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Bao-cun Zhang[1], Ramya Nandakumar[1*], Line Reinert[1*], Anders Laustsen[1], Søren Beck Jensen[1], Sonia Assil[1], Martin F. Bertehlsen[1,2], Carsten Scavenius[3,4], Samuel J Windross[1], Chenglong Sun[1], Chiranjeevi Bodda[1], Ryo Narita[1,4], Yujia Cai[1], Harald Stenmark[5], Christine M. Doucet[6], Takeshi Nod[7], Raphaela Goldbach-Mansky[8], Rune Hartmann[4], Jan J Enghild[3,4], Rasmus O Bak[1], Martin K. Thomsen[1,2], Søren R Paludan[1]

[1] Department of Biomedicine, Aarhus University, DK-8000 Aarhus, Denmark

[2] Department of Clinical Medicine, Aarhus University, DK-8000 Aarhus, Denmark

[3] Interdisciplinary Nanoscience Center, Aarhus University, DK-8000 Aarhus, Denmark

[4] Department of Molecular Biology and Genetics, Aarhus University, DK-8000 Aarhus, Denmark.

[5] Department of Molecular Cell Biology, Institute for Cancer Research, Oslo University Hospital, Montebello, N-0379 Oslo, Norway.

[6] Centre de Biochimie Structurale (CBS), INSERM, CNRS, Université de Montpellier , France.

[7] Graduate School of Frontier Biosciences, Osaka University, Osaka, Japan.

[8] Translational Autoinflammatory Diseases Section, National Institute of Allergy and Infectious Diseases (NIAID), National Institutes of Health (NIH), Bethesda, MD, USA

* These two people contributed equally to the work

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Correspondence to be addressed to Søren R Paludan (srp@biomed.au.dk)

Abstract

STING is essential for control of infections and for tumor immunosurveillance, but can also drive pathological inflammation. STING resides on the ER, and traffics following stimulation to ERGIC/Golgi where signaling occurs. Although STING ER exit is the rate-limiting step in STING signaling, the mechanism that drives this process is not understood. Here we identify STEEP as a positive regulator of STING signaling. STEEP was associated with STING and promoted STING traffic from the ER. This was mediated through stimulation of phosphatidylinositol-3-phosphate (PI3P) production, which promotes ER membrane curvature formation and facilitates COPII-mediated ER-Golgi traffic of STING. Depletion of STEEP impaired STING-driven gene expression in response to virus infection in brain tissue and in cells from SAVI patients. Interestingly, SAVI-associated STING mutants interacted strongly with STEEP leading to increased ER PI3P levels and membrane curvature. Thus, STEEP promotes STING signaling by allowing ER exit.

Introduction

Cytosolic DNA is a potent danger signal, leading to activation of innate immune responses, most notably expression of the cytokines type I interferon (IFN) and interleukin 1β[1]. Induction of type I IFN by cytosolic DNA is mediated by cyclic GMP-AMP (cGAMP) synthase (cGAS), which produces 2'3'cGAMP upon DNA binding[2]. 2'3'cGAMP in turn binds the endoplasmic reticulum (ER)-resident adaptor protein stimulator of IFN genes (STING), which then undergoes conformational changes in a dimeric form and recruits the kinase TBK1[3, 4, 5]. Upon activation, TBK1 phosphorylates STING at serine 366, thus allowing recruitment of the latent transcription factor IFN regulatory factor 3 (IRF3), and positioning of IRF3 for TBK1-mediated phosphorylation[6, 7]. Upon phosphorylation, IRF3 dissociates from STING to form dimers, and translocate to the nucleus to activate transcription. In addition to type I IFN, the cGAS-STING pathway also induce expression of inflammatory gene, autophagy, and apoptosis[8, 9].

The cGAS-STING pathway is essential for control of a wide panel of human pathogens[10, 11, 12] and for tumor immunosurveillance[13, 14]. Moreover, several inflammatory diseases are driven by excessive activation of STING signaling[15, 16, 17, 18, 19]. Therefore, pharmacological activation of the STING pathway is currently being tested as vaccine adjuvants as well as in cancer immunotherapy[20, 21], and cGAS-STING antagonism holds promise for treatment of inflammatory diseases[22, 23]. Given the clinical importance of the cGAS-STING pathway in human diseases, there is an urgent need to obtain in-depth understanding of the mechanisms governing its activity during defense and disease.

Although STING is an ER-resident protein in the resting state, active STING signaling occurs in the Golgi/ERGIC[4, 24, 25]. Gain-of-function STING mutants from patients with S*TING*-associated

vasculopathy with onset in infancy (SAVI) translocate to the Golgi and signal independent of cGAMP binding[18, 19, 25]. In fact, it was shown that STING translocation is a rate-limiting event in STING signaling, and proposed that STING is actively retained in the ER, and released through a specific mechanism, which involves the Ca^{2+} sensor STIM1 [25, 26]. Despite this, there is limited knowledge on the mechanisms that govern STING ER exit. Components described to be involved in STING ER exit include translocon proteins[4], the autophagy-associated phosphatidylinositol 3-kinase VPS34[27], inactive rhomboid protein 2 (iRhom2)[28], Sorting nexin 8 (SNX8)[29], the ubiquitin regulatory X domain-containing proteins 3B (UBXN3B) [30], transmembrane emp24 protein transport domain containing 2 (TMED2)[31]. However, the actual mechanism of STING ER exit is not known.

Here we identify CxORF56 as a protein that interacts with STING, and demonstrate that this protein is essential for STING ER exit, and for initiation of signaling and induction of autophagy as well as expression of interferon and inflammatory cytokines. We propose the name STING ER exit protein (STEEP) for CxORF56. STEEP mediates DNA-induced STING-dependent increase in ER phosphatidylinositol-3-phosphate (PI3P) production, ER membrane curvature, and COPII-dependent STING ER-to-Golgi Trafficking. STEEP deficiency inhibits STING-dependent signaling by dsDNA and human pathogenic microorganisms, and also prevents ligand-independent signaling by STING mutants from patients with SAVI. Thus, STEEP is essential for the critical ER exit step in STING signaling.

