSV-1 or transfected with ISD45, 90 bp dsDNA (DNA90), or 120 bp HSV genomic dsDNA (HSV120). Tmed2 knockdown significantly inhibited the transcription of the cytosolic DNA-induced cytokines *Ifnb1*, *Ifna4*, and *Tnfa* (Figures 1I and 1J), suggesting that mouse Tmed2 plays a similar role to its human homolog.

### TMED2 Targets MITA

Because TMED2 deficiency impaired the phosphorylation of TBK1, we further examined the molecular place of TMED2 in the IFN-I-inducing pathway. Reducing TMED2 expression by RNAi inhibited cGAS-MITA-induced ISRE activation but hardly affected TBK1-induced ISRE activation (Figure 2A). Moreover, TMED2 knockdown had no marked effect on ISD45-induced production of cGAMP, which is catalyzed by cGAS (Figure 2B). In addition, TMED2 knockdown had no marked effect on the expression level of cGAS upon HSV-1 infection (Figure S2A). These findings suggest that TMED2 functions upstream of TBK1 and downstream of cGAS. The co-immunoprecipitation

(C and D) Stable TMED2 knockdown THP-1 cells were infected with HSV-1 for the indicated time. Cell lysates were separated by native (upper panel of C) and SDS (bottom panels of C and D) PAGE and analyzed by immunoblotting with the indicated antibodies.
(E) TMED2-deficient THP-1 cells were generated by CRISPR/Cas9 technology and verified by immunoblots.
(F and G) TMED2-deficienct THP-1 cells were infected with HSV-1 (F) or transfected with ISD45 (2 μg/mL) (G) and analyzed by immunoblotting with the indicated antibodies.
(H) TMED2-deficient THP-1 cells were infected with HSV-1 (MOI = 1) for 36 hr. Supernatants were then collected for plaque assays to determine the viral titer.
(I and J) MLFs transfected with negative control (N.C.) or *Tmed2* siRNAs were infected with HSV-1 (I) or transfected with ISD45 (2 μg/mL), DNA90 (2 μg/mL), or HSV120 (2 μg/mL) (J) for the indicated time before qPCR analysis. The graphs show mean ± SD, n = 3. *p < 0.05; **p < 0.01, ns, no significance.
See also Figure S1.

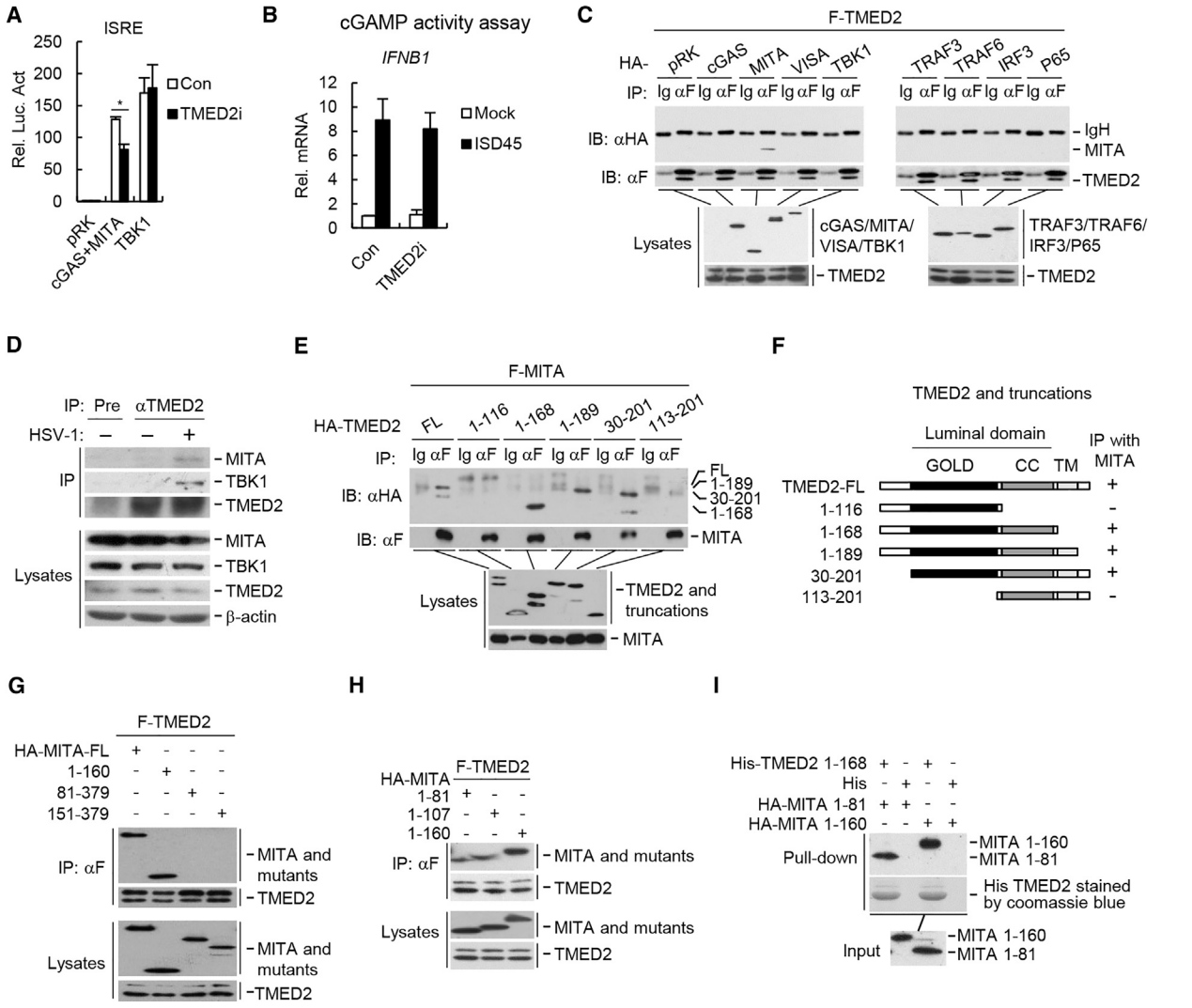



**Figure 2. TMED2 Targets MITA**

(A) Dual-luciferase reporter assays were performed using HEK293 cells transfected with the indicated plasmids. The graphs show mean ± SD, n = 3. *p < 0.05; **p < 0.01.

