A conserved PLPLRT/SD motif of STING mediates the recruitment and activation of TBK1

Baoyu Zhao1,7, Fenglei Du1,7, Pengbiao Xu1, Chang Shu1, Banumathi Sankaran2, Samantha L. Bell3, Mengmeng Liu4, Yuanjiu Lei3, Xinxeng Gao5, Xiaofeng Fu5, Fanxiu Zhu5, Yang Liu6, Arthur Laganowsky6, Xueyun Zheng6, Jun-Yuan Ji4, A. Phillip West3, Robert O. Watson5 & Pingwei Li1,8

Nucleic acids from bacteria or viruses induce potent immune responses in infected cells1–4. The detection of pathogen-derived nucleic acids is a central strategy by which the host senses infection and initiates protective immune responses5,6. Cyclic GMP-AMP synthase (cGAS) is a double-stranded DNA sensor7,8. It catalyses the synthesis of cyclic GMP-AMP (cGAMP)9–12, which stimulates the induction of type I interferons through the STING–TBK1–IRF-3 signalling axis13–15. STING oligomerizes after binding of cGAMP, leading to the recruitment and activation of the TBK1 kinase8,16. The IRF-3 transcription factor is then recruited to the signalling complex and activated by TBK117–20. Phosphorylated IRF-3 translocates to the nucleus and initiates the expression of type I interferons21. However, the precise mechanisms that govern activation of STING by cGAMP and subsequent activation of TBK1 by STING remain unclear. Here we show that a conserved PLPLRT/SD motif within the C-terminal tail of STING mediates the recruitment and activation of TBK1. Crystal structures of TBK1 bound to STING reveal that the PLPLRT/SD motif binds to the dimer interface of TBK1. Cell-based studies confirm that the direct interaction between TBK1 and STING is essential for induction of IFN3 after cGAMP stimulation. Moreover, we show that full-length STING oligomerizes after it binds cGAMP, and highlight this as an essential step in the activation of STING-mediated signalling. These findings provide a structural basis for the development of STING agonists and antagonists for the treatment of cancer and autoimmune disorders.

Mass spectrometry analysis showed that several residues within the C-terminal tail (CTT) of STING are phosphorylated by TBK1 (Extended Data Fig. 1a, b). To investigate the roles of these residues, we generated several STING mutants and conducted IFN3 luciferase reporter assays. After stimulation with cGAMP, the reporter is activated in cells transfected with wild-type STING, but not in the control cells (Fig. 1a). Although the expression of STING alone induces 1–2-fold induction of the reporter, stimulation with cGAMP induces approximately ten times higher signals (Fig. 1a, Extended Data Fig. 1c). Consistent with previous studies16, the Ser366Ala mutation abolishes activation of the IFN3 reporter and phosphorylation of IRF-3 (Fig. 1a, b). By contrast, the Thr376Ala and Ser379Ala mutations do not affect activation of TBK1 or IRF-3 (Fig. 1b). Notably, deletion of the nine C-terminal residues of STING (ΔC9) abolishes activation of the reporter and phosphorylation of IRF-3 (Fig. 1a, b). In addition, deletion of these residues impairs activation of TBK1 (Fig. 1b). Confocal microscopy analysis revealed that the truncated STING mutant still translocates to perinuclear punctate structures after cGAMP stimulation (Fig. 1c, Extended Data Fig. 1d). However, TBK1 does not co-translocate into these puncta with ΔC9 STING (Fig. 1c). Furthermore, immunoprecipitation assays indicate that both wild-type STING and the Ser366Ala mutant bind TBK1; however, deletion of the nine C-terminal residues abolishes TBK1 binding and phosphorylation (Fig. 1d).

To test whether STING binds TBK1 directly, we expressed several biotin-labelled STING truncation mutants (Extended Data Fig. 2a, b) and conducted TBK1 binding studies by surface plasmon resonance (SPR) analysis (Extended Data Table 1). We observed that unphosphorylated STING binds TBK1 directly with relatively low affinity (Extended Data Fig. 2c, d). The binding affinity does not change notably in the presence of cGAMP (Extended Data Fig. 2e, f). However, after phosphorylation, STING binds to TBK1 at almost 20 times higher affinity (Fig. 1e, Extended Data Fig. 2g). The binding affinity does not change notably in the absence of cGAMP (Extended Data Fig. 2h, i).

In addition, phosphorylation of TBK1 does not affect its binding affinity with STING (Extended Data Fig. 2j). To map the TBK1-binding site, we conducted binding studies with several truncated forms of STING (Extended Data Fig. 2a, b). We observed that truncation of the C-terminal 9 or 37 residues of STING abolishes TBK1 binding (Fig. 1e, f, Extended Data Fig. 2g, k). By contrast, peptides containing the C-terminal 37 or 14 residues of STING are fully capable of binding TBK1 (Fig. 1f, Extended Data Fig. 2k, l).