Results

Identification of STEEP as a STING-interacting protein promoting IFNβ induction

To identify factors that govern early STING signaling, STING was immunoprecipated from lysates of resting Myc-STING iBMDCs, and interacting proteins were identified by Mass Spectrometry (Fig. 1a). This lead to identification of a number of STING-interacting proteins (Supplementary Table 1). From this list, 9 proteins were selected for further analysis, using overexpression in HEK293T-STING cells and IFNβ promoter activation as the evaluation system. Only one of the tested candidate proteins, CxORF56, elevated cGAMP-mediated IFNβ promoter activation, while several of the candidates inhibited activation (Fig. 1b). We propose the name STEEP for CxORF56. STEEP is highly conserved through evolution from mammals to nematodes, and is more conserved than STING (Supplementary Fig. 1a). The protein belongs to the uncharacterized UPF0428 family, and is highly expressed in most tissues (Supplementary Fig. 1b). STEEP can occur in 3 isoforms, and the amino acid sequence contains both nuclear entry and nuclear exit signals (Supplementary Fig. 1c-d). Consistently STEEP was found in both the nuclear and cytoplasmic fraction of THP1 cell lysates (Supplementary Fig. 1e). Finally, STEEP is predicted not to contain transmembrane regions, unlike STING, which contains four membrane-spanning regions (Supplementary Fig. 1f).

Further analyses revealed that STEEP overexpression elevated cGAS- and cGAMP-driven IFNβ promoter activity in HEK293T-STING cells in a dose-dependent manner (Fig. 1c-d), whereas STEEP overexpression did not affect TBK1-, IRF3-5D-, MAVS- or TRIF- mediated IFNβ promoter activity (Fig. 1e-f, Supplementary Fig. 2a). Co-immunoprecipitation of endogenous STING and STEEP in THP1 cells confirmed that STEEP and STING interact constitutively, and this is further elevated early after stimulation (Fig. 1g). We could also detect Flag-tagged STING to co-precipitate with STEEP as well (Supplementary Fig. 2b). Consistently, we observed significant colocation

between STEEP and STING in Hela cells before and after cGAMP stimulation (Fig. 1h). Taken together, these data suggest that STEEP associates with STING at the early stages of signaling to promote type I IFN induction.

STEEP deficiency impairs STING-dependent signaling

To further characterize the importance of STEEP in the cGAS-STING pathway, two different guide RNAs (gRNAs) were designed targeting two different *STEEP* exons, and clonal THP1 cell lines were generated using CRISPR-Cas9 technology (Supplementary Fig. 3a-b). Interestingly *STEEP*$^{-/-}$ cells exhibited significantly attenuated the expression of *IFNB*, *CXCL10*, and *IL6* after stimulation with dsDNA and cGAMP (Fig. 2a-d). In addition, STEEP deficiency significantly affected the expression of *IFNB* induced by the human pathogenic DNA viruses HSV-1 and HSV-2 (Fig. 2e), and the induction of type I bioactivity by HSV1 infection (Fig. 2f). By contrast, IFNB expression in response to the RNA virus Sendai virus was not affected in *STEEP*$^{-/-}$ cells (Fig. 2g). In addition to IFN responses, STING signaling can also induce NF-κB activation, autophagy, and apoptosis[32]. As shown in Fig. 2H, STEEP deficient cells have reduced LC3 conversion, p62 degradation, and p65 phosphorylation??, but not cleaved caspase 3 after dsDNA-stimulation. This suggests that STEEP is essential for STING-mediated induction of IFN, autophagy, and NF-kB, but not apoptosis.

STEEP is essential for signaling events between STING dimerization and TBK1 recruitment

To start to identify how STEEP regulates cGAS-STING signaling, we compared the activation of different steps in the pathway following stimulation in wild type (WT) cells and *STEEP*$^{-/-}$ cells. Consistent with the reduced IFN induction in STEEP-deficient cells, the phosphorylation STING and TBK1 was clearly reduced in *STEEP*$^{-/-}$ THP1 cells after stimulation with dsDNA or cGAMP (Fig. 3a-b). This was paralleled by reduced recruitment of TBK1 to STING in STEEP deficient

cells after cGAMP stimulation (Fig. 3c). The observed impairment of STING and TBK1 phosphorylation was also observed in polulations of Hela and HFF cells transduced with Cas9-STEEP gRNA expressing lentivirus (Supplementary Fig. 4a-b). However, STEEP-deficiency did not affect STING dimerization after cGAMP stimulation (Fig. 3d).

Given the essential role of ubiquitination in the STING pathway [33], we explored whether STEEP could influence STING ubiquitin levels. By TUBE assay, we found that K63-linked ubiquitination of STING was markedly inhibited in *STEEP*$^{-/-}$ cells following cGAMP stimulation (Fig. 3e). In HEK 293T cells, we contransfected Flag-tagged-STING, untagged STEEP, and HA-tagged ubiquitin mutants with all lysines mutated into arginines except for the one indicated. After immunoprecipitation and re-immunoprecipitation with anti-Flag, we observed that STEEP coexpression significantly enhanced K27- and K63-linked poly-ubiquitination chains on STING, and slightly promoted K48-linked poly-ubiquitination (Fig. 3f). Collectively, these data suggest that STEEP acts in the STING activation pathway downstream of STING dimerization and upstream of STING ubiqutination, phosphorylation and recruitment of TBK1, all of which occur after ER exit of STING[24, 34].

STEEP promotes STING trafficking from ER to Golgi

To evaluate whether STEEP promotes STING trafficking, microsomes from *STEEP*$^{-/-}$ THP1 cells were mixed with cytoplasm from wt and *STEEP*$^{-/-}$, and we evaluated co-precipitation of STING with small vesicles (Fig. 4a). Interestingly, we observed that more STING was found in the vesicle fraction when STEEP was present in the cytoplasmic fraction (Fig. 4b). When STING localization was monitored, we observed that expression of STEEP in HEK293T-STING cells reduced the levels of STING in the ER while increasing the localization of STING to the Golgi (Fig. 4c-d).

Similarly, confocal microscopy revealed that STING failed to traffic efficiently from ER to Golgi in cells lacking STEEP (Fig. 4e). Sar1 is a protein involved in COPII vesicle formation on the ER [31, 35]. Overexpression of WT, but not an inactive form of Sar1 (Sar1-H79G), led to STING activation and promoted STING trafficking in HEK293T-STING cells (Supplementary Fig. 5a-c). Additionally, we found that the COPII complex protein SEC24 interacted with STING and the interaction could not be inhibited by treatment with brefeldin A (BFA), which blocks ER-to-Golgi trafficking of COPII vesicles (Supplementary Fig. 5d). When evaluating for Sar1 recruitment to the ER, we observed that this cytoplasmic protein was indeed abundantly localized to the ER following STING activation in Wt THP1, but not in *STEEP*$^{-/-}$ cells (Fig. 4f and Supplementary 5e). Based on the knowledge that Sar1 induces membrane curvature through the amphipathic N-terminal helix[36], we wanted to examine ER membrane curvature in STING-activated cells, and the dependence on STEEP. Using the ALPS (Amphipathic Lipid Packing Sensors) motif GFP[133] membrane curvature probe together with ER-specific staining, we found that ER membrane curvature is indeed induced by cGAMP stimulation and STEEP overexpression compared to controls (Fig. 4g, Supplementary Fig. 5f-g). In addition, we found STING to cluster at ER curvature sites after cGAMP stimulation (Supplementary Fig. 5h). These data suggest that STEEP promotes STING trafficking from ER to Golgi by inducing ER membrane curvature, which enables loading into COPII complexes.

