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iRhom2 is essential for innate immunity to DNA viruses by mediating trafficking and stability of the adaptor STING

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STING is a central adaptor in the innate immune response to DNA viruses. However, the manner in which STING activity is regulated remains unclear. We identified iRhom2 ('inactive rhomboid protein 2') as a positive regulator of DNA-virus-triggered induction of type I interferons. iRhom2 deficiency markedly impaired DNA-virus- and intracellular-DNA-induced signaling in cells, and iRhom2-deficient mice were more susceptible to lethal herpes simplex virus type 1 (HSV-1) infection. iRhom2 was constitutively associated with STING and acted in two distinct processes to regulate STING activity. iRhom2 recruited the translocon-associated protein TRAPβ to the STING complex to facilitate trafficking of STING from the endoplasmic reticulum to perinuclear microsomes. iRhom2 also recruited the deubiquitination enzyme EIF3S5 to maintain the stability of STING through removal of its K48-linked polyubiquitin chains. These results suggest that iRhom2 is essential for STING activity, as it regulates TRAPβ-mediated translocation and EIF3S5-mediated deubiquitination of STING.

The innate immune response is initiated following sensing of non-self microbial products called pathogen-associated molecular patterns (PAMPs) by the pattern recognition receptors (PRRs), which triggers signaling pathways that lead to induction of type I interferons and pro-inflammatory cytokines. These cytokines subsequently induce transcription of a wide range of antiviral and inflammatory genes, which mediate innate antiviral immune and inflammatory responses[1–3].

Viral nucleic acids act as classic PAMPs to initiate innate immune responses. Although it is well known that viral RNA is recognized by the endosomal receptor TLR3 and the cytosolic receptors RIG-I and MDA5, the PRRs that sense viral DNA are less well defined. Previously, it has been demonstrated that TLR9 (ref. 4), AIM2 (ref. 5), DAI (ref. 6), RNA polymerase III[7,8], IFI16 (ref. 9), DDX41 (ref. 10) and LSm14A[11] can detect viral DNA in particular cell types and mouse models. However, these proteins are not universally required for detecting viral DNA in distinct cell types or *in vivo*[12]. A nucleotidyltransferase family member, cyclic GMP-AMP (cGAMP) synthase (cGAS), was identified as a cytoplasmic DNA sensor in various cell types and mice[13]. Gene-deletion studies have suggested that cGAS is essential for innate immune responses against various DNA viruses[14].

After sensing viral DNA, cGAS catalyzes synthesis of the second messenger molecule cGAMP, which binds to the adaptor STING (MITA) in the endoplasmic reticulum (ER)[13,15]. The cGAMP-binding STING traffics from the ER to the Golgi apparatus via the translocon-associated protein (TRAP) complex TRAPβ (SSR2)[16], and then further to the Sec5-containing perinuclear microsomes or punctuate structures[17]. During the trafficking processes, STING recruits TBK1 and IRF3, leading to induction of type I interferons[16–18]. These findings suggest that innate immune responses to DNA viruses are regulated by STING trafficking.

In addition to trafficking, the functions of STING are also regulated by several post-translational mechanisms. The E3 ubiquitin ligases TRIM32 and TRIM56 can catalyze K63-linked polyubiquitination of STING and promote the recruitment of TBK1 to STING, thereby positively regulating innate immune responses[19,20]. In addition, the ER-associated E3 ligase AMFR mediates K27-linked polyubiquitination of STING, providing a scaffold to recruit TBK1 and IRF3 (ref. 21). Conversely, the E3 ubiquitin ligase RNF5 negatively regulates virus-triggered signaling by targeting STING for K48-linked polyubiquitination and degradation[22], whereas another E3 ubiquitin ligase, RNF26, competes with RNF5 by catalyzing K11-linked polyubiquitination of STING at the same lysine residue[23]. These studies collectively suggest that innate immune responses to DNA viruses are regulated by polyubiquitination of STING. However, other post-translational regulatory mechanisms remain elusive. A thorough investigation on the regulation of STING activities is important because inappropriate activation of STING causes severe auto-inflammatory diseases[24–26].

Rhomboids are a conserved subfamily of proteins related to rhomboid intramembrane serine proteases that lack key catalytic residues, but exhibit a wide range of functions involved in growth factor signaling, mitochondrial remodeling, apoptosis and protein secretion[27–29]. Rhomboids have been associated with various human diseases, such

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as cancer, inflammatory arthritis, tylosis and Alzheimer's disease[30]. iRhom2 has been shown to facilitate lipopolysaccharide (LPS)- and *Listeria*-induced tumor necrosis factor (TNF) production by promoting the maturation and trafficking to the cell surface of TNF convertase (TACE)[28,29]. We identified iRhom2 as a positive regulator of DNA-virus-trigged innate immune responses by regulating STING protein stability and activation-induced trafficking from the ER to perinuclear microsomes.

RESULTS

iRhom2 positively regulates DNA-virus-triggered signaling

To identify candidate molecules involved in virus-triggered innate immune responses, we screened ~10,000 independent human cDNA expression plasmids for their ability to regulate interferon-stimulated response element (ISRE) activity by expression reporter assays and identified iRhom2 as a candidate. iRhom2 has also been reported as a critical pathogenic mediator of inflammatory arthritis, which is often linked to situations of pathologically enhanced innate immune sensing of DNA[31]. Similar to iRhom2-mediated TACE trafficking, STING is translocated from the ER via Golgi apparatus to perinuclear microsomes in responses to DNA viruses. Thus, we investigated whether iRhom2 is also involved in the regulation of innate immune responses to DNA viruses. We first analyzed the dynamic expression of the gene encoding iRhom2 (*RHBDF2* in humans and *Rhbdf2* (called '*iRhom2*' here) in mice) in human moncytic THP-1 cells and murine bone-marrow-derived macrophages (BMDMs). We found that both human and murine mRNAs encoding iRhom2 were induced by Sendai virus (SeV) and HSV-1, but not by cytokines such as TNF, IL-1β, IFN-β and IFN-γ (**Fig. 1a**), which further indicates a potential link between iRhom2 and antiviral immune responses. Consistent with this reporter assays revealed that overexpression of iRhom2 activated the *IFNB* promoter by itself and potentiated HSV-1-induced activation of the *IFNB* promoter in a dose-dependent manner in HeLa cells (**Fig. 1b**). We carried out quantitative PCR (qPCR) and found that overexpression of iRhom2 potentiated HSV-1-induced and transfected interferon stimulatory DNA (ISD)-induced (double-stranded 45-base-pair oligonucleotides lacking CpG sequences) expression of *IFNB1*, *ISG56* and *IL6* genes in human monocytic THP-1 cells (**Fig. 1c**). These results suggest that iRhom2 is involved in cytoplasmic-DNA- and DNA-virus-triggered induction of downstream antiviral genes.