(B) Stable TMED2 knockdown THP-1 cells and control cells were left untreated or treated with ISD45 (4 μg/mL) for 6 hr, then cell extracts containing cGAMP were delivered to digitonin-permeabilized THP-1 cells for 4 hr before qPCR.

(C) HEK293 cells were transfected with Flag-tagged TMED2 (F-TMED2) and HA-tagged plasmids as indicated followed by co-immunoprecipitation and immunoblotting analysis.

(D) THP-1 cells were infected with HSV-1 for 6 hr and then subjected to co-immunoprecipitation with mouse serum (pre) or mouse anti-TMED2 antiserum (αTMED2).

(E and F) HEK293 cells were transfected with F-MITA and HA-TMED2 or its truncations followed by co-immunoprecipitation and immunoblotting analysis (E), and the results were shown in the schematic diagram (F).

(G and H) HEK293 cells were transfected with F-TMED2 and full length of HA-MITA (G) or MITA truncations (residue 1–160, 81–379, and 151–379 for G; residue 1–81, 1–107, and 1–160 for H) followed by co-immunoprecipitation and immunoblotting analysis.

(I) Bacterially expressed His-tagged TMED2 truncation (His-TMED2) was coupled to BeaverBeads, followed by incubation with HEK293 cell extracts expressing F-MITA truncation. Proteins bound to beads were eluted by boiled with SDS loading buffer, and analyzed by immunoblotting or Coomassie blue staining as indicated.

See also Figure S2.

assay results showed that TMED2 interacted specifically with MITA, but not with other key components in the IFN-I-inducing pathway, such as cGAS, TBK1, TRAF3, TRAF6, IRF3, p65, or virus-induced signaling adaptor (VISA) (Figure 2C). No obvious interactions were detected between MITA and TMED1, TMED3, TMED4, or TMED5, suggesting a specific role for TMED2 in MITA signaling (Figure S2B). Indeed, when we reduced the expression of TMED1 or TMED3 in THP-1 cells, neither IRF3

phosphorylation nor the HSV-1 titer changed compared with those in the control cells upon HSV-1 infection (Figures S2C–S2E). We then performed endogenous co-immunoprecipitation assays to examine the time course of the TMED2-MITA interaction. Endogenous TMED2 did not interact with MITA until the cells were infected with HSV-1 for 6 hr (Figure 2D). Moreover, the antibody against TMED2 pulled down TBK1 upon HSV-1 infection, suggesting that TMED2 becomes a component of the MITA-TBK1 complex only after HSV-1 stimulation (Figure 2D).

TMED2 is a type I membrane protein that contains a transmembrane (TM) domain, a coil-coiled (CC) domain, and a Golgi dynamics (GOLD) domain. The GOLD domain is a β strand-rich domain involved in Golgi dynamics and intracellular protein trafficking (Carney and Bowen, 2004; Luo et al., 2007; Strating and Martens, 2009). Both the CC domain and the GOLD domain of TMED2 extrude to the vesicle lumen. To determine the minimal regions of TMED2 and MITA responsible for their interaction, we constructed truncation plasmids of TMED2 or MITA, and performed co-immunoprecipitation assays. The results showed that the luminal part of TMED2 containing the GOLD and CC domain interacted with MITA, and TMED2 truncations lacking either the GOLD domain or the CC domain failed to interact with MITA (Figures 2E and 2F); these data suggested that both the GOLD and CC domain are required for the interaction between TMED2 and MITA. Domain mapping assays also revealed that the N-terminal TM1–TM2 fragment of MITA (residue 1–81) interacted with TMED2 (Figures 2G, 2H, and S2F). Consistently, the truncated MITA lacking TM1–TM2 (residue 81–379) failed to interact with TMED2. Moreover, the interaction of TMED2 with the intact TM1–TM4 domain of MITA (residue 1–160) was stronger than that with the TM1–TM2 or TM1–TM3 of MITA (residue 1–81 or 1–107) (Figures 2G, 2H, and S2F), suggesting that TM3 and TM4 domain of MITA reinforce the basic association of TM1–TM2 of MITA with TMED2. To confirm this interaction, we performed an *in vitro* binding assay. Bacterially expressed and affinity-purified His-TMED2 truncation (residue 1–168) protein was incubated with ectopic HA-MITA truncation-expressing whole-cell extracts. As shown in Figure 2I, both MITA truncations were pulled down by His-TMED2. Because only short linker region of MITA between predicted TM1 and TM2, or TM3 and TM4, protrudes into the luminal domain, we then mutated the possible key amino acid LAS in the luminal side loop1 between TM1 and TM2, and the amino acid AVGPPF in the luminal side loop2 between TM3 and TM4 into alanines. The mutant with LAS to AAA was designated as MITA-mut1, with AVGPPF to AAAAAA was designated as MITA-mut2, and with both LAS and AVGPPF was designated as MITA-mut1/2. The immunoprecipitation assay results showed that the interactions between MITA mutants and TMED2 were much weaker than the interaction between WT MITA and TMED2 (Figure S2G). In addition, MITA-mut1/2 could hardly activate the ISRE reporter, and MITA-mut1 or MITA-mut2 had less effect than that of WT MITA in cGAS-mediated ISRE activation (Figure S2H), suggesting that the luminal loops between transmembrane domains of MITA are indeed important for MITA functions. All these data indicate that TMED2 targets MITA, and the association between TMED2 and MITA occurs in the lumen of ER.