STEEP augments ER PI3P production to promote membrane curvature and STING ER exit

Based on the observation that STEEP promotes STING ER exit and ER membrane curvature, we wanted to explore how this is mediated. It was reported that PI3P but not PI4P is enrichment at the ER, and suppression of the PI3P kinase Vps34 has been reported to reduce DNA-induced type I IFN expression [27, 37]. Accumulation of negatively charged PI3P in one lipid layer of a lipid bilayer increases repulsive forces between polar heads, thus promoting membrane curvature [38]. First, we observed that STING agonist treatment elevated PI3P levels on the ER and that STING can bind to

PI3P (Fig. 5a, Supplementary Fig. 6a-c). In agreement with this, PI3P exhibited significant colocalization with STING (Fig. 5b, Supplementary Fig. 6d). Second, the PI3P addition to cells promoted STING activation (Fig. 5c), STING vesicles formation (Fig. 5d), and ER membrane curvature (Fig. 5e) [to be repeated]. Consistently, overexpression of a PI3P hydrolase MTMR3, but not an inactive mutant, inhibits activation of the STING pathway (Supplementary Fig. 6e) correlating with impaired STING trafficking from the ER to the Golgi (Supplementary Fig. 6f-g), reduced Sar1 recruitment to the ER (Supplementary Fig. 6h), and significantly decreased the level of ER membrane curvature induced by cGAMP stimulation (Supplementary Fig. 6i).

Importantly, the accumulation of PI3P on the ER following STING activation was strongly reduced in STEEP-deficient cells (Fig. 5f-g). Altogether, these data demonstrate that STEEP promotes ER PI3P accumulation, which induces ER membrane curvature to reinforce COPII-mediated STING ER exit.

STEEP recruits the VPS34 complex I to ER to produce PI3P

We confirmed previous findings that inhibition of the PI3P kinase VPS34 can suppress the STING activation (Supplementary Fig 7a-b). VPS34 can engage in two different complexes to produce PI3P; could be produced by two VPS34 complexes including VPS34 complex I (VPS34, Beclin1, VPS15, and ATG14) and Complex II (VPS34, Beclin1, VPS15, and UVRAG)[39]. Through overexpression in HEK293T cells, we found that enforced expression of VPS34 and ATG14 alone promotes *IFNB* promoter activity (Supplementary Fig. 7c). In addition, endogenous STEEP associated with ATG14 and VPS34 but not UVRAG, and cGAMP stimulation can enhance these interactions (Fig. 6a). Notably, recruitment of VPS34 complex I to the ER after STING activation was reduced in *STEEP*$^{-/-}$ cells compered to controls (Fig. 6b, Supplementary Fig. 7d). Finally, we found that overexpression of vps34 complex I proteins promote STING trafficking from ER to

Golgi (Fig. 6c-d), increase PI3P levels at the ER (Fig. 6e), and induce ER membrane curvature (Fig. 6f). These observations demonstrate that the VPS34 complex I is recruited to the ER in a STEEP-dependent manner ER to produce PI3P, which is essential for STING trafficking.

STEEP is important for STING activation in primary human cells and mice

To explore whether STEEP contributes the activation of STING signaling in primary human cells we targeted the STEEP gene with gRNAs in primary macrophages and fibroblasts (Supplementary Fig. 3a, c, d). Under these conditions, we found that suppression of STEEP expression impaired induction of *IFNB* mRNA expression (Fig. 7a-b). To test whether STEEP was important for immune responses at the tissue level, we targeted the STEEP gene in brain slices from Cas9+ mice using AAV-mediated delivery of gRNA (Supplementary Figs. 3f-h, and 8a). We have previously reported that cGAS and STING are essential for control of HSV1 in the mouse brain[10]. Interestingly, reduced STEEP expression in the brain led to elevated HSV replication in the tissue and impaired ISGs response (Fig. 7c, d). Thus, STEEP promotes STING-dependent antiviral immune responses.

SAVI patient STING activation is dependent on STEEP

SAVI-associated STING gain-of-function mutations drive constitutive type I IFN production without requirement for STING agonists[18]. Given the positive role for STEEP in STING activation, we wondered whether STEEP was important for the activity of SAVI-associated STING mutants. When the STEEP gene was targeted in SAVI patient cells (Supplementary Fig 3e), we observed that STING signaling and expression of *IFNB* and ISG genes was reduced (Fig. 7e-g). In addition, STEEP tended to have stronger interaction with SAVI STING variants compared to WT STING (Fig. 7h). Moreover, cells expressing SAVI STING variants had higher levels of PI3P at the ER,

and increased ER membrane curvature (Fig. 7i-j). Removal of PI3P with MTMR3 reversed this difference between the variants (Supplementary Fig 8b). These data demonstrate that the STEEP-STING interaction facilitates ligand-independent signaling by SAVI-associated STING mutants by promoting PI3P production, ER membrane curvature, and STING trafficking.

Discussion

The cGAS-STING pathway is central for defense against a large panel of infections, and for efficient anti-cancer immune responses [10, 11, 12, 13, 14, 33]. On the other hand, this pathway is also responsible for the excessive immune response that cause development of some autoinflammatory diseases [15, 16, 17, 18, 19]. Thus, tight control of STING signaling is essential for ensuring stimulus specific signaling and avoidance of undesired stimulus-independent activity. STING ER-to-golgi traffic is an essential early step in STING signaling[24], and has been suggested to be a rate-limiting step in activation of the pathway[25]. Recently it was reported that STIM1 plays an essential role in retaining STING in the ER[26]. Despite this, it is not known what mechanisms govern STING exit from the ER to reach the ERGIC/golgi where TBK1 is recruited and signaling is activated. We report here that STING interacts with STEEP, and this promotes VPS34-mediated production of PI3P at the ER. This leads to recruitment of the membrane bending proteins Sar1, membrane curvature, and association of STING with the COPII vesicle machinery, and traffic out of the ER. STEEP-dependent STING ER exit is essential for STING signaling in antiviral defense and in inflammatory diseases.