We next determined whether endogenous iRhom2 is required for DNA-virus-triggered signaling. We constructed two human iRhom2-RNAi plasmids that could efficiently inhibit the expression of transfected and endogenous iRhom2 (**Fig. 1d**). Knockdown of iRhom2 significantly inhibited HSV-1-induced transcription of downstream genes such as *IFNB1*, *ISG56* and *IL6* (**Fig. 1e**), but not IFN-γ-induced transcription of *IRF1* (**Fig. 1f**). Consistently, the phosphorylation of TBK1, IRF3 and IκBα following HSV-1 infection, which is hallmark of virus-triggered signaling, were markedly inhibited by iRhom2 knockdown in THP-1 cells (**Fig. 1g**). In contrast, knockdown of iRhom2 had no marked effects on IFN-γ-induced phosphorylation

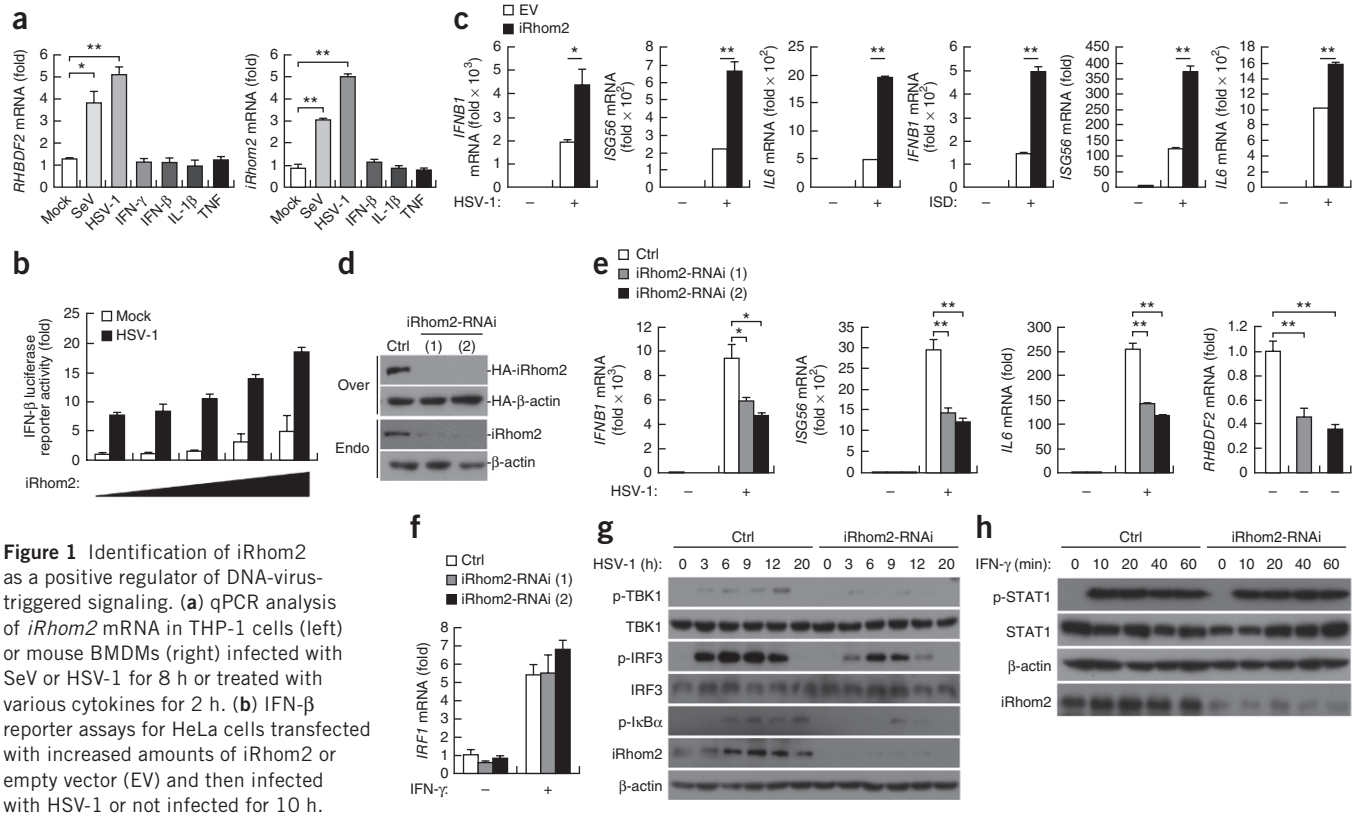

Figure 1 Identification of iRhom2 as a positive regulator of DNA-virus-triggered signaling. (**a**) qPCR analysis of *iRhom2* mRNA in THP-1 cells (left) or mouse BMDMs (right) infected with SeV or HSV-1 for 8 h or treated with various cytokines for 2 h. (**b**) IFN-β reporter assays for HeLa cells transfected with increased amounts of iRhom2 or empty vector (EV) and then infected with HSV-1 or not infected for 10 h. (**c**) qPCR analysis of mRNA abundance of the indicated genes in iRhom2-stable-expressing THP1 cells infected with HSV-1 or transfected with ISD for 6 h. (**d**) Immunoblot analysis of iRhom2 in HEK293 cells transfected with iRhom2-RNAi plasmids and an iRhom2 expression or control (ctrl) plasmid. (**e**) qPCR analysis of mRNA abundance of the indicated genes in iRhom2-RNAi stable knockdown THP-1 cells un-infected or infected with HSV-1 for 6 h. (**f**) qPCR analysis of *IRF1* mRNA in iRhom2-knockdown HEK293 cells treated with IFN-γ for 2 h. (**g**) Immunoblot analysis of the indicated proteins in iRhom2 stable knockdown THP-1 cells un-infected or infected with HSV-1 for the indicated times. (**h**) Immunoblot analysis of the indicate proteins in iRhom2 stable knockdown THP-1 cells un-treated or treated with IFN-γ for the indicated times. $^*P < 0.05$, $^{**}P < 0.01$ (unpaired *t* test). Data are representative of three experiments with similar results (mean and s.d. in **a**–**c**,**e**,**f**).