## TMED2 Reinforces the Dimerization of MITA upon HSV-1 Infection

MITA dimerization is required for the subsequent downstream signaling (Sun et al., 2009; Tsuchida et al., 2010; Wang et al., 2016). HSV-1, but not SeV, infection reinforced the dimerization of MITA. However, TMED2 knockdown inhibited the HSV-1-induced dimerization of MITA (Figures 3A, 3B, and S3A). Furthermore, HSV-1, but not SeV, infection triggered the recruitment of TBK1 and IKKβ to MITA (Figure 3C). The depletion of TMED2 markedly impaired the recruitment of TBK1 and IKKβ to MITA, suggesting that TMED2 is important for the assembly of the MITA signalosome (Figure 3D). Phosphorylation of MITA in Ser366 by recruited TBK1 is critical for the activation of IRF3 (Liu et al., 2015; Tanaka and Chen, 2012). In stable TMED2 knockdown cells, the phosphorylation of MITA in Ser366 by HSV-1 infection was much weaker than that in control THP-1 cells (Figure 3E). These results indicate that TMED2 regulates MITA activation by reinforcing MITA dimerization and recruiting downstream molecules. In cells under physiological conditions, TMED proteins exist as monomers, dimers, or oligomers (Fligge et al., 2000; Jenne et al., 2002). It has been reported that the dimerization of TMED proteins favors its interaction with COP complex proteins (Barr et al., 2001; Gommel et al., 1999; Strating and Martens, 2009). Our results using native-PAGE gels showed that HSV-1 infection promoted the oligomerization of TMED2 (Figures 3F and S3B). The luminal domain of TMED2 (residue 1–168), but not the GOLD domain only (residue 1–116), interacted with full-length TMED2 (Figure 3G). Consistently, the Flag-tagged luminal domain of TMED2 interacted with the HA-tagged luminal domain (Figure 3H), suggesting that TMED2 forms oligomers through its luminal domain. Moreover, the HSV-1-induced transcription of *Ifnb1* was potentiated by the truncations containing intact luminal domain (residue 1–168 and 1–189), but not the GOLD domain only (Figure 3I). All these results suggest that the oligomerization of TMED2, mediated by its luminal domain, is required for its function in cGAS-MITA signaling.

## TMED2 Facilitates Both the Translocation of MITA into the ER and the Trafficking of MITA out of the ER to the Golgi

Previous reports showed that translocon-associated protein β (TRAPβ) and the translocon subunit Sec61β associate with MITA and may be able to influence the induction of IFNs (Ishikawa and Barber, 2008; Ishikawa et al., 2009). Inactive rhomboid protein 2 (iRhom2) enhances the interaction between TRAPβ and MITA, thus promoting protein translocation into the ER following translation (Luo et al., 2016). Thus, we investigated whether TMED2 plays a role in MITA translocation.

We overexpressed TMED2 plasmids with MITA, iRhom2, TRAPβ, Sec61β, or Sec5, a component of the exocyst 8 subunit complex that participates in the vesicular transport process (Chien et al., 2006; Ishikawa et al., 2009). Immunoprecipitation results showed that TMED2 interacted with TRAPβ and iRhom2 but not Sec5 or Sec61β (Figure 4A). TMED2 knockdown statistically impaired the synergy effect of TRAPβ or iRhom2 on cGAS-MITA-mediated ISRE activation (Figure 4B). TMED2 promoted the association of MITA with