Several proteins have been reported to interact constitutively with STING and to be essential for STING traffic including iRhom2[28], SNX8[29], UBXN3B [30], and TMED2[31]. Despite this, in-depth understanding on the key question as to how STING leaves the ER to start assembly of the signaling complex at the ERCIG/Golgi has remained unresolved. We found that STEEP interacts constitutively with STING, that this interaction is augmented following DNA stimulation and also for SAVI-associated STING mutants. The STING-STEEP interaction in turn promotes STING trafficking. The cryo-EM structure of cGAMP-bound STING was recently reported and revealed a 180° rotation of the ligand-binding domain relative to the transmembrane domain upon ligand

binding[40]. This rotation involves the region where SAVI-associated mutations have been identified, and it was speculated that these STING mutants have lower threshold for undergoing the domain rotation spontaneously. The regions of STING and STEEP remain to be determined, but it is tempting to speculate that the altered surface of the STING dimer following rotation of the ligand-binding domain relative to the transmembrane domain may increase the affinity of STING for STEEP.

The trafficking of proteins from the ER can occur through ER-associated protein degradation (ERAD) or COPII. While the former targets misfolded proteins for proteasomal degradation[41], the latter mediates traffic of proteins from the ER to the golgi through vesicular transport[42]. COPII-mediated vesicular budding is protein transport is dependent on the GTPase Sar1 and the coat protein complex consisting of Sec23/24, and Sec13/31. In addition, an ER-associated pathway for control of vesicles in the perinuclear area has been described[43]. Recent publications reported that STING exits the ER through COPII-mediated export[31, 44, 45], and accordingly we found that STING activation lead to recruitment of Sar1 to the ER in a STEEP-dependent manner upstream of STING ER exit. Sar1 is recruited from the cytoplasm to the ER, and it's recruitment and GTPase activity is dependent on membrane curvature[35]. High local concentration of PI3P in a membrane is well-described in promote membrane curvature, via repulsive forces between the negatively charged polar heads. We observed that the PI3K VPS34 complex I was recruited to STING in a STEEP-dependent manner, and that STEEP promoted PI3P accumulation on the ER. Moreover, Sar1 was recruited to the ER following STING activation in a STEEP-dependent manner and STING ER-exit was prevented by inhibition of PI3K activity. Collectively, these data demonstrate that STEEP promotes formation of ER membrane curvature to enable COPII-mediated trafficking by promotion of PI3P accumulation in the ER membrane (Supplementary Fig. 9).

STEEP is evolutionarily highly conserved, with strong homology between all vertebrates, and with clear orthologs to human STEEP in arthopods and nematodes. By contrast, nematodes do not have STING and only few arthopods do. This suggests that STEEP is involved in an ancient cellular process, which STING took advantage of as the cGAS-STING pathway developed. Based on the data presented in this work, we suggest that STEEP is involved in ER membrane modulation, including the interaction with the COPII trafficking machinery. It should be mentioned that STEEP has a predicted nuclear localization signal, and a pool of STEEP localizes to the nucleus. Thus, it is likely that STEEP also has other functions, which have yet to be discovered. The tight connection between the STING pathway and membrane modulatory systems is further highlighted by the finding that STING induces autophagy[45, 46], and that several autophagy-related proteins are involved in both activation and regulation of STING signaling[8, 24, 27]. This includes VPS34, the activity of which we here show is augmented by STEEP upon DNA stimulation. We observed that STING-induced expression of IFN and IL6 as well as activation of autophagy were all dependent on STEEP, suggesting a requirement for STING to exit from the ER for these processes. However, STING dependent caspase 3 cleavage is independent of STEEP suggests that not all STING-driven activities are dependent on STING to leave the ER. We did not explore the role of STEEP in STING dependent ER stress[47]. Unraveling the determinants, which governing whether STING relays signaling to IFN expression or cell death pathways in different immunological contexts deserves urgent attention.

A key observation of the present study is that depletion of STEEP in mouse brain tissue reduces activation of STING signaling and impairs antiviral defense. In addition, we observed that signaling by SAVI-associated STING was dependent on STEEP. In parallel, STEEP interacts more strongly

with SAVI-associated than wt STING, and the disease variants also stimulated ER accumulation of PI3P production as well as ER membrane curvature more potently than wt STING. SAVI-associated STING variants can signal independent of cGAMP binding[18], Our data suggest that the elevated STEEP interaction of SAVI-associated STING variants, which leads to elevated PI3P production and ER membrane curvature, could allow STING ER exit, and hence signaling independent of cGAMP binding. Therefore, pharmacological targeting of the STING-STEEP interaction holds potential for treatment of SAVI and other STING-dependent inflammatory diseases.

Subcellular trafficking is of central importance for activation and gearing of signaling by innate immune receptors, including TLRs, RIG-I, and cGAS[48, 49]. In this work we identify STEEP as a key factor in STING ER exit, which is the rate-limiting step in STING signaling[25]. STEEP acts by inducing PI3P production on the ER, thus promoting ER membrane curvature, and sorting of STING into COPII vesicles for trafficking to the Golgi where active signaling takes place.

Methods

Cells, Viruses, and Reagents

The cell lines used for the study were iBMDC-MycSTING, THP1, HEK293T cells, HaCaT, Hela cells, and Human foreskin fibroblasts (HFF) cells. SAVI patient-derived fibroblasts cells were obtained as described previously[18]. Human blood-derived monocytes and primary fibroblasts were obtained XXXXX? (Rasmus/Anders). The virus used were HSV-1 (Strain F+, and McKrae), HSV-2 (strain 333), Sendai virus (strain Cantrell). The reagents used were 2'3'-cGAMP (BIOLOG), 60‐mer dsDNA (DNA technology), PI(3)P diC8 (Echelon), phorbol 12-myristate 13-acetate (PMA, Sigma), Brefeldin A (BFA, Sigma), Bafilomycin A1(BafA1, InvivoGen), 3-Methyladenine (3-MA, InvivoGen), Vps34 Inhibitor 1 (VPS34 IN1, Cayman).

Cell line culture and transfection

HEK293T, Hela and HFFs were cultured in DMEM with 10% Fetal bovine serum (FBS) and 100 units/ml penicillin, 100 μg/ml streptomycin. THP1 cells were cultured in RPMI 1640 media with L-glutamine supplemented with 10% Fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin. THP1 cells were differentiated into macrophage cells using PMA (150nM) overnight. The PMA was removed and cells were used for experiments 24 h later. The 60mer double-stranded DNA (dsDNA) was transfected into cells by Lipofectamine 2000 according to manufacturer's instructions. The 2',3'-cGAMP was transfected into cells by digitonin according to previous study[50].