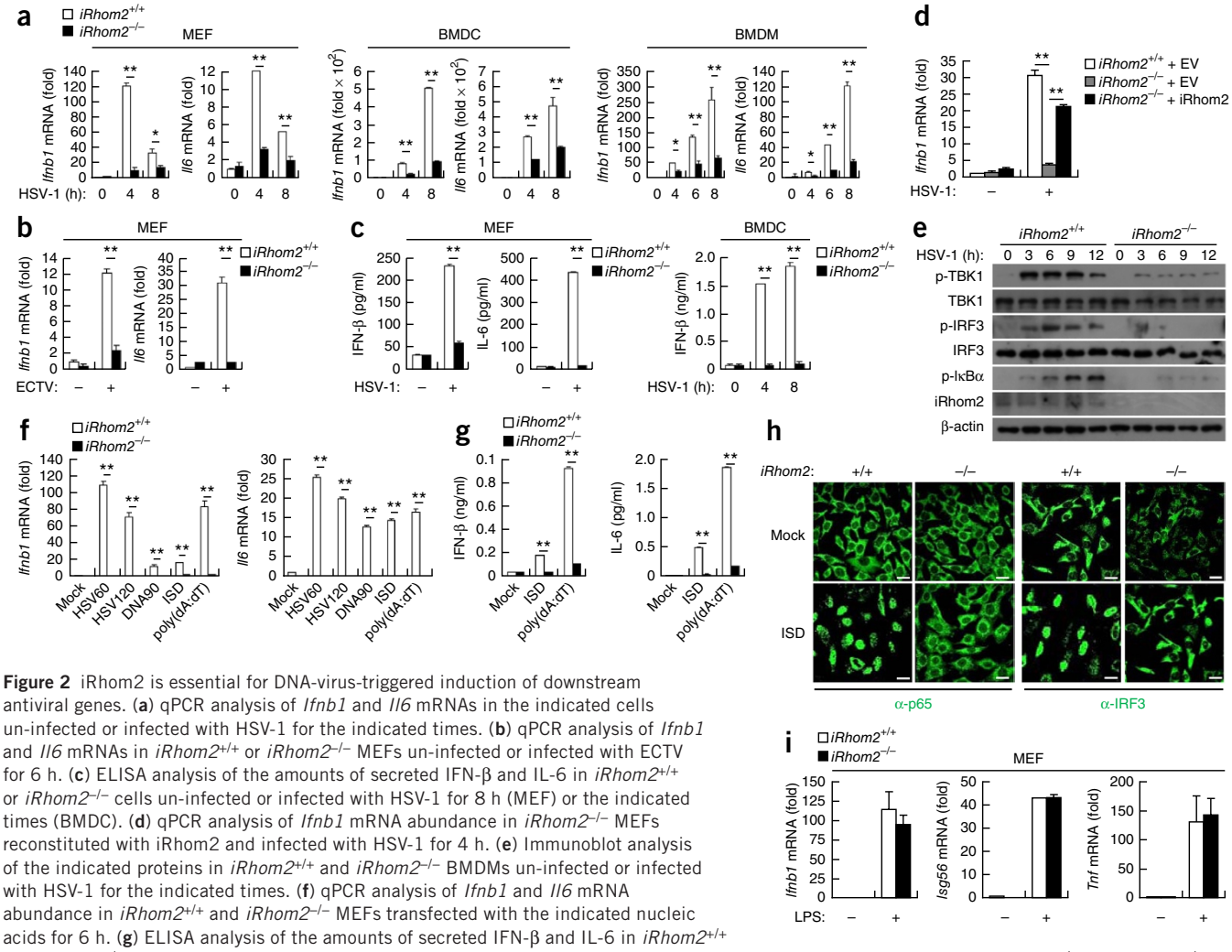

Figure 2 iRhom2 is essential for DNA-virus-triggered induction of downstream antiviral genes. (**a**) qPCR analysis of *Ifnb1* and *Il6* mRNAs in the indicated cells un-infected or infected with HSV-1 for the indicated times. (**b**) qPCR analysis of *Ifnb1* and *Il6* mRNAs in *iRhom2*$^{+/+}$ or *iRhom2*$^{-/-}$ MEFs un-infected or infected with ECTV for 6 h. (**c**) ELISA analysis of the amounts of secreted IFN-β and IL-6 in *iRhom2*$^{+/+}$ or *iRhom2*$^{-/-}$ cells un-infected or infected with HSV-1 for 8 h (MEF) or the indicated times (BMDC). (**d**) qPCR analysis of *Ifnb1* mRNA abundance in *iRhom2*$^{-/-}$ MEFs reconstituted with iRhom2 and infected with HSV-1 for 4 h. (**e**) Immunoblot analysis of the indicated proteins in *iRhom2*$^{+/+}$ and *iRhom2*$^{-/-}$ BMDMs un-infected or infected with HSV-1 for the indicated times. (**f**) qPCR analysis of *Ifnb1* and *Il6* mRNA abundance in *iRhom2*$^{+/+}$ and *iRhom2*$^{-/-}$ MEFs transfected with the indicated nucleic acids for 6 h. (**g**) ELISA analysis of the amounts of secreted IFN-β and IL-6 in *iRhom2*$^{+/+}$ and *iRhom2*$^{-/-}$ MEFs transfected with the indicated nucleic acids for 18 h. (**h**) Immunostaining showing p65 and IRF3 in *Rhom2*$^{+/+}$ and *iRhom2*$^{-/-}$ MEFs transfected with ISD for 6 h. Scale bars represent 200 μm. (**i**) qPCR analysis of mRNA abundance of the indicated genes in *iRhom2*$^{+/+}$ and *iRhom2*$^{-/-}$ MEFs un-treated or treated with LPS for 4 h. $*P < 0.05$; $**P < 0.01$ (unpaired *t* test). Data are representative of three experiments with similar results (mean and s.d. in **a**–**d**,**f**,**g**,**i**).

of STAT1 (**Fig. 1h**). These data suggest that endogenous iRhom2 is important for DNA-virus-triggered induction of downstream antiviral genes.

iRhom2 deficiency suppresses DNA virus-triggered signaling

To further investigate the functions of iRhom2 *in vivo*, we obtained iRhom2-deficient mice (*Rhbdf2*$^{-/-}$; called '*iRhom2*$^{-/-}$' here) (**Supplementary Fig. 1a**,**b**). qPCR analysis confirmed that *iRhom2*$^{-/-}$ cells were defective at producing *iRhom2* mRNA, whereas *iRhom2*$^{+/-}$ cells produced an intermediate abundance of *iRhom2* mRNA (**Supplementary Fig. 1c**). Homozygous *iRhom2*$^{-/-}$ mice were born at the Mendelian ratio (**Supplementary Fig. 1d**) and did not display any developmental abnormality, which suggest that iRhom2 is dispensable for the survival and development of mice. The numbers and compositions of cells in the lymph nodes, spleen and thymus were similar between *iRhom2*$^{-/-}$ mice and their wild-type littermates (**Supplementary Fig. 1e**), suggesting that iRhom2 is not required for the development of various types of immune cells.

To determine whether iRhom2 is required for virus-triggered signaling, we examined expression of antiviral genes induced by HSV-1 in *iRhom2*$^{-/-}$ murine embryonic fibroblasts (MEFs), bone-marrow-derived dendrite cells (BMDCs) and BMDMs. We found that induction of *Ifnb1* and *Il6* mRNAs were severely impaired in *iRhom2*$^{-/-}$ MEFs, BMDCs and BMDMs in comparison with their wild-type counterparts (**Fig. 2a**). In similar experiments, induction of *Ifnb1* and *Il6* mRNAs following infection with another DNA virus, ectromelia virus (ECTV), was also severely impaired in *iRhom2*$^{-/-}$ cells (**Fig. 2b**). In addition, secretion of IFN-β and IL-6 were abolished in *iRhom2*$^{-/-}$ MEFs or BMDCs following HSV-1 infection (**Fig. 2c**). Moreover, reconstitution of iRhom2 markedly rescued HSV-1-induced *Ifnb1* expression in *iRhom2*$^{-/-}$ MEFs (**Fig. 2d**). Consistently, phosphorylation of TBK1, IRF3 and IκBα induced by HSV-1 infection was markedly inhibited in *iRhom2*$^{-/-}$ BMDMs (**Fig. 2e**).

Previously, it was shown that transfected nucleic acid mimics such as double-stranded DNA (dsDNA, 60- or 120-mers) representing the genomes of HSV-1 (HSV60 or HSV120), dsDNA of approximately 90 bp (dsDNA90), ISD and poly(dA:dT) are efficient at inducing the expression of downstream antiviral cytokines. We found that expression of *Ifnb1* and *Il6* genes induced by transfection of these synthetic DNAs were barely detectable in *iRhom2*$^{-/-}$ MEFs, whereas their expressions were normally induced in wild-type MEFs (**Fig. 2f**). Consistently, iRhom2 deficiency resulted in over 90% less production

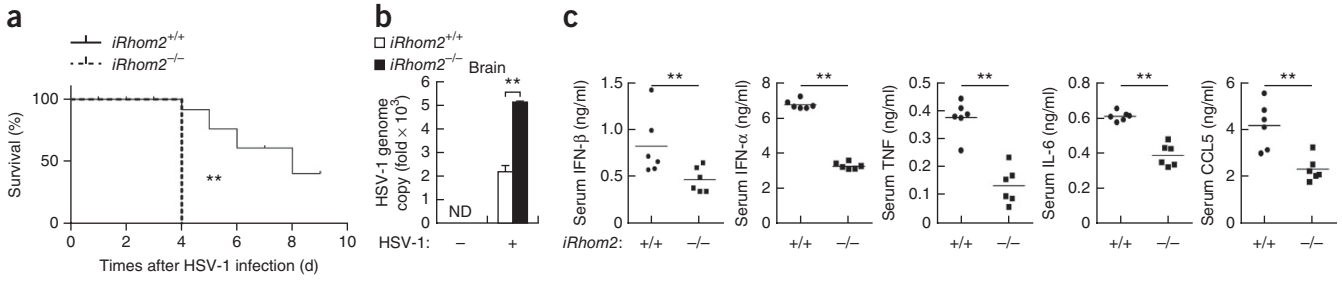

Figure 3 iRhom2 is essential for host defense against HSV-1 infection in mice. (**a**) Survival of *iRhom2*$^{+/+}$ and *iRhom2*$^{-/-}$ mice ($n = 7$ per strain, 8 weeks old) after intravenous injection with HSV-1 (1×10^7 PFU per mouse). (**b**) qPCR analysis of HSV-1 genomic copy in brains of *iRhom2*$^{+/+}$ and *iRhom2*$^{-/-}$ mice ($n = 3$ per strain, 8 weeks old) after intravenous injection of HSV-1 (1×10^7 PFU per mouse). (**c**) EILSA analysis of the indicated cytokines in sera of *iRhom2*$^{+/+}$ and *iRhom2*$^{-/-}$ mice ($n = 6$ per strain, 8 weeks old) injected i.v. with HSV-1 (1×10^7 PFU per mouse) for 12 h. $^{**}P < 0.01$ (unpaired *t* test (**b**,**c**) or log-rank test (**a**)). Data are representative of two experiments with similar results (mean and s.d. in **a**–**c**).