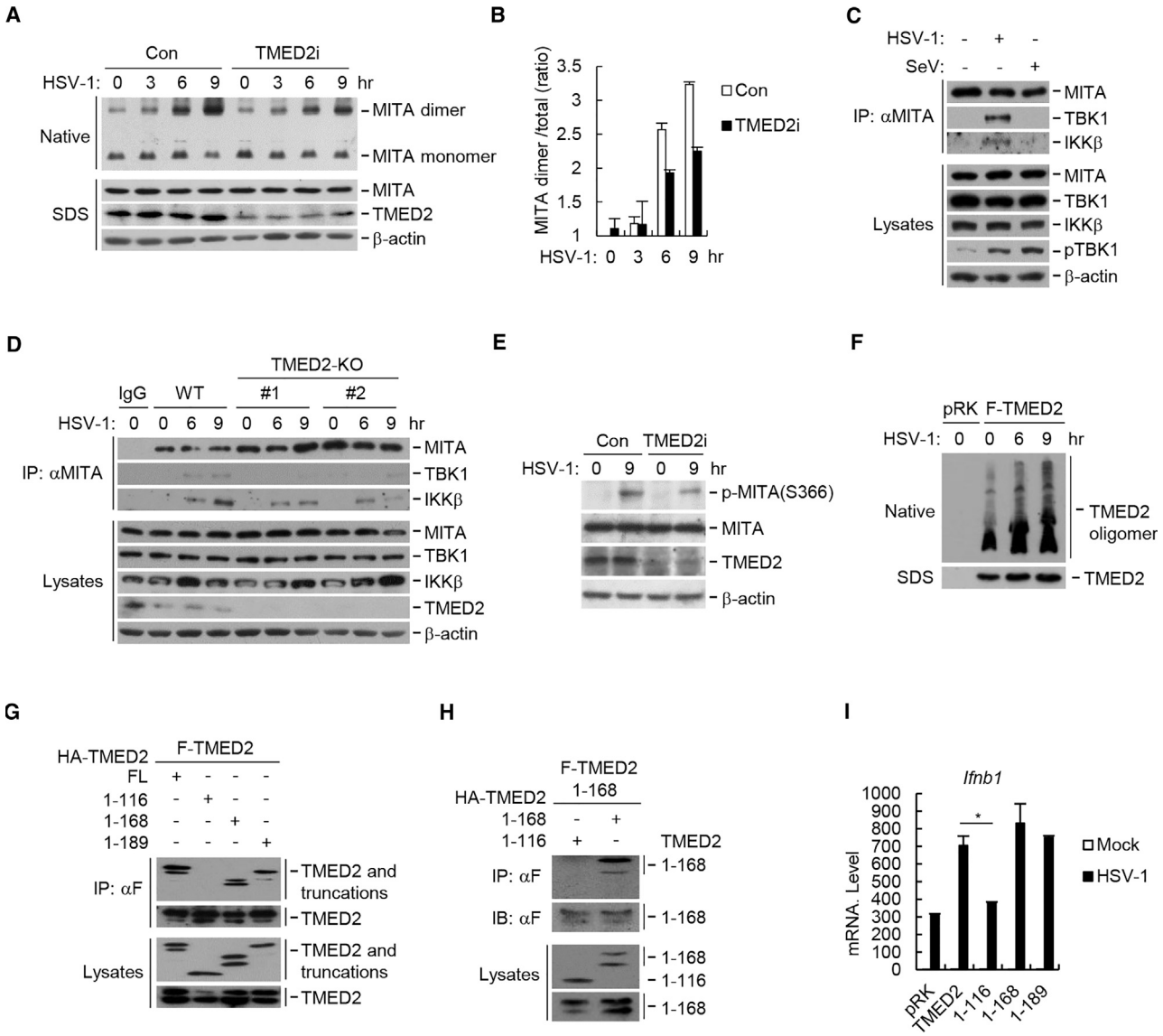



**Figure 3. TMED2 Reinforces the Dimerization of MITA upon HSV-1 Infection**

(A) Stable TMED2 knockdown THP-1 cells were infected with HSV-1 for the indicated time. Cell lysates were separated by native (top) or SDS (bottom) PAGE followed by immunoblotting analysis.

(B) The grayscale values of bands of MITA in (A) were quantified and shown as the ratio of MITA dimer to total amount in the histogram.

(C) THP-1 cells were infected with HSV-1 or SeV for 6 hr. Cell lysates were immunoprecipitated with anti-MITA and analyzed by immunoblotting.

(D) Stable TMED2-deficient THP-1 cells were infected with HSV-1 for the indicated time. Cell lysates were immunoprecipitated with anti-MITA followed by immunoblotting analysis.

(E) Stable TMED2 knockdown THP-1 cells were infected with HSV-1 for 9 hr. Cell lysates were analyzed by immunoblotting with the indicated antibodies.

(F) TMED2 stable expression THP-1 cells were infected with HSV-1 for the indicated time. Cell lysates were separated by native (top) or SDS (bottom) PAGE and analyzed by immunoblotting with the indicated antibodies.

(G and H) HEK293 cells were transfected with F-TMED2 and full length of HA-TMED2 (FL) (G) or TMED2 truncations (residue 1–116, 1–168, and 1–189 for G; residue 1–116 and 1–168 for H), followed by co-immunoprecipitation and immunoblotting analysis.

(I) qPCR analysis of *Ifnb1* mRNA levels in TMED2 truncation-transfected MLFs with HSV-1 infection. The graphs show mean ± SD, n = 3. *$p < 0.05$; **$p < 0.01$. See also Figure S3.

TRAPβ but had little effect on MITA-Sec5 or MITA-iRhom2 interaction (Figure 4C). Furthermore, TMED2 interacted with TRAPβ through its intact luminal domain (Figure 4D). These data suggested TMED2 facilitates the translocation of MITA to the ER by enhancing the interaction of MITA with translocon subunit TRAPβ.

As full MITA activation occurs in the perinuclear microsomal compartments, the proper trafficking of MITA from the ER

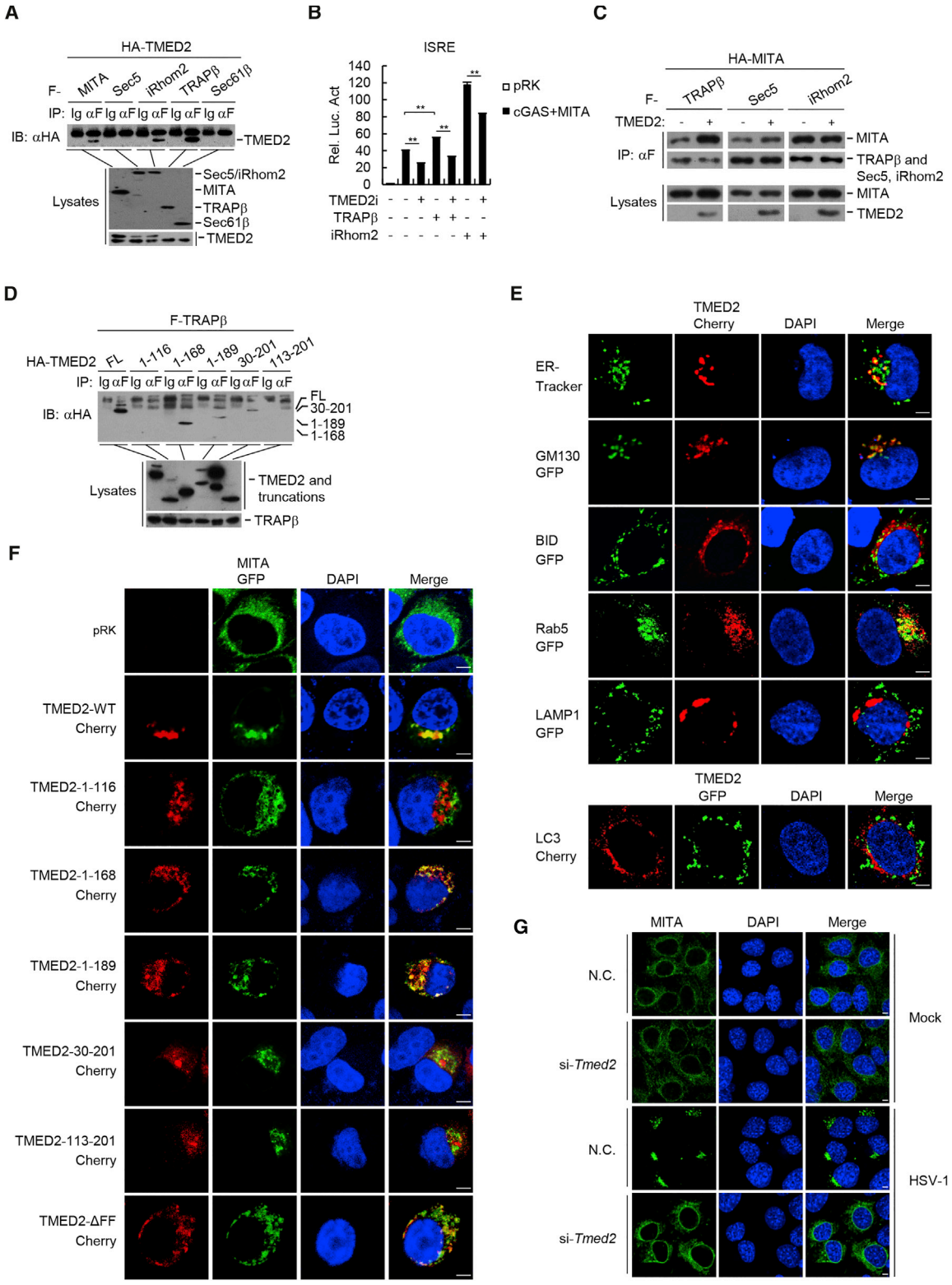



**Figure 4. TMED2 Facilitates Both the Translocation of MITA into the ER and the Trafficking of MITA out of the ER to the Golgi**

(A) HEK293 cells were transfected with the indicated plasmids and subjected to co-immunoprecipitation and immunoblotting.