Mass spectometry

Myc was immunoprecipitated from whole cell lysates from iBMDC-MycSTING cells using the protocol described under "Co-Immunoprecipitation" below. The immunoprecipitate was subjected to mass spectrometry analysis. Briefly, XXXXX (Carsten, Jan).

Generation of genome-edited cell lines

The STEEP knockout in THP1, Hela, and HFF cells was generated by CRISPR/Cas9 technology as previously described[51]. The gRNAs were used in human cell lines STEEP gRNA#1, 5′- GGAGACTATGTATCTGCGG- 3′; STEEP gRNA#2, 5′- gAACAGGAGCATTCTTTGGC- 3′ (Supplementary Fig. 3a). The cells were transduced by lentivirus packaged with the targeted gRNA. After the selection by 1ug/ml Puromycin, the knockout efficiency was analyzed by westernblot.

Genome-editing in primary human primary cells

CRISPR sgRNAs directed against AAVS1 (5'-GGGGCCACUAGGGACAGGAU-3') and CXorf56 (#1: 5'-GGAGACUAUGUAUCUGCGG-3', #3: 5'-UUUGGCAGCAUCAAUCACAC-3') were purchased from Synthego with three terminal nucleotides in both ends chemically modified with 2'-O-methyl-3'-phosphorothioate[52]. Cas9 protein (Alt-R S.p. Cas9 Nuclease 3NLS) was purchased from Integrated DNA Technologies (IDT). Ribonucleoprotein (RNP) complexes were made by complexing sgRNA at a molar ratio of 1:2.5 (Cas9:sgRNA) at 25 °C for 15 min prior to nucleoporation. Cells were resuspended in OptiMEM (Gibco, Thermo Fisher Scientific) and electroporated with a concentration of 300 µg/mL Cas9 protein using the Lonza 4D-Nucleofector System (program CM-138). For primary fibroblasts, $1x10^5$ cells were electroporated for each condition, using the 20 µL Nucleocuvette Strip format (Lonza), and were subsequently seeded into

a 24-well plate. For the generation of genetically modified primary macrophages, CD14+ monocytes were initially purified from PBMCs by negative immunomagnetic selection, according to the manufacturer's instructions (EasySep Human Monocyte Enrichment kit, STEMCELL Technologies). $4x10^6$ isolated monocytes were electroporated for each condition, using the 100 µL Nucleocuvette Vessel format (Lonza), and were subsequently distributed into 8 wells of a 24-well plate (5x105 cells/well). To induce differentiation of monocytes into macrophages, cells were cultured in DMEM supplemented with 10% heat inactivated human AB-positive serum for seven days in the presence of 15 ng/mL M-CSF (R&D Systems). Medium was replenished every third day. Seven days after transfection, Indel frequencies were quantified using TIDE (Tracking of Indels by Decomposition)[53] or ICE software (http://ice.synthego.com). For this, genomic DNA was extracted from the cells and PCR amplicons spanning the sgRNA target sites were generated using Phusion high-fidelity DNA polymerase (Thermo Fisher Scientific). The following primers were used: AAVS1 forward: 5'-ATTCGGGTCACCTCTCACTCC-3'; AAVS1 reverse: 5'-CAGGGTGGCCACTGAGAACC-3'; CXorf56 forward: 5'-ACCAGGGTAGTAAGAGAGCTGG-3'; CXorf56 reverse: 5'-GGCCGACATGGTGAAACTGC-3'. Purified PCR products were then Sanger-sequenced and Indel frequencies quantified with a reference WT sequence (mock-transfected sample) used as a control.

RNA extract and quantitative RT–PCR

The RNA isolation and qPCR was performed as manufacture's protocol with the following primers from Applied Biosystems: IFNB1 (Hs01077958_s1), ISG15 (Hs01921425_s1), IL6 (Hs00174131_m1), and CXCL10 (Hs01124251_g1). The mRNA level of the genes were normalized by β- actin using the formula $2^{Ct(\beta actin) - Ct(mRNA\ X)}$. The resulting normalized ratio (NR) was either presented directly in the figures.

Western blotting

Whole- cell extracts or immunoprecipitation samples were diluted in XT sample buffer and XT reducing agent, and loaded into 4-20% SDS–PAGE gel (Bio- Rad). The proteins were transferred from gel to PVDF membranes through Trans- Blot Turbo™ Transfer System® (Bio- Rad). The membrane was blocked in 5% nonfat skim milk (Sigma) for 1 hour at room temperature. For the STING dimerization assay, cell lysates were prepared by IP lysis buffer (Thermo Fisher Scientific) mixed with XT sample buffer but without XT reducing agent and complete mini inhibitor (Sigma). The information of antibodies used for immunoblotting is listed as follows: anti- STING (Cell Signaling, D2P2F/#13647, 1:1,000), sheep anti- STING (R&D Systems, AF6516, 1:500), rabbit anti- pSTING (S366) (Cell Signaling Technology, #85735, 1:1,000), rabbit anti- TBK1 (Cell Signaling, 3504, 1:1,000), rabbit anti- pTBK1 (Ser172) (Cell Signaling, D52C2/#5483, 1:1,000), mouse anti- p62 (Cell Signaling, # 88588, 1:1,000), rabbit anti- LC3 (Cell Signaling, #3868, 1:1,000), mouse anti-ERGIC53/LMAN1 (Santa Cruz Biotechnology, sc398893, 1:1000), rabbit anti-calreticulin (Abcam, #ab2907,1:1000), rabbit anti-GM130 (Cell Signaling, #12480, 1:1000), rabbit anti-cleaved caspase3 (Cell Signaling, #9664,1:1000), mouse anti- FLAG M2 (Sigma- Aldrich, F3165, 1:1,000), rabbit anti-ACTB (Cell Signaling, #4970,1:1000), rabbit anti- HA (Cell Signaling, #3274, 1:1,000), rabbit anti-VPS34 (MBL, #4263,1:1000), mouse anti-ATG14 (MBL, #M184-3, 1:1000), rabbit anti-UVRAG (MBL, # M160-3, 1:1000), mouse anti-Viperin Antibody (Millipore, #, 1:1000), rabbit anti-ISG15 (Cell Signaling, #2743, 1:1,000) and mouse anti- vinculin (Sigma, #V9131 , 1:10,000).