of IFN-β and IL-6 induced by transfected ISD and poly(dA:dT) (**Fig. 2g**). In addition, nuclear translocation of IRF3 and p65 induced by transfected ISD was markedly deceased in *iRhom2*$^{-/-}$ MEFs (**Fig. 2h**). In similar experiments, the abundance of *Ifnb1*, *Isg56* and *Tnf* mRNAs induced by LPS was comparable between *iRhom2*$^{-/-}$ and wild-type MEFs (**Fig. 2i**), whereas the secretion of TNF, but not IFN-β, induced by LPS was impaired in *iRhom2*$^{-/-}$ cells (**Supplementary Fig. 2a**). Moreover, we found that both the mRNA and protein abundance of TNF induced by HSV-1 were impaired by iRhom2 deficiency (**Supplementary Fig. 2b**). Collectively, these data suggest that iRhom2 is essential for efficient induction of downstream antiviral genes by DNA virus and cytosolic DNA in murine fibroblasts and immune cells.

iRhom2 is essential for antiviral response *in vivo*

To evaluate the importance of iRhom2 in host defense against viral infection *in vivo*, we infected 2–3-month-old wild-type and *iRhom2*-deficient mice with HSV-1 intravenously (i.v.). All infected *iRhom2*$^{-/-}$ mice developed discrepant lethargy and ataxia within 3 d of HSV-1 infection and died within 1 d of the appearance of symptoms. In contrast, only approximately half of the infected wild-type mice exhibited these symptoms, which then died over a period of 5–10 d after the symptoms (**Fig. 3a**). Given that HSV-1 is a neurotropic virus and the leading cause of sporadic viral encephalitis, we measured HSV-1 genomic DNA copy numbers in the brains extracted from wild-type and *iRhom2*$^{-/-}$ mice on day 3 after infection. We found that significantly higher amounts of HSV-1 genomic DNA copies existed in the brains of *iRhom2*$^{-/-}$ than wild-type mice (**Fig. 3b**). Consistently, sera from *iRhom2*$^{-/-}$ mice infected with HSV-1 for 6 h showed significantly lower concentrations of IFN-α, IFN-β, TNF, IL-6 and CCL5 compared with that from the wild-type mice (**Fig. 3c**). Collectively, these data suggest that iRhom2 is essential for host defense against HSV-1 infection in *vivo*.

iRhom2 interacts with STING

To determine the mechanisms by which iRhom2 is involved in DNA-virus-triggered signaling, we first examined whether cGAMP-induced downstream activation is affected by iRhom2 deficiency. We found that expression of *Ifnb1* and *Il6* mRNAs, as well as the secretion of these cytokines induced by transfected cGAMP, was almost completely abrogated in *iRhom2*$^{-/-}$ in comparison with wild-type MEFs (**Fig. 4a**). In addition, phosphorylation of TBK1, IRF3 and IκBα induced by transfected intracellular cGAMP was also impaired in *iRhom2*$^{-/-}$ MEFs (**Supplementary Fig. 3a**). These results suggest that iRhom2 acts downstream of cGAMP and upstream of TBK1-IRF3. Consistently, knockdown of iRhom2 inhibited cGAS- and STING-mediated, but not TBK1- and IRF3-5D-mediated (an active mutant of IRF3), activation of the *Ifnb* promoter in a dose-dependent manner (**Fig. 4b**). Recently, it was reported that 5,6-Dimethylxanthenone-4-acetic acid (DMXAA) acts as a ligand for STING in murine cells[32,33]. We found that iRhom2 deficiency substantially inhibited DMXAA-triggered expression of *Ifnb1*, *Ifna4*, *Isg56* and *Cxcl10* (**Supplementary Fig. 3b**). Collectively, these results suggest that iRhom2 functions at the STING level.

We next determined whether iRhom2 is associated with signaling components in DNA-virus-triggered pathways. Transient transfection and co-immunoprecipitation experiments revealed that iRhom2 was associated with STING, but not with DDX41, IFI16, cGAS, TRAF3, TRAF6, IRF3 or IRF7 (**Fig. 4c**). Using co-immunoprecipitation of endogenous proteins, we found that iRhom2 was constitutively associated with STING in unstimulated cells and that this association was increased during the first 3–6 h after HSV-1 infection and decreased by 9–12 h (**Fig. 4d**). These data suggest that iRhom2 is dynamically associated with STING and the association is negatively regulated in the late phase of viral infection.

Similar to STING, iRhom2 is a membrane protein containing seven transmembrane domains. To investigate the domains responsible for iRhom2-STING interaction, we performed co-immunoprecipitation experiments with a series of truncations of iRhom2 and STING. We found that the first transmembrane domain of iRhom2 (amino acids 410–430) and the N-terminal transmembrane domains of STING (1–190) were required for their interaction (**Fig. 4e**,**f**). Reporter assays revealed that the truncations of iRhom2 that could interact with STING potentiated STING-mediated activation of the *IFNB* promoter, whereas the iRhom2(1–409) truncation, which lost its ability to interact with STING, inhibited STING-mediated activation of the *IFNB* promoter (**Fig. 4g**). These results suggest that iRhom2 is associated with STING through their respective transmembrane domains and that their association is necessary for the functions of iRhom2 in STING-mediated signaling.

iRhom2 is essential for the trafficking of STING

STING traffics from the ER to Golgi and then to endoplasmic-associated microsomes following DNA virus infection[16]. We investigated whether iRhom2 is involved in the trafficking of STING. Confocal microscopy revealed that iRhom2 was mostly localized in the ER and partially in the Golgi apparatus and lysosomes, but was undetectable in other examined intracellular compartments such as mitochondria, the ER-Golgi intermediate compartment (ERGIC), autophagosomes and endosomes in rest HeLa cells (**Fig. 5a**). Notably,