(B) Stable TMED2-knockdown HEK293 cells were transfected with indicated plasmids followed by dual-luciferase reporter assays. The graphs show mean ± SD, n = 3. *p < 0.05; **p < 0.01.

*(legend continued on next page)*

through the Golgi to the perinuclear microsomal compartments is important. We next investigated whether TMED2 is involved in MITA trafficking. The results from the immunofluorescent assays showed that TMED2 colocalized with an ER tracker, the Golgi matrix protein GM130, and the endosomal marker Rab5 but not with the mitochondria marker BID, the lysosomal marker LAMP1, and the autophagosomal marker LC3 (Figure 4E). The TMED2 truncations containing the N-terminal signal peptide, such as fragment 1–116, 1–168, and 1–189, colocalized with ER tracker, whereas those truncations without N-terminal signal peptide (30–201 and 113–201) did not (Figure S4), suggesting that the N-terminal signal peptide of TMED2 is required for its correct localization. Cherry-tagged full length (FL) of TMED2, TMED2 1–168, and 1–189 truncation colocalized with GFP-MITA, whereas TMED2 1–116, 30–201, and 113–201 truncations did not (Figure 4F). These results further suggested that the N-terminal signal peptide (residue 1–29) and intact luminal region of TMED2 (residue 1–168) are required for the colocalization of TMED2 and MITA inside cells.

Moreover, GFP-MITA was dispersed in the cytosol, while co-transfection with full-length TMED2, but not any of the TMED2 truncations or deletion mutant lacking di-hydrophobic motif, led to MITA congregation in the perinuclear compartments (Figure 4F). HSV-1 infection also induced MITA congregation in the perinuclear compartments as reported before (Fu et al., 2017; Luo et al., 2016), whereas TMED2 suppression markedly inhibited HSV-1-induced MITA congregation (Figure 4G). These data suggested that TMED2 facilitates MITA trafficking from the ER through the Golgi apparatus to the perinuclear microsomal compartments.

## TMED2 Promotes the Recruitment of MITA into the COPII Complex

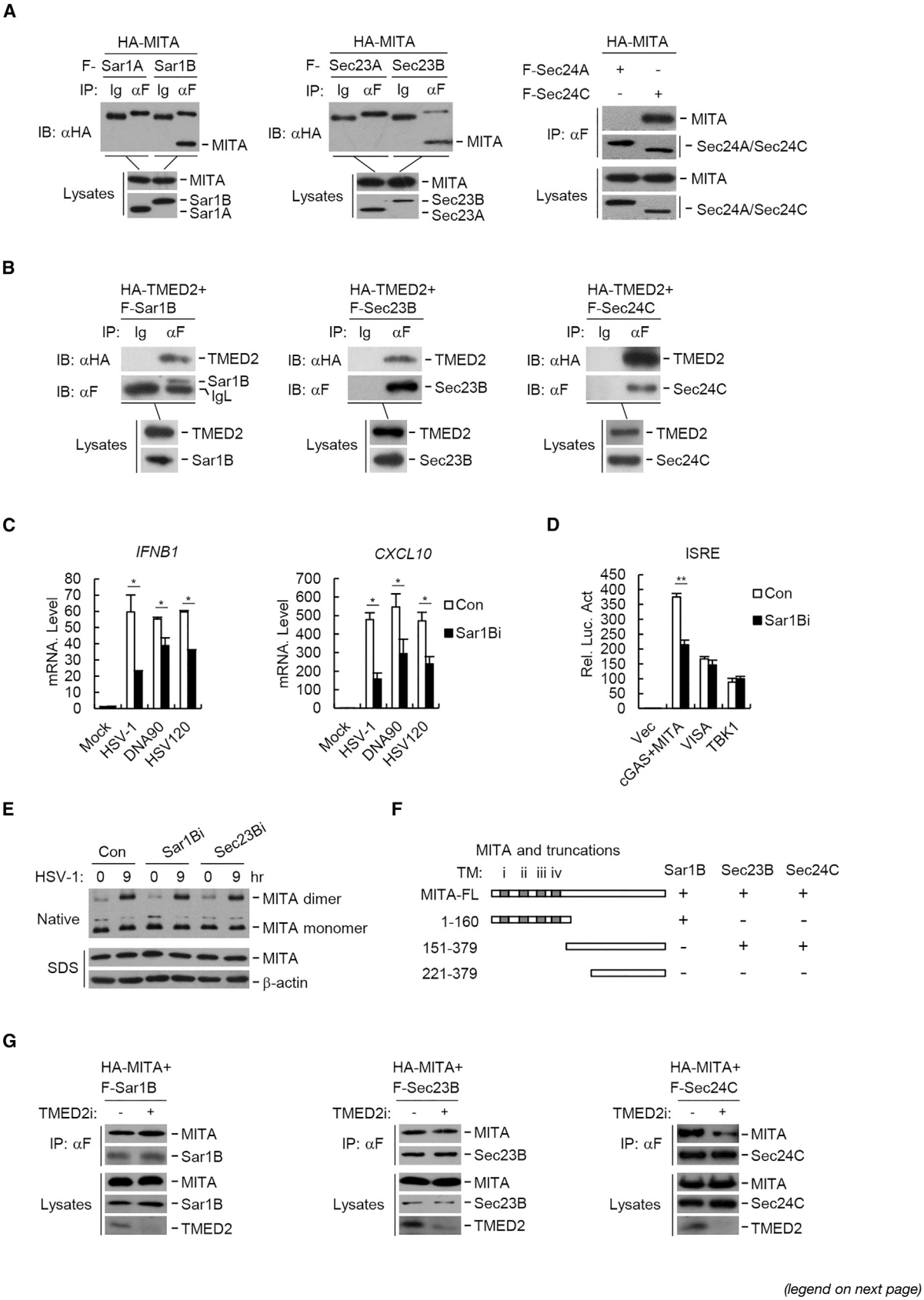
Secretory protein trafficking from the ER to the Golgi apparatus usually depends on the COPII complex to drive vesicle formation. COP II complex assembly starts with the insertion of a small GTPase, Sar1, to the ER. Then, activated Sar1 recruits the GTPase-activating protein Sec23. Sec24 is also recruited by interacting with Sec23 and is responsible for engaging cargo proteins. The Sec13-31 hetero-tetramer is further captured to establish COP II coat architecture and promotes COP II vesicle fission from the ER (Gürkan et al., 2006; Kuge et al., 1994; Schekman and Orci, 1996; Stagg et al., 2006).