Co-Immunoprecipitation

For the Co-Immunoprecipitation of endogenous protein, the cells were lysis in IP lysis buffer with 1xProtease Inhibitor Cocktail (Sigma) and 10mM NaF, and then the cell lysates were centrifuged 2,500g for 10min at 4 ^{o}C. The cleared cell lysates were incubated with primary antibody against sheep anti-STING or rabbit anti-STEEP overnight at 4°C. Dynabeads^{TM} Protein G (Invitrogen) was added into the mixtures for extra 2 hours. After 4-6 times wash by IP lysis buffer, the immunoprecipitated complexes were eluted by glycine buffer (200 mM glycine, pH 2.5) with Protease Inhibitor Cocktail (Sigma) and 10mM NaF, and the elutes were evaluated by Western blotting.

For the Co-Immunoprecipitation of overexpressed proteins, the HEK293T cells were transfected for 24 hours by lipofectamine 2000, and then were lysis by IP lysis buffer with 1xProtease Inhibitor Cocktail and 10mM NaF. After 14000g centrifugation for 10min at 4 ^{o}C, the cleared cell lysates were incubated with FLAG® M2 Magnetic Beads (Sigma) for 2 hours at RT. After 4 times wash by 1xTBS buffer with 0.05% tween 20 (TBST), the immunoprecipitated complexes were eluted by 3X FLAG® tag peptide (Sigma-Aldrich) with Protease Inhibitor Cocktail (Sigma) and 10mM NaF, and the elutes were evaluated by Western blotting.

Purification of ubiquitin conjugates

The endogenous total- or K63 ubiquitylated proteins were isolated through Tandem Ubiquitin Binding Entities (TUBEs, LifeSensors) according to the manufacture's protocol. The whole cell lysates were centrifuged 14,000g for 10min at 4 ^{o}C. The cleared cell lysates were incubated with TUBE magnetic beads for 2 hours or overnight followed 4 times wash by TBST. For Western blot analysis, add ~25 µl of 2X SDS reducing sample prep buffer to the resin, and heat at ~95ºC for 5 min. Let stand for 3-5 minutes on the magnetic rack. Collecting the eluted supernatant carefully to avoid disturbing the beads.

For the ectopic ubiquitin, two-step immunoprecipitation was performed described previously[54]. Briefly, clear cell lysates after 14000g centrifugation were incubated with FLAG® M2 Magnetic Beads (Sigma) for 2 hours at RT. After 4 times wash by 1xTBST, the immunoprecipitated complexes were eluted by 3X FLAG® tag peptide (Sigma) with Protease Inhibitor Cocktail and 10mM NaF. and the elutes were evaluated by Western blotting. Then, the elutes were boiled for 5 min followed diluting in 1:10 with Lysis buffer. The diluted elutes were re-immunoprecipiated with the FLAG® M2 Magnetic Beads. After washing for 4 times, the immunoprecipitates were eluted by heating at 95ºC for 5 min in SDS reducing sample buffer and then subjected to immunoblot analysis.

ER isolation

The ER fractions were purified using Endoplasmic Reticulum Isolation Kit (Sigma). THP1 cells from ten 15cm dishes were harvested and homogenized by passing through a 22 G needle until ~85% lysis. The homogenates were centrifuged at 1,000×*g* for 10 min to remove cell debris and nuclear and then centrifuged at 12,000×*g* for 15 min to get a post mitochondrial fraction (PMF). The PMFs were centrifuged at 100,000 g for 1 hour to remove cytosol proteins and the pellets (crude microsome) were re-suspended in 1× Isotonic Extraction Buffer (10 mM HEPES, pH 7.8, 250 mM sucrose, 25 mM potassium chloride, and 1 mM EGTA). The crude microsomes were loaded onto 15-23% Opti-Prep gradient made by Gradient Master. Centrifuge in an ultracentrifuge for 18 hours at 80,000×g. At the end of the run, withdraw fractions of from the top of the gradient downwards using the 4-inch blunt ended needle. Westernblot was applied to identify the ER fraction.

Budding assay

The cytosol preparation was carried out following previously published work study[55]. The WT or STEEP ko THP1 cells were collected and re-suspended in B88 buffer (20 mM HEPES-KOH, pH 7.2, 250 mM sorbitol, 150 mM potassium acetate and 5 mM magnesium acetate) with protease inhibitors cocktail, phosphatase inhibitors (Roche) and 0.3 mM DTT. The cells homogenized by passing a 22 G needle for 60 times and the cell homogenates were centrifuged at 160,000×g for 30 min for 4 times. Collect the clarified supernatant fractions.

For the membrane fraction preparation, STEEP ko THP1 cells were lysis in the buffer containing 20 mM HEPES-KOH, pH 7.2, 400 mM sucrose and 1 mM EDTA. Homogenates were centrifuged at 1,000×g for 10 min and then the supernatant was further centrifuged at 100,000×g for 1 hour. After centrifugation, the pellets were dissolved in B88 buffer which was adjusted into OD600=10 for total membrane.

The budding reaction was performed at 30°C for 1 hr with membrane (OD5600=1), WT/STEEPko THP1 cytosol (2 mg/ml final concentration), ATP regeneration system (40 mM creatine phosphate, 0.2 mg/ml creatine phosphokinase, and 1 mM ATP), and GTP (0.15 mM)[56]. After reaction, the solution was centrifuged at 20,000g for 20min and the collected supernatant was centrifuged at 100,000g for 30min. The pellets were dissolved in the 2X SDS reducing sample prep buffer for Westernblot. All of the above centrifugation was carried out at 4°C.

Confocal microscopy

Hela or PMA- differentiated THP1 cells were seeded on coverslips and stimulated as indicated. The cells were fixed with methanol for 5 min at -20℃. Cells were blocked in 1xPBS with 1%BSA. Cells were stained with primary antibodies for 1 h/overnight and then stained by secondary antibody (all 1:300, Alexa Fluor; Invitrogen) for 1 h.

For the experiments related with PI3P, the cells were fixed with 4% paraformaldehyde (Sigma), permeabilized with Saponin (Sigma), and blocked with 10% Goat serum (Sigma). 1xTBS was used instead of 1xPBS. Images were acquired on Zeiss LSM 780 confocal microscope and processed with the Zen Blue software (Zeiss).

The antibodies used were sheep anti- STING (1:50), rabbit anti- calreticulin (1:100), mouse anti- GM130 (1:3000), mouse anti-Flag (1:300), rabbit anti-HA (1:50) and mouse anti-PtdIns(3)P (5ug/ml, echelon #Z-P003).