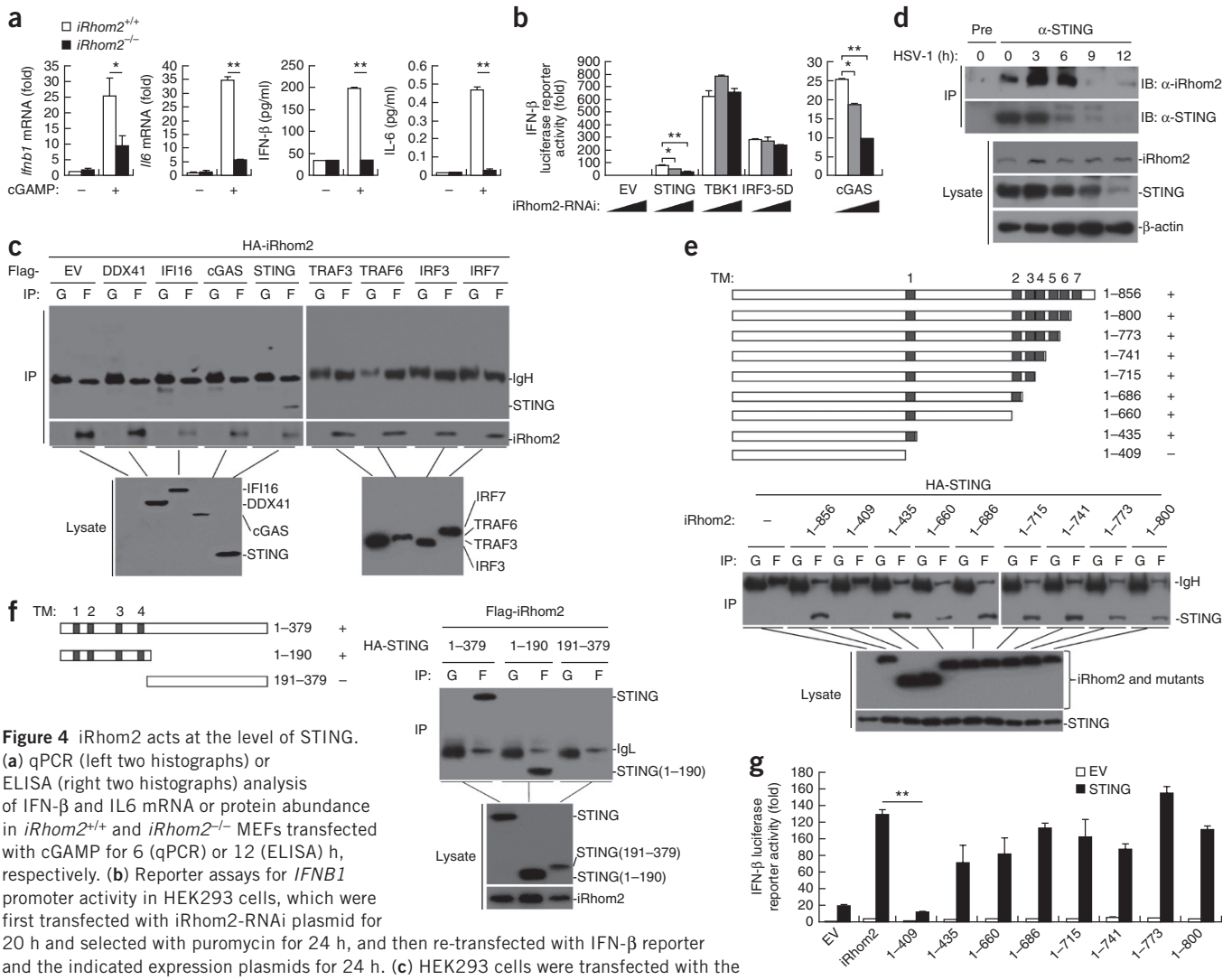

Figure 4 iRhom2 acts at the level of STING. (**a**) qPCR (left two histographs) or ELISA (right two histographs) analysis of IFN-β and IL6 mRNA or protein abundance in *iRhom2*$^{+/+}$ and *iRhom2*$^{-/-}$ MEFs transfected with cGAMP for 6 (qPCR) or 12 (ELISA) h, respectively. (**b**) Reporter assays for *IFNB1* promoter activity in HEK293 cells, which were first transfected with iRhom2-RNAi plasmid for 20 h and selected with puromycin for 24 h, and then re-transfected with IFN-β reporter and the indicated expression plasmids for 24 h. (**c**) HEK293 cells were transfected with the indicated plasmids before co-immunoprecipitation and immunoblot analysis were performed with the indicated antibodies. G, IgG; F, anti-Flag. (**d**) MEFs were left un-infected or infected with HSV-1 for the indicated times before endogenous co-immunoprecipitation and immunoblot analysis with the indicated antibodies. (**e**,**f**) Domain mapping of the iRhom2 and STING interaction. HEK293 cells were transfected with the indicated truncations before co-immunoprecipitation and immunoblot analysis with the indicated antibodies. The schematic representations of iRhom2 and STING truncations were showed at the top. (**g**) Reporter assays for *IFNB1* promoter activity in HEK293 cells transfected with iRhom2 and its mutants for 24 h. $^{*}P < 0.05$; $^{**}P < 0.01$ (unpaired *t* test). Data are representative of three experiments with similar results (mean and s.d. in **a**,**b**,**g**).

following HSV-1 infection, iRhom2 was disassociated from the ER and translocated to and was enriched in the ERGIC, Golgi apparatus and lysosomes, but not endosomes (**Fig. 5a**). These results suggest that HSV-1 infection induces trafficking of iRhom2 from the ER to ERGIC, Golgi apparatus and lysosomes.

Confocal microscopy revealed that iRhom2 and STING were well colocalized in the cytoplasm (**Fig. 5b**). Notably, HSV-1 infection or transfection of synthetic ISD caused accumulation of STING at the endoplasmic-associated microsomes in wild-type MEFs, but this accumulation was completely absent in *iRhom2*$^{-/-}$ MEFs (**Fig. 5c–e**). Consistent with previous findings that translocation of STING is necessary for the activation of downstream events, Brefeldin A (BFA), an inhibitor of protein transport, inhibited the synergetic effects of iRhom2 on STING-mediated activation of the *IFNB* promoter in reporter assays (**Fig. 5f**). These results suggest that iRhom2 is required for the trafficking of STING from the ER to perinuclear microsomes.

iRhom2 promotes STING-TRAPβ complex assembly

Previous studies have identified TRAPβ, Sec61β and Sec5 as the STING-associated translocator in the ER that enhances STING-mediated signaling[16,17]. We found that iRhom2 was associated with TRAPβ and Sec5, but not with Sec61β and derlin-1 (**Fig. 5g**). Confocal microscopy confirmed that iRhom2 was colocalized with TRAPβ and Sec5, but not with LC3 (**Supplementary Fig. 4**). These findings and previous results prompted us to investigate whether iRhom2 facilitates STING trafficking by regulating the association of STING with the TRAPβ or Sec5 translocator. Using co-immunoprecipitation, we found that iRhom2 promoted the association STING with TRAPβ, but not with Sec5 (**Fig. 5h**), whereas knockdown of TRAPβ markedly inhibited ISD-induced STING trafficking (**Fig. 5i**). In addition, domain mapping revealed that amino acids 1–430, but not 1–409, of iRhom2 were able to interact with TRAPβ, suggesting that amino acids 1–430 of iRhom2, which contain the first transmembrane domain (410–430), are important for its association with both STING and TRAPβ

(**Supplementary Fig. 5**). Notably, knockdown of TRAPβ inhibited the synergetic effects of iRhom2 on STING-mediated expression of *IFNB1* and *IL6* genes (**Fig. 5j**). These results suggest that iRhom2 promotes assembling of a STING-TRAPβ translocation complex, which is required for the trafficking and activation of STING.

iRhom2 maintains stability of STING

In our experiments, we repeatedly observed that STING protein abundance was markedly downregulated in *iRhom2*$^{-/-}$ cells. These clues prompted us to investigate the effects of iRhom2 on the expression of STING protein. The abundance of STING, but not cGAS, TBK1 and