To analyze the possible mechanism behind MITA trafficking, we first examined the role of COPII complex components in MITA signaling. Isoforms of Sar1, Sec23, and Sec24 were co-transfected with MITA or TMED2. The immunoprecipitation assay results showed that both MITA and TMED2 interacted specifically with Sar1B, Sec23B, and Sec24C but not with Sar1A, Sec23A, or Sec24A (Figures 5A and 5B), suggesting that TMED2 may form complexes with MITA, Sar1B, Sec23B, and Sec24C. Sar1B knockdown by RNAi statistically inhibited HSV-1-induced transcription of *IFNB1* and *CXCL10* in THP-1 cells (Figures 5C and S5A). Similar results were obtained with DNA90 or HSV120 transfection (Figure 5C). Furthermore, Sar1B knockdown inhibited cGAS and MITA-mediated, but not TBK1- or VISA-mediated, ISRE activation (Figure 5D), suggesting that Sar1, as the initiator of COP II complex formation, is required for IFN signaling, and it functions at the level of MITA. Unlike TMED2, knockdown of either Sar1B or Sec23B knockdown had no marked effect on the stabilization of MITA dimeric complex upon HSV-1 infection (Figure 5E). Domain mapping analyses indicated that Sar1B interacted with the transmembrane domain of MITA, while Sec23B and Sec24C interacted with the cytosolic domain of MITA (Figures 5F and S5B). TMED2 knockdown had no marked effect on the interaction of MITA with Sar1B or Sec23B, but clearly attenuated the interaction between MITA and Sec24C (Figure 5G). These findings suggested that TMED2 enhances the assembly of the cargo protein MITA into the COP II complex via strengthening the interaction between MITA and Sec24C. All these results indicate that the COP II complex is critical for MITA-mediated IFN signaling, and TMED2 mediates the recruitment of MITA to COP II complex.

## TMED10 Participates in MITA Signaling

It has been reported that TMED2 can form heterodimer with TMED7, TMED9, or TMED10 (Jenne et al., 2002; Jerome-Majewska et al., 2010; Strating and Martens, 2009). We then examined the possible role of these TMEDs in MITA signaling. Overexpressed TMED7 and TMED10, but not TMED9, amplified cGAS-MITA-mediated ISRE activation, although they were not as strong as TMED2 did (Figure 6A). Stable TMED7 or TMED10-knockdown cells were then established and infected with HSV-1. TMED10 knockdown, but not TMED7 knockdown, impaired the transcription of *CXCL10* induced by HSV-1 (Figures 6B and 6C), suggesting that TMED10 is involved in MITA signaling. However, no marked interaction between MITA and TMED10 were detected by co-immunoprecipitation assays (Figure 6D). Furthermore, the results from dual luciferase reporter assays showed that TMED2 knockdown inhibited the synergy effect of TMED10 on cGAS-MITA-mediated ISRE activation (Figure 6E), but TMED10 knockdown could hardly affect the synergy effect of TMED2 on cGAS-MITA-mediated ISRE activation (Figure 6F). These results suggested that TMED10, but not TMED7 or TMED9, participates in MITA signaling through TMED2, but its function is limited. Because cellular TMED2 exists as both

(C) HEK293 cells were transfected with HA-MITA and the indicated plasmids with or without TMED2. Cell lysates were subjected to co-immunoprecipitation and immunoblotting.
(D) HEK293 cells were transfected with F-TRAPβ and the indicated plasmids, followed by co-immunoprecipitation and immunoblotting.
(E) HeLa cells were transfected with Cherry-TMED2 and the indicated organelle markers, and observed under confocal microscopy. Scale bars represent 25 μm.
(F) HeLa cells were transfected with GFP-MITA and Cherry-TMED2 or TMED2 mutants, and observed under confocal microscopy. TMED2-ΔFF: TMED2 deletion mutant lacking di-hydrophobic motif (residue 194 and 195). Scale bars, 25 μm.
(G) *Mita*$^{-/-}$-MLFs reconstituted with MITA-Flag were transfected with *Tmed2* siRNAs for 48 hr before HSV-1 infection. The cells were stained with FITC-anti-mouse IgG, and observed under confocal microscopy. Scale bars, 25 μm.
See also Figure S4.

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homodimers and heterodimers, and HSV-1 infection reinforced the formation of TMED2 oligomers, we speculated that HSV-1-induced TMED2 homo-oligomer is the main functional form that participates in MITA signaling.