Imagestream

The colocation of STING-ER, STING-Golgi, SAR1-ER, and STING-PI3P was determined by the ImageStream MK II Imaging Flow Cytometer (Amnis, Co., Seattle, WA, USA). The cells transfected with the indicated plasmids for 24 hours, were fixed by 4% formalin for 20 min at RT, and then pre-permeabilized by 0.2% Triton X 100 for 6 min. The cells were incubated with primary antibodies for 1 hour on ice, and then incubated with the Alexa-Fluor-labeled secondary antibodies for 1 hour. After every step, the cells were washed by 1xPBS for 3 times. At last, the cells were resuspended in 1xPBS with 2mM EDTA and 3%BSA. A 60X magnification was used for all samples. The images of 7,000-10,000 single cells with different channels were acquired in the ImageStream and the data was analyzed through IDEAS software v6.2 (Amnis Corporation). The antibodies used were mouse anti- Flag (1:300), sheep anti- STING (1:50), rabbit anti-Calreticulin (1:50), rabbit anti-GM130 (1:3000), and rabbit anti-HA (1:50).

Preparation and genome-editing of mice brain slices.

Brain slices were isolated from Cas9+ mice and cultured according to the method previously described[10]. On the next day of isolation, the brain slices were infected with $1x10^{11}$ PFU Adeno-

Associated Virus (AAV) carrying mouse STEEP targeted gRNAs for 6 days. Then, the culture medium with AAV was removed and changed into the one with HSV-1 (Mckrae; $5x10^3$PFU in 3,5ml per brain slice). Two and five days post infection culture medium were collected and the viral loads were determined by plaque assay on Vero cells following the steps from previous study[10]. Meanwhile, at five days post infection, brain slices were homogenized and the tissue proteins were extracted by RIPA buffer (Thermo) with Protease inhibitor for the western blot as described above.

Statistics

The data are shown as means of biological replicates ± SD. Statistically significant differences between groups were determined using two- tailed Student's t- test when the data exhibited normal distribution and Wilcoxon rank- sum test when the data set did not pass the normal distribution test. The data shown are from single experiments. All experiments were performed at least 3 times with similar results.

AUTHOR CONTRIBUTIONS

B.C.Z. and S.R.P. conceived the idea and designed the experiments. B.C.Z., R.N., L.R., A.L., S.B.J., S.A., M.F.B., C.S., S.J.W., C.S., C.B., R.N., and Y.C. performed the experiments. H.S., C.M.D., T.D., and R.G-M provided materials, R.H., J.J.E., R.O.B., M.K.T., and S.R.P. supervised experiments. B.C.Z. and S.R.P. wrote the manuscript.

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Figure legends

Figure 1. Identification of STEEP as a positive regulator of STING-dependent signaling. (**a**) Graphical summary of identification of STING-interacting proteins. (**b**) Reporter gene assays for *IFNB1* promoter activity in HEK293T cells with STING stable expression co-transfected with *IFNB1* luciferase reporter, β-actin Renilla reporter, STING, and the indicated expression plasmids for 24 h and then stimulated by 100nM cGAMP for 6 hours (n= 3). (**c**) Reporter gene assays for HEK293T cells with STING stable expression transfected with 50ng STEEP or empty vector, and *IFNB1* promoter luciferase reporter, β-actin Renilla reporter for 24 hours and then stimulated by increased concentration of cGAMP (n= 3). (**d-f**) Reporter gene assays for HEK293T cells transfected with 50ng STEEP or empty vector, *IFNB1* promoter luciferase reporter, β-actin Renilla reporter, and increased concentration of cGAS, TBK1 or IRF3-5D as indicated for 24 hours (n= 3). (**g**) Endogenous STEEP and STING were immunoprecipitated from THP1 cell lysates isolated after cGAMP (100nM) with stimulation for the indicated time. Precipitates and lysates were immunoblotted with antibodies against STEEP, STING, and vinculin (n= 3). (**h**) Confocal microscopy of HeLa cells transfected with HA-STEEP and Flag-STING for 24 h followed by mock treatment or cGAMP stimulation (n= 3). Data in panel **b-f** are shown as means of biological triplicates +/- st.dev. * $p<0.05$; ** $p<0.01$.

Figure 2. STEEP is essential for IFN induction through the cGAS-STING pathway. (**a-g**) Wt and *STEEP*$^{-/-}$ THP1 cells were treated for 6 h with (**a-c**) dsDNA (500ng/ml), (d) for 2h with 2'3' cGAMP (50nM), (**e-f**) for 12h with HSV1 (MOI 1)/HSV2 (MOI 1), or (**g**) Sendai virus (MOI 1). Total RNA was isolated 6h post treatment, and *IFNB*, *CXCL10*, and *IL6* mRNA were measured as indicated by RT-qPCR. (**h**) Wt and *STEEP*$^{-/-}$ THP1 cells were treated with dsDNA (2ug/ml) for the indicated time intervals, and levels of LC3, p62, cleaved caspase 3 (CC3), p-p65??, STEEP and

actin were determined by immunoblotting. Data in panel **a-g** are shown as means of biological triplicates +/- st.dev. * $p<0.05$; ** $p<0.01$. (n= 3 for all data sets in the figure).

Figure 3 STEEP is essential for STING ubiquitination and recruitment of TBK1. (**a, b**) Immunoblot analysis of the indicated proteins from the whole cell lysates of WT or *STEEP*$^{-/-}$ THP-1 cells after stimulation with (**a**) dsDNA or (**b**) cGAMP for the indicated time intervals. (n= 3). (**c**) Immunoblot analysis of proteins co-immunoprecipitated with endogenous STING probed with the indicated antibodies. The material used for the immunoprecipitation was whole cell lysates from WT or *STEEP*$^{-/-}$ THP-1 cells stimulated with cGAMP for 1 h. (n= 3). (**d**) STING dimerization assay. Immunoblot analysis with anti-STING probing of lysates from WT or *STEEP*$^{-/-}$ THP-1 cells after stimulation with cGAMP for the indicated time intervals. The lysates were run on non-reducing SDS-PAGE prior to blotting. (n= 2). (**e**) K63-linkage ubiquitin TUBE assay and immunoblotting using anti K63-linkage ubiquitin and anti-STING antibodies. The material used was whole cell lysates from WT or *STEEP*$^{-/-}$ THP-1 cells stimulated with cGAMP for 1 h. (n= 3). (**f**) HEK293T cells were transfected with Flag-tagged STING, STEEP, HA-tagged Ub-K11, Ub-K27, Ub-K29, Ub-K33, Ub-K48 or Ub-K63 before co-immunoprecipitation and immunoblot analysis were performed with the indicated antibodies. (n= 2).