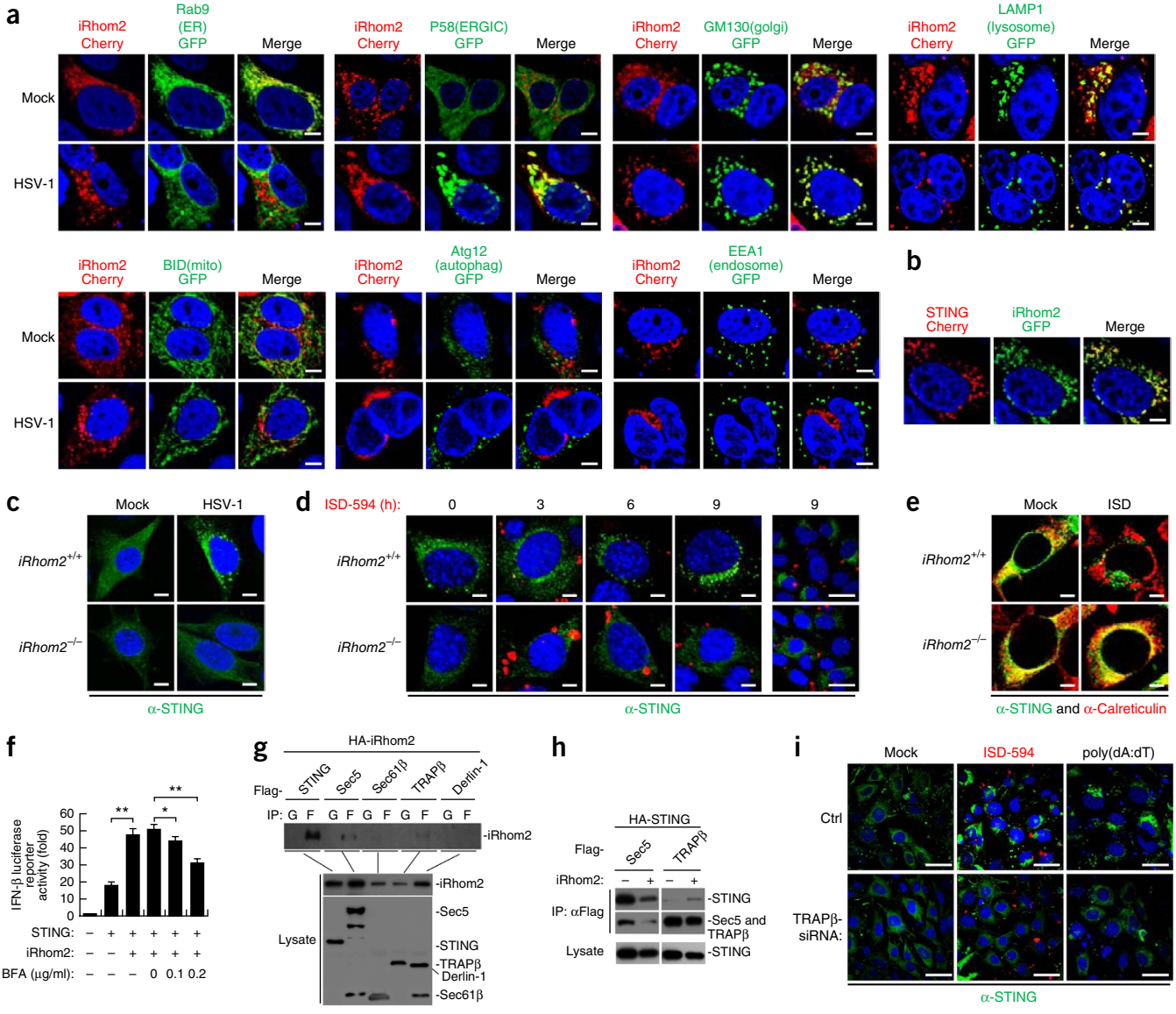

Figure 5 iRhom2 facilitates the trafficking of STING. (**a**) Confocal microscopy of HeLa cells transfected with iRhom2-Cherry and GFP-tagged Rab9 (ER marker), p58 (ERGIC marker), GM130 (Golgi marker), BID (mitochondria marker), Atg12 (autophagosome marker), and EEA1 (endosome marker), and then un-infected or infected with HSV-1 for 6 h. mito, mitochondria; autophag, autophagosome. Scale bars, 50 μm. (**b**) Confocal microscopy of HeLa cells transfected with iRhom2-Cherry and STING-GFP for 24 h. Scale bars represent 50 μm. (**c**,**d**) Immunostaining of STING (green) in *iRhom2*$^{+/+}$ and *iRhom2*$^{-/-}$ MEFs stably transduced with STING and infected with HSV-1 for 6 h (C) or transfected with Alexa-Fluor-594-labeled ISD (ISD-594) for the indicated times. Scale bars represent 50 μm (**c** and left in **d**) and 200 μm (right in **d**). (**e**) Immunostaining of STING (green) and calreticulin (red) in *iRhom2*$^{+/+}$ and *iRhom2*$^{-/-}$ MEFs transfected with ISD for 6 h. Scale bars represent 50 μm. (**f**) Reporter assays for *IFNB1* promoter activity in HEK293 cells transfected with the indicated plasmids and treated with the indicated doses of BFA for 2 h. (**g**,**h**) HEK293 cells were transfected with the indicated plasmids before co-immunoprecipitation and immunoblot analysis were performed with the indicated antibodies. G, anti-IgG; F, anti-Flag. (**i**) Immunostaining of STING (green) in MLFs transfected with TRAPβ-siRNA for 2 d and then re-transfected with ISD-594 or poly(dA:dT) for 6 h. Scale bars represent 200 μm. (**j**) qPCR analysis of *IFNB1*, *IL6* and *SSR2* mRNA abundance in HEK293 transfected with TRAPβ-siRNA for 30 h and then re-transfected with STING or STING+iRhom2 for 24 h. $^{*}P < 0.05$; $^{**}P < 0.01$ (unpaired *t* test). Data are representative of three experiments with similar results (mean and s.d. in **f**,**j**).

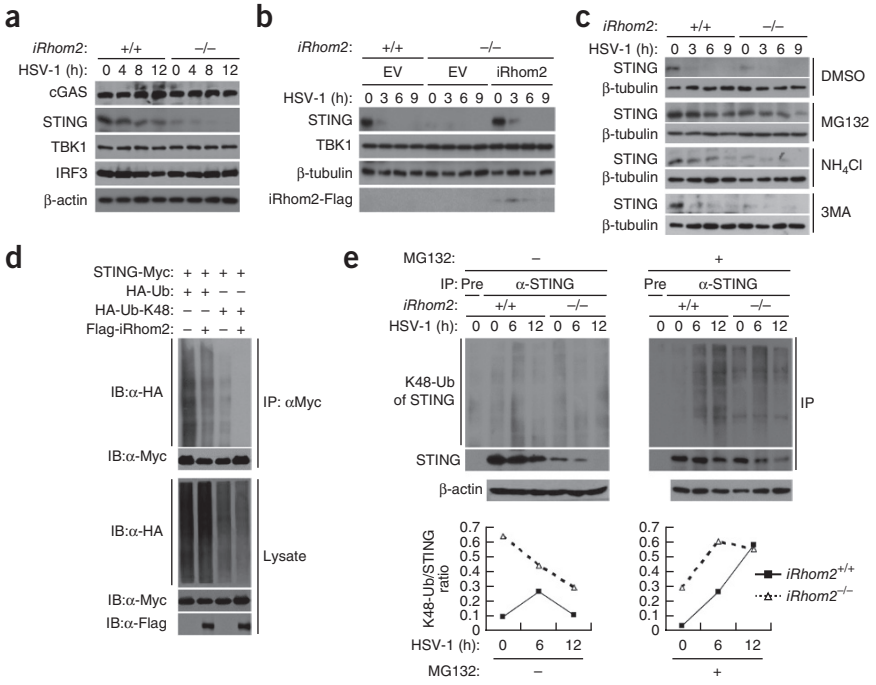

Figure 6 iRhom2 maintains the stability of STING. (**a**) Immunoblot analysis of the indicated proteins in *iRhom2*$^{+/+}$ and *iRhom2*$^{-/-}$ MEFs infected with HSV-1 for the indicated times. (**b**) Immunoblot analysis of STING and TBK1 in *iRhom2*$^{-/-}$ MEFs reconstituted with murine iRhom2 and infected with HSV-1 for the indicated times. (**c**) Immunoblot analysis of STING in *iRhom2*$^{+/+}$ and *iRhom2*$^{-/-}$ MEFs treated with MG132 (100 μM), NH_4Cl (25 mM) or 3-MA (500 ng/ml) for 1 h and then infected with HSV-1 for the indicated times. (**d**) HEK293 cells were transfected with C-terminal Myc-tagged STING, Flag-tagged iRhom2, HA-tagged ubiquitin (Ub) or Ub-K48 before co-immunoprecipitation and immunoblot analysis were performed with the indicated antibodies. (**e**) Co-immunoprecipitation and immunoblot analysis for detection of endogenous K48-linked polyubiquitination of STING in *iRhom2*$^{+/+}$ and *iRhom2*$^{-/-}$ MEFs infected with HSV-1 for the indicated times (left). Right, the experiments were similarly performed except that cells were pre-treated with MG132 for 1 h before HSV-1 infection. Data are representative of three experiments with similar results.