## DISCUSSION

Upon aberrant DNA stimulation, MITA, which is a critical adaptor, is delicately regulated to trigger a proper and controlled immune response. Many studies have focused on the various post-translational regulation of MITA, including phosphorylation and ubiquitination, but little is known about how the dimerization, translocation, and trafficking of MITA are triggered. In the present study, we identify TMED2 as an amplifier in anti-DNA virus immune responses by enhancing MITA-dependent IFN induction. Several pieces of biochemical evidence support our findings. First, TMED2 knockdown or knockout impairs HSV-1-induced IRF3 activation and subsequent IFN and inflammatory cytokine transcription. TMED2-knockout cells harbor more HSV-1 titer than WT cells. Second, TMED2 interacts specifically with MITA but not other key components in the DNA-sensing pathway, and this interaction is dependent on HSV-1 infection. Third, TMED2 knockdown impairs HSV-1-induced augment of MITA dimerization as well as the assembly of the MITA signalosome. Fourth, TMED2 promotes the translocation of MITA into the ER following translation by enhancing the interaction between MITA and TRAPβ. Last, TMED2 facilitates the trafficking of MITA from the ER to the Golgi by enhancing the association of MITA with the principle adaptor Sec24 for the COP II coat. We also provide the first evidence that the COP II coat is indeed critical for the MITA-mediated IFN-inducing pathway.

The TMED family plays ubiquitous roles in vesicular transport machinery (Strating and Martens, 2009). Deletions or mutations of Tmed genes in yeast and *Drosophila* results in ER-stress and activation of the unfolded protein response (Belden and Barlowe, 2001; Ma and Hendershot, 2004; Marzioch et al., 1999). Indeed, TMED2 knockdown enhances the phosphorylation of inositol-requiring protein 1α (IRE1α) and the expression level of BiP (Figures S5C and S5D). The TMED family also plays roles in innate immune signaling (e.g., TMED1 and TMED7 are involved in IL-33 and TLR4 signaling, respectively) (Connolly et al., 2013; Doyle et al., 2012; Liaunardy-Jopeace et al., 2014). In the present study, TMED2, but not TMED1, TMED3, TMED7, and TMED9, participates in MITA signaling to control HSV-1 infection. TMED2 knockdown impairs the HSV-1-induced, but not SeV-induced, transcription of *IFNB1* and inflammatory cytokines. TMED2 knockout cells harbor more HSV-1 titer than control cells. TMED2 knockdown impairs the phosphorylation of MITA, TBK1, and IRF3, but has no marked effect on the production of cGAMP and the TBK1-induced activation of ISRE, suggesting that TMED2 functions downstream of cGAMP and upstream of TBK1.

TMED2 is a transmembrane protein located in multiple cellular locations, including the ER, Golgi, and endosomes. HSV-1 infection induces the association of endogenous TMED2 with MITA. TMED2 knockdown impairs HSV-1-induced augment of MITA dimerization and subsequent recruitment of TBK1 and IKKβ. Interestingly, the intact luminal domain of TMED2, but not its TM domain is responsible for the interaction with MITA. Consistently, the intact luminal domain of TMED2 is important for HSV-1-induced production of IFNs and the oligomerization of TMED2. These results indicate that the oligomerization of TMED2 through its luminal domain promotes the spatial gathering of MITA, and thus reinforces the dimerization of MITA. This interference is further supported by our immunofluorescent assay results that TMED2 overexpression changes the spatial distribution of MITA from dispersion to congregation.

Several groups reported that the translocon-related molecules TRAPβ, Sec61β, and iRhom2 regulate MITA signaling (Ishikawa and Barber, 2008; Ishikawa et al., 2009; Luo et al., 2016). TMED2 interacts with TRAPβ through its luminal domain as well, and promotes the association of MITA and TRAPβ. These findings indicate another function of TMED2—its facilitation MITA translocation from the ribosome into the ER through strengthening the interaction of MITA with translocon. In addition, we also tested the relation of TMED2 with other two positive regulators in MITA signaling, ZDHHC1 and ZDHHC11, both of which are members of DHHC palmitoyl transferase family. TMED2 knockdown impaired the synergy effect of ZDHHC11, but not ZDHHC1, on cGAS-MITA-mediated ISRE activation (Figure S5E). As the detail role of either ZDHHC11 or ZDHHC1 is still obscure, we can only infer that ZDHHC11 is possibly involved in the translocation of MITA from the ribosome into the ER, and TMED2 plays its role downstream of ZDHHC11 and upstream of ZDHHC1.

HSV-1 infection triggered the trafficking of MITA from the ER through the Golgi to the perinuclear regions. In the perinuclear regions, the transcription factor IRF3 is recruited to MITA and further activated (Dobbs et al., 2015; Ishikawa et al., 2009; Luo et al., 2016). Thus, the trafficking process of MITA is critical for the signaling cascade. Evidence that treatment with brefeldin A abrogates DNA virus-induced IFN production also demonstrate the importance of MITA trafficking (Konno et al., 2013). The

**Figure 5. TMED2 Promotes the Recruitment of MITA into the COP II Complex**

(A and B) The interaction of MITA (A) or TMED2 (B) with the indicated component of COPII complex was examined by co-immunoprecipitation and immunoblotting.

(C) Stable Sar1B-knockdown THP-1 cells were infected with HSV-1 or transfected with DNA90 (2 μg/mL) or HSV120 (2 μg/mL) for 6 hr before qPCR.

(D) Dual-luciferase reporter assays were performed in HEK293 cells transfected with indicated plasmids.

(E) Stable Sar1B or Sec23B knockdown THP-1 cells were infected with HSV-1 for the indicated time. Cell lysates were separated by native (top) or SDS (bottom two panels) PAGE and analyzed by immunoblotting with the indicated antibodies.

(F) Mapping of the interaction between MITA truncations and Sar1B, Sec23B, or Sec24C.

(G) Stable TMED2 knockdown HEK293 cells were transfected with the indicated plasmids and subjected to co-immunoprecipitation and immunoblotting. The graphs show mean ± SD, n = 3. *$p < 0.05$; **$p < 0.01$.

See also Figure S5.