Figure 4. STEEP is essential for STING trafficking from ER to Golgi. (**a**) Diagram of the *in vitro* membrane budding reaction. (**b**) Immunoblot analyses of budded material from the *in vitro* membrane budding reactions using cytosolic fractions from WT or *STEEP*$^{-/-}$ THP1 cells and membrane fractions from *STEEP*$^{-/-}$ THP1 cells. LMAN1/ERGIC53 is involved in COPII cargo loading whose incorporation into budded vesicles. (n= 3). (c, d) Imagestream analysis of (**c)** STING-ER, and (**d**) STING-Golgi colocation. HEK293T cells were transfected with STEEP (or

empty vector) and Flag-tagged STING for 24 hours. After fixation and pre-permeabilization, the cells were incubated by mouse anti-flag and either rabbit anti-calreticulin (ER marker) or anti-GM130 (Golgi marker). (n= 3). (**e**) Confocal microscopy of WT and *STEEP*$^{-/-}$ Hela cells stimulated with cGAMP for 0.5 h. The cells were immunostained with anti-STING (red), anti-calreticulin (green), anti-GM130 (purple) in. Sections were counterstained with DAPI to visualize nuclei. (n= 2). (**f**) Imagestream analysis of SAR1-ER colocation. Flag-tagged SAR1 was transfected into WT or *STEEP*$^{-/-}$ Hela cells for 24 hours, and stimulated with cGAMP (100 nM) for 30min. After fixation and pre-permeabilization, the cells were incubated by rabbit anti-calveticulin (ER marker) and mouse anti-flag. (n= 3). (**g**) Imagestream analysis of the colocation between ER and ER curvature probe. GFP-tagged ALPS (GFP^{133}) was co-transfected with empty vector (control) or *STEEP* and Flag-tagged STING into HEK293T cells for 24 hours. After fixation and pre-permeabilization, the cells were incubated by rabbit anti-calveticulin (ER marker).(n=3).

Figure 5. STEEP governs PI3P accumulation on ER to promote STING ER exit. (**a**) Imagestream analysis of PI3P and ER colocation. HEK293T cells were co-transfected with GFP-tagged FYVE domain and Flag-tagged STING for 24 hours and stimulated cGAMP (150 nM) for 30min. After fixation and pre-permeabilization, ER was marked using anti-calreticulin. (n= 3). (**b**) Confocal microscopy of Hela cells transfected with GFP-FYVE and Flag-tagged STING and then treated for 20 min with vehicle or cGAMP (100 nM) and immunostained with and anti-Flag (red). Cells were stained with DAPI to visualize nuclei (n= 2).

(**c**) Immunoblot analyses of budded material from the *in vitro* membrane budding reactions incubated with increase PI3P does. LMAN1/ERGIC53 is involved in COPII cargo loading whose incorporation into budded vesicles. (n= 3).

(**d**) Immunoblot analysis of the indicated proteins from the whole cell lysates of THP-1 cells treated with 100uM PI3P after stimulation with cGAMP for the indicated time intervals. (n= 3).

(**e**) XXXXXXX

(**f, g**) Imagestream and Confocal microscopy analysis of PI3P-ER colocation in WT and *STEEP*$^{-/-}$ Hela cells. The cells were transfected by GFP-tagged FYVE and Flag-tagged STING for 24 hours and stimulated with cGAMP for 30 min. After fixation and pre-permeabilization, the cells were stained with anti-Flag, anti-calreticulin relevante secondary antibodies. (n= 3). Data in panel **a, e, f** are shown as means of biological triplicates +/- st.dev. * $p<0.05$; ** $p<0.01$.

Figure 6. STEEP recruits the VPS34 complex I to ER to produce PI3P. (**a**) Immunoblot analysis of proteins co-immunoprecipitated with endogenous STEEP probed with the indicated antibodies. The material used for the immunoprecipitation was whole cell lysates from THP-1 cells stimulated with 100nM cGAMP for 0, 0.5, and 1 h. (n=2). (**b**) Immunoblot analysis of the indicated proteins from the ER fraction of THP1 cells treated with cGAMP for 1 h. (n= 2). (**c, d**) Imagestream analysis of STING trafficking from ER (c) to Golgi (d). HEK293T cells were transfected by Control (VPS34, Beclin1, VPS15 and empty vector), Complex I (VPS34, Beclin1, VPS15 and ATG14) or Complex II (VPS34, Beclin1, VPS15 and UVRAG) and Flag-tagged STING for 24. After fixation and pre-permeabilization, flag and ER were labelled using rabbit anti-calreticulin (ER marker) and mouse anti-flag, and relevant secondary antibodies. (n= 3). (**e**) Imagestream analysis of PI3P-ER colocation. HEK293T cells were transfected by Control, Complex I or Complex II, GFP-FYVE and Flag-tagged STING for 24h. After fixation and pre-permeabilization, ER was labelled using rabbit anti-calreticulin (ER marker), and relevant secondary antibodies. (n= 3). (**f**) Imagestream analysis of ER membrane curvature. HEK293T cells were transfected by Control, Complex I or Complex II,

GFP^{133} and Flag-tagged STING for 24h. After fixation and pre-permeabilization, ER was labelled using rabbit anti-calreticulin (ER marker), and relevant secondary antibodies. (n= 3).

Figure 7. STEEP facilitates STING-dependent antiviral defense and pathological signaling. (**a, b**) Primary human monocyte-derived macrophages and fibroblasts treated with Cas9 and STEEP gRNA were stimulated with vehicle or cGAMP for 2 hours (100 nM). Total RNAs were harvested and *IFNB* levels was measured. (n= 3). (**c, d**) Brain slices from Cas9+ mice treated with *Steep*-specific gRNAs were infected with 5x10^{3}PFU HSV1 (**c**) Viral load in the culture supernatants were determined by plaque assay (n=4). (**d**) Immunoblot analysis of ISGs response by indicative proteins in HSV infected wt or steep ko mouse brain splice (n= 2). (**e-g**) Fibroblasts from SAVI patients were treated with Cas9 and control gRNAs or STEEP-specific gRNAs. Whole-cell lysates and total RNA was isolated, and analysed by immunoblot or RT-qPCR, respectively, with the indicated antibodies and PCR primers. (n= 3). (**h**) Lysates from HEK293T cells transfected with STEEP together with wt and SAVI-associated STING mutants were immunoprecipitated with anti-STING, and immunoblotted with antibodies directed against STEEP and STEEP. (n=2). (**i, j**) Imagestream analysis of PI3P-ER (**i**) and ER-GFP^{133} colocation (**j**). HEK293T cells were transfected with GFP-FYVE/GFP^{133} and STING/STING-SAVI mutants for 24 hours. After fixation and pre-permeabilization, the cells were incubated with rabbit anti-calreticulin. Data in panel **i-j** are shown as means of biological triplicates +/- st.dev. * $p<0.05$; ** $p<0.01$.