IRF3, was obviously downregulated in *iRhom2*$^{-/-}$ in comparison with wild-type MEFs or BMDMs (**Fig. 6a** and **Supplementary Fig. 6a**). Furthermore, reconstitution of iRhom2 was able to rescue the decrease of STING protein in *iRhom2*$^{-/-}$ MEFs (**Fig. 6b**). Similarly, knockdown of iRhom2 in human THP-1 cells also led to downregulation of endogenous STING (**Supplementary Fig. 6b**). However, qPCR analysis revealed that knockdown of iRhom2 did not affect STING mRNA abundance (**Supplementary Fig. 6c**), suggesting that downregulation of STING following iRhom2 deficiency occurs at the protein level. Using cycloheximide-chase assays, we found that iRhom2 retarded the degradation of STING (**Supplementary Fig. 6d**). These results suggest that iRhom2 maintains the stability of STING protein.

To investigate the mechanisms responsible for the role of iRhom2 on the stability of STING, we treated *iRhom2*$^{+/+}$ and *iRhom2*$^{-/-}$ cells with various inhibitors for protein degradation pathways. MG132, a proteasome inhibitor, but not the lysosome inhibitor ammonium chloride (NH_4CI) or autophagosome inhibitor 3-methyladenine (3-MA), markedly inhibited the degradation of STING in *iRhom2*$^{-/-}$ cells (**Fig. 6c**), suggesting that iRhom2 deficiency leads to degradation of STING through a proteasome-dependent pathway. In an overexpression system, iRhom2 inhibited K48-linked, but not K6-, K11-, K27-, K29-, K33- or K63-linked, polyubiquitination of STING (**Fig. 6d** and **Supplementary Fig. 6e**). In *iRhom2*$^{-/-}$ MEFs,

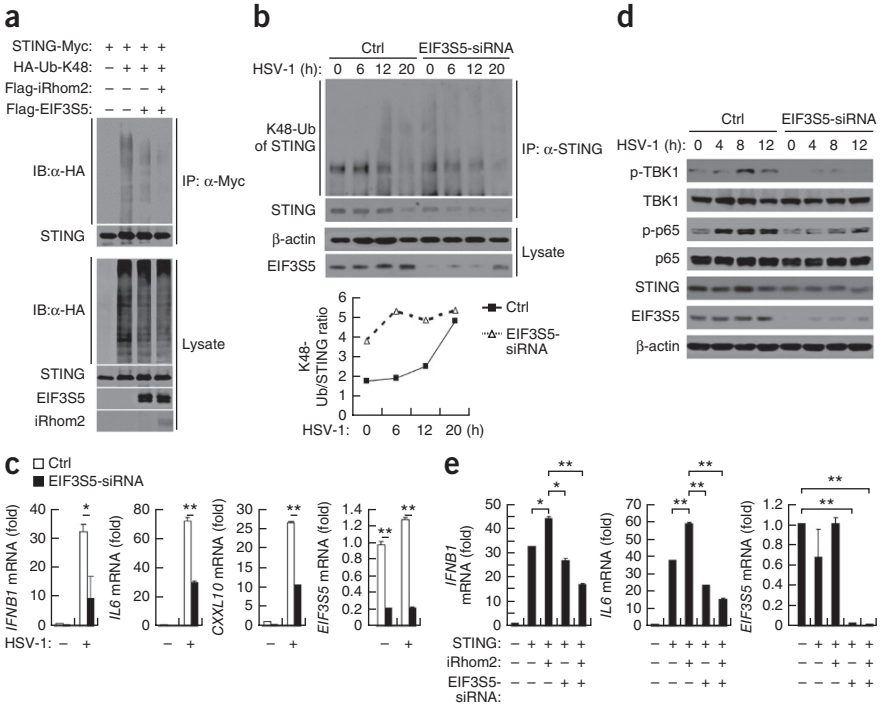

Figure 7 iRhom2-EIF3S5 axis deubiquitinates STING. (**a**) HEK293 cells were transfected with the indicated plasmids before co-immunoprecipitation and immunoblot analysis with the indicated antibodies. (**b**) Detection of endogenous K48-linked polyubiquitination of STING in HFFs transfected with EIF3S5-siRNA plasmid for 2 d and then infected with HSV-1 for the indicated times. (**c**) qPCR analysis of mRNA abundance of the indicated genes in HFFs transfected with EIF3S5-siRNA plasmid for 2 d and then infected with HSV-1 for 6 h. (**d**) Immunoblot analysis of the indicated proteins in HFFs transfected with EIF3S5-siRNA plasmid for 2 d and then infected with HSV-1 for the indicated times. (**e**) qPCR analysis of *IFNB1*, *IL6* and *EIF3S5* mRNA abundance in HEK293 transfected with EIF3S5-siRNA plasmid for 30 h and then re-transfected with STING or STING+iRhom2 plasmids for 24 h. $^*P < 0.05$, $^{**}P < 0.01$ (unpaired *t* test). Data are representative of three experiments with similar results (mean and s.d. in **c**,**e**).

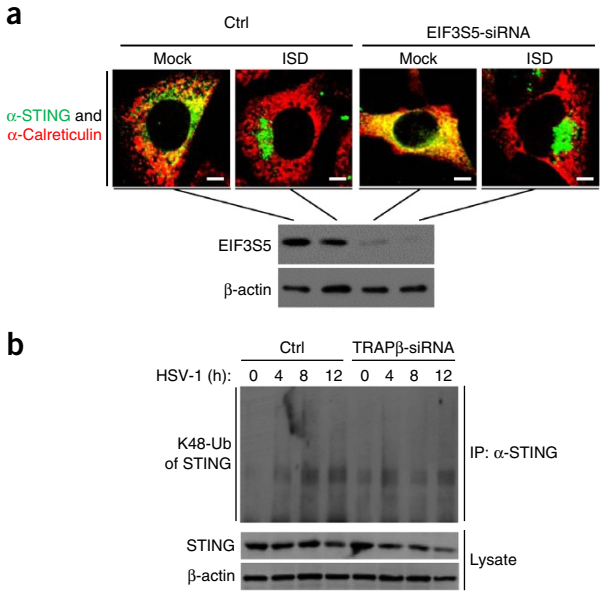

Figure 8 iRhom2-mediated translocation and stability of STING are two independent processes. (**a**) Immunostaining of STING (green) and Calreticulin (red) in MLFs transfected with EIF3S5-siRNA plasmid for 2 d and then re-transfected with ISD-594 for 6 h. Scale bars represent 50 μm. (**b**) Co-immunoprecipitation and immunoblot analysis of endogenous K48-linked polyubiquitination of STING in HFFs transfected with EIF3S5-siRNA plasmid for 2 d and then infected with HSV-1 for the indicated times. Data are representative of three experiments with similar results.

although the total amount of K48-linked polyubiquitinated STING was not markedly increased before or after HSV-1 infection, quantitative analysis revealed that the ratios of K48-linked polyubiquitinated STING to un-ubiquitinated STING were ~5.5- and ~1–2 fold higher before and after HSV-1 infection, respectively, in *iRhom2*$^{-/-}$ compared with *iRhom2*$^{+/+}$ MEFs (**Fig. 6e**). Furthermore, pretreatment of the cells with MG132 markedly increased the amount of K48-linked polyubiquitination of STING in uninfected as well as early infected *iRhom2*$^{-/-}$ in comparison with *iRhom2*$^{+/+}$ MEFs (**Fig. 6e**). These results suggest that iRhom2 may mediate the deubiquitination of STING in uninfected cells as well as cells at an early phase of infection.

iRhom2-EIF3S5 axis deubiquitinates STING

Given that iRhom2 is not a deubiquitinase, we reasoned that iRhom2 might function as an adaptor to recruit a deubiquitinase to STING. To identify candidate deubiquitinases that target STING, we screened for deubiquitinases that could interact with both iRhom2 and STING in a mammalian overexpression system. These screens identified 10 candidates from 60 deubiquitinates (**Supplementary Fig. 7a**). However, only the interaction between STING and the deubiquitinase EIF3S5, and not the other nine candidates, was increased by the addition of iRhom2 (**Supplementary Fig. 7b**). Conversely, knockdown of iRhom2 impaired the interaction of STING with EIF3S5, but not with other candidates (**Supplementary Fig. 7c**). These results suggest that iRhom2 functions as an adaptor to recruit EIF3S5 to STING. Confocal microscopy revealed that, similar to STING, EIF3S5 was localized at the ER and Gogli apparatus, but was undetectable at autophagosomes in both uninfected and HSV-1-infected cells (**Supplementary Fig. 7d**). Notably, EIF3S5 was colocalized with STING and iRhom2 in uninfected cells and at condensed perinuclear microsomes in HSV-1-infected cells (**Supplementary Fig. 7e**).