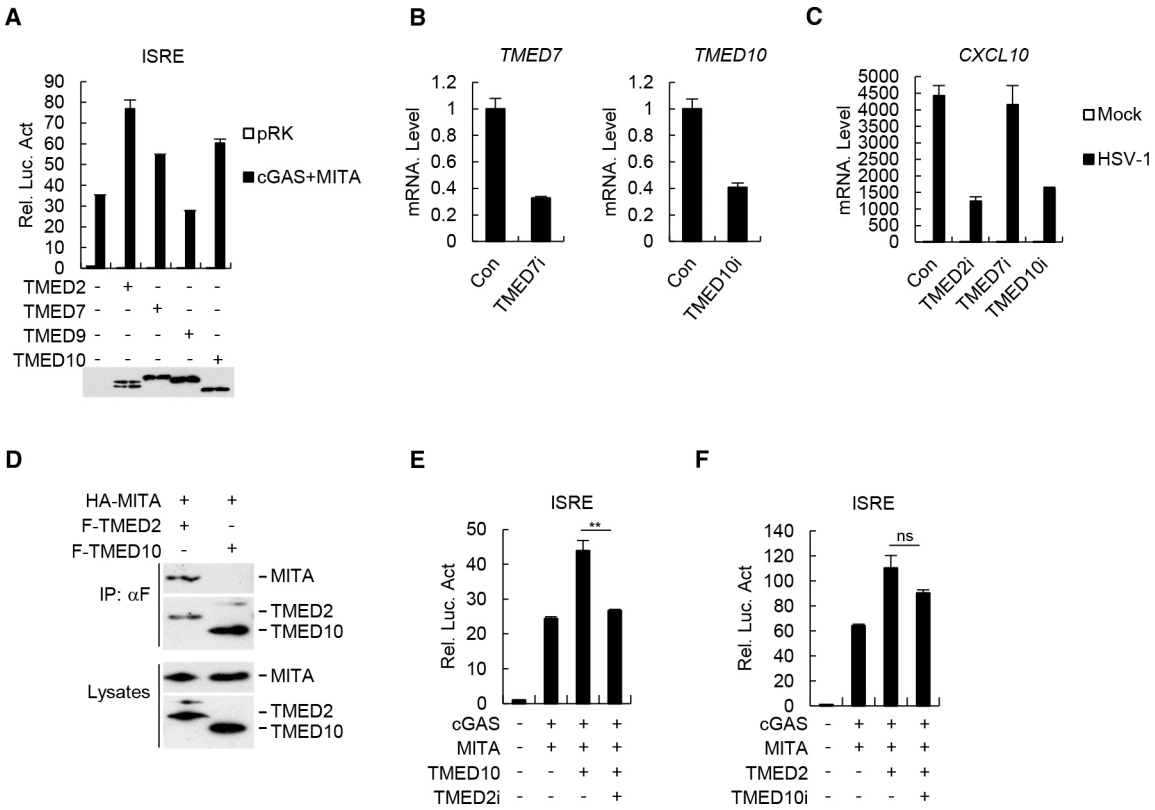


**Figure 6. TMED10 Participates in MITA Signaling**

(A) Dual-luciferase reporter assays were performed in HEK293 cells transfected with indicated plasmids.

(B) Knockdown efficiencies of TMED7i and TMED10i were analyzed by qPCR.

(C) HSV-1 induced transcription of *CXCL10* in stable TMED2, TMED7, and TMED10 knockdown THP-1 cells were determined by qPCR.

(D) HEK293 cells were transfected with the indicated plasmids and subjected to co-immunoprecipitation and immunoblotting.

(E and F) Stable TMED2 (E) or TMED10 (F) knockdown HEK293 cells were transfected with the indicated plasmids and subjected to dual-luciferase reporter assays. The graphs show mean ± SD, n = 3. *p < 0.05; **p < 0.01; ns, no significance.

formation of cargo protein-containing vesicles trafficking from the ER to the Golgi is usually dependent on the COP II complex (Kuge et al., 1994; Lederkremer et al., 2001; Sato and Nakano, 2007; Schekman and Orci, 1996; Stagg et al., 2006; Yoshihisa et al., 1993). In this process, how the cargo proteins are recognized and designated for specific compartments remains unclear. Our results showed that Sar1B knockdown impairs the aberrant DNA-induced transcription of *IFNB1* and *CXCL10* and thus provides direct evidence that the COP II complex is responsible for MITA signaling and demonstrates the importance of MITA trafficking by COPII pathway in the IFN-inducing pathway. Our immunofluorescent assays showed that TMED2 knockdown greatly impaired HSV-1-induced MITA congregation around the nucleus. Furthermore, TMED2 knockdown attenuated the interaction between MITA and the selective receptor Sec24C, but not Sar1B or Sec23B. These data indicate that TMED2 promotes the selection of MITA into COPII complex, strengthens the assembly of MITA-COPII complex, and therefore enhances MITA trafficking.

In summary, we present biochemical evidence and molecular mechanistic explanations that TMED2 potentiates anti-DNA virus immune responses by regulating MITA signaling in several ways, including enhancing MITA dimerization, translocation into the ER, and trafficking out of the ER into the perinuclear region. Our findings provide insight into the subtle regulation of innate immune responses induced by DNA viruses.

## STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- CONTACT FOR REAGENT AND RESOURCE SHARING
- EXPERIMENTAL MODEL AND SUBJECT DETAILS
  - Cell culture
- METHOD DETAILS
  - Retroviral gene transfer
  - CRISPR/Cas9- mediated genomic editing
  - Co-immunoprecipitation and immunoblot analysis
  - *In vitro* His pull-down assays
  - Measurement of cGAMP activity
  - Transfection and reporter assays
  - RNA extraction and quantitative real-time PCR

- RNA interference
- Immunofluorescence and confocal microscopy
- Viral infection and plaque assay

- QUANTIFICATION AND STATISTICAL ANALYSIS
- DATA AND SOFTWARE AVAILABILITY

## SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures and four tables and can be found with this article online at https://doi.org/10.1016/j.celrep.2018.11.048.

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## AUTHOR CONTRIBUTIONS

Y.L., H.-B.S., and M.-S.S. designed research. M.-S.S., J.Z., L.-Q.J., Y.-X.P., J.-Y.T., and F.Y. performed the experiments. L.Y. and C.S. provided technical support. M.-S.S., L.G., H.-B.S., and Y.L. analyzed data. M.-S.S. and Y.L. wrote the paper. All of the authors discussed the results.

## DECLARATION OF INTERESTS

The authors declare no competing interests.

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