In a mammalian overexpression system, iRhom2 substantially increased the ability of EIF3S5 to remove K48-linked polyubiquitin moieties from STING (**Fig. 7a**). Conversely, knockdown of EIF3S5 enhanced K48-linked polyubiquitination of STING in uninfected as well as cells infected at early phase (**Fig. 7b**). Notably, knockdown of EIF3S5 had minimal effects on K48-linked polyubiquitination of STING at the late phase of HSV-1 infection (**Fig. 7b**). These results suggest that EIF3S5 functions as a deubiquitinase for K48-linked polyubiquitin moieties of STING in uninfected and early infected cells, and this function of EIF3S5 is diminished at a late phase of viral infection.

We next determined whether EIF3S5 is involved in innate antiviral responses. We found that knockdown of EIF3S5 markedly abrogated HSV-1- or ISD-triggered expression of the *IFNB1*, *ISG56* and *CXCL10* genes (**Fig. 7c** and **Supplementary Fig. 7f**). Consistently, knockdown of EIF3S5 also inhibited HSV-1-triggered phosphorylation of TBK1 and p65 (**Fig. 7d**). In these experiments, knockdown of EIF3S5 caused the downregulation of STING both before and after HSV-1 infection (**Fig. 7b**,**d**). In addition, knockdown of EIF3S5 inhibited the synergetic effects of iRhom2 on STING-mediated expression of *IFNB1* and *IL6* genes (**Fig. 7e**). Collectively, these data suggest that EIF3S5 is involved in innate immune responses to DNA viruses by deubiquitinating K48-linked polyubiquitinated STING and preventing its degradation by the proteasome pathway.

iRhom2 mediates two independent processes

Given that iRhom2 modulates both the trafficking and stability of STING, we investigated whether these two processes are interdependent. Confocal microscopy revealed that knockdown of EIF3S5 did not affect ISD-induced STING trafficking (**Fig. 8a**). On the other hand, the protein abundance and K48-linked polyubiquitination of STING were not markedly changed in TRAPβ knockdown in comparison with control cells (**Fig. 8b**). These results suggest that iRhom2-mediated trafficking and stability of STING are independent.

DISCUSSION

We found that iRhom2 acts in two ways to regulate STING-mediated innate antiviral response: by mediating TRAPβ-dependent translocation and activation of STING, and by linking the deubiquitinase EIF3S5 to STING and facilitating the deubiquitination and stability of STING.

iRhom2 deficiency markedly impaired DNA-virus- and intracellular-DNA-induced signaling in cells, and *iRhom2*$^{-/-}$ mice were more susceptible to lethal HSV-1 infection. Notably, iRhom2 deficiency markedly inhibited, but not completely abolished, HSV-1-induced transcription of downstream cytokine genes, such as *Ifnb* and *Il6*. In contrast, the secretion of IFN-β and IL-6 following HSV-1 infection were completely abolished in iRhom2-deficient cells. As it has been shown that iRhom2 is required for the secretion of TNF[28,29], the simplest explanation is that iRhom2 is not only important for virus-triggered transcription of downstream antiviral genes, but is also required for maturation and secretion of the cytokines induced by the virus. In addition, we noticed that transcriptional induction of downstream genes by transfected nucleic acid mimics were abolished in *iRhom2*$^{-/-}$ cells, whereas induction of these antiviral genes following HSV-1 infection was markedly, but not completely, inhibited in *iRhom2*$^{-/-}$ cells. It is possible that iRhom2 is required for induction of downstream antiviral genes mediated by viral nucleic acid, but not by other viral components.

Our results suggest that iRhom2 acts as a critical link for TRAPβ-mediated translocation and activation of STING. iRhom2 was colocalized with STING in the ER and Golgi apparatus in resting cells and both

proteins were translocated from the ER to the ERGIC, Golgi apparatus and perinuclear microsomes following HSV-1 infection. The formation of the perinuclear microsomes of STING following HSV-1 infection or DNA transfection were completely impaired in *iRhom2*$^{-/-}$ cells. These data suggest that iRhom2 is essential for the trafficking of STING from the ER to Golgi apparatus and perinuclear microsomes. We found that iRhom2 was associated with TRAPβ and promoted the association STING with TRAPβ. In addition, knockdown of TRAPβ markedly inhibited ISD-induced STING trafficking. Notably, knockdown of TRAPβ markedly abrogated expression of downstream antiviral genes induced by STING and iRhom2. These results suggest that iRhom2 links STING to the TRAPβ translocation complex and facilitates the trafficking of STING from the ER to the ERGIC, Golgi apparatus and the perinuclear microsomes.

Various studies have demonstrated that STING is regulated by different types of polyubiquitination[34]. However, little is known about the deubiquitination of STING. We identified EIF3S5 as a deubiquitinase for K48-linked polyubiquitin chains conjugated to STING. iRhom2 promoted the association of STING with EIF3S5, whereas knockdown of iRhom2 severely impaired their association. In addition, EIF3S5 inhibited K48-linked polyubiquitination and degradation of STING, whereas knockdown of EIF3S5 had the opposite effect. Consistent with this knockdown of EIF3S5 markedly inhibited DNA-triggered expression of downstream genes. These results suggest that EIF3S5 is a deubiquitinase for STING and that the iRhom2-EIF3S5 axis is required for maintaining the stability of STING.

It is interesting to note that, although iRhom2 mRNA is upregulated by viruses, the expression of iRhom2 is decreased at the late phase of viral infection. In addition, we also found that iRhom2 was constitutively associated with STING in unstimulated cells, and this association was increased at the early phase and then decreased at the late phase of viral infection. These observations indicate that the positive feedback regulation of iRhom2 is turned down at the late phase of viral infection. It is possible that iRhom2 is directly degraded by K48-linked polyubiquitination, lysosomal or other mechanisms at the late phase of viral infection. Alternatively, the activation of the iRhom2-mediated virus-triggered pathways may be terminated independently of targeting iRhom2. For example, it has been demonstrated that Atg9a negatively regulates DNA-induced immune response by inhibiting the dynamic translocation of STING[35]. It has also been shown that ULK1(ATG1) phosphorylates STING at S366 and causes degradation of STING, thereby preventing the persistent innate immune response[36].

On the basis of our findings and previously published results, we propose a working model of iRhom2-mediated regulation of STING activities in innate antiviral responses to DNA viruses. In resting cells, iRhom2 constitutively interacts with STING and EIF3S5 in the ER. In these complexes, EIF3S5 constitutively deubiquitinates STING and prevents its degradation by the proteasomal pathways. Following infection by DNA virus, iRhom2 links STING to the TRAPβ translocator, which facilitates the trafficking of STING to the Golgi apparatus and to the perinuclear microsomes, as well as recruitment of downstream TBK1 and IRF3 to the STING complex, leading to induction of downstream antiviral genes. Our results provide important insights into the molecular mechanisms of innate immune responses to DNA viruses.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

H.-B.S., S.L. and W.-W.L. conceived and designed the study. S.L. performed the human cDNA expression plasmid screens. W.-W.L., C.L., H.L. and Q.Y. performed the majority of experiments. H.B.S., S.L., W.-W.L., C.L., H.L. and B.Z. analyzed the data. H.-B.S. and W.-W.L. wrote the manuscript. All of the authors discussed the results and commented on the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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