To determine the exact STING residues that contribute to binding of TBK1, we generated several STING mutants (Fig. 2a, Extended Data Fig. 3a) and conducted binding studies (Fig. 2a, Extended Data Fig. 3b–l). These studies showed that the Leu374Ala mutation disrupts binding of TBK1 (Fig. 2a, Extended Data Fig. 3f). However, this mutation does not affect binding of IRF-3 (Extended Data Fig. 3m). In addition, the Arg375Ala and Phe378Ala mutations reduce the binding affinity by 30–20-fold, respectively (Fig. 2a, Extended Data Fig. 3g, j). The Leu372Ala and Pro373Ala mutations reduce the binding affinity by about tenfold (Fig. 2a, Extended Data Fig. 3d, e). The Pro371Ala, Thr376Ala, Asp377Ala and Ser379Ala mutations only reduce the binding affinity by 3–6-fold (Fig. 2a, Extended Data Fig. 3c, h, i, k). By contrast, mutations Lys370Ala and Ser366Ala do not affect binding of TBK1 (Fig. 2a, Extended Data Fig. 3l). Sequence alignment of STING from 60 species (Extended Data Table 2) reveals that the C-terminal residues of STING are highly conserved (Extended Data Fig. 3n). Therefore, these mutagenesis and binding studies identified a highly conserved PLPLRT/SD motif that mediates the recruitment of TBK1 by STING (Extended Data Fig. 3o). On the basis of these results, we also identified a high-affinity phosphomimetic mutant of STING (Thr376Glu/Phe378Met/Ser379Thr, referred to hereafter as EMW; Extended Data Fig. 3o, Extended Data Table 1).

To determine the functional roles of the nine C-terminal residues of STING, we mutated each of them to alanine and conducted IFN3 reporter assays (Fig. 2b). These assays showed that truncation of the nine C-terminal residues of STING (ΔC9) or mutation Leu374Ala disrupts the activation of the luciferase reporter (Fig. 2b). Mutation Arg375Ala reduces the reporter activity by about 50% (Fig. 2b). Mutations Pro371Ala, Thr376Ala and Asp377Ala reduce the reporter signal by about 30% (Fig. 2b). By contrast, mutations Lys370Ala,
Leu372Ala, Pro373Ala, Phe378Ala and Ser379Ala only reduce the signals slightly (Fig. 2b). Notably, the high-affinity EMW mutant stimulates a similar level of signal as wild-type STING (Fig. 2b). As a control, mutation Ser366Ala disrupts activation of the reporter (Extended Data Fig. 3p). In addition, the reporter assays showed that wild-type STING induces a faster response to cGAMP than the Thr376Ala mutant, indicating that phosphorylation of the PLPLRT/SD motif is needed for optimal signalling by STING (Extended Data Fig. 3q).

Next, we tested how these mutations affect STING-mediated signalling in cells. Western blot analysis showed that TBK1, STING and IRF-3 are phosphorylated after cGAMP stimulation of cells expressing wild-type STING (Fig. 2d). Truncation of the nine C-terminal residues of Leu372Ala and Ser379Ala only abolished IRF-3 phosphorylation, but did not affect activation of TBK1 (Extended Data Fig. 3r). The Arg375Ala mutation markedly reduces the phosphorylation of TBK1, STING and IRF-3 (Fig. 2e). By contrast, the Pro373Ala mutation only abolished IRF-3 phosphorylation, but did not affect activation of TBK1 (Extended Data Fig. 3s). The Arg375Ala mutation markedly reduces the phosphorylation of TBK1 (Extended Data Fig. 3t). By contrast, the Pro373Ala mutation only abolished IRF-3 phosphorylation, but did not affect activation of TBK1 (Extended Data Fig. 3u). The Arg375Ala mutation markedly reduces the phosphorylation of TBK1, STING and IRF-3 (Fig. 2e).

By contrast, the Leu372Ala, Pro373Ala, Phe378Ala and Ser379Ala mutations only slightly reduce TBK1, STING and IRF-3 phosphorylation (Fig. 2e). Mutations Lys370Ala, Pro373Ala, Thr376Ala and Ser379Ala, and the EMW mutation, do not affect the activation of TBK1, IRF-3 or STING (Fig. 2c). Consistent with these results, immunoprecipitation showed that these mutations affect binding of TBK1 by STING in cells (Fig. 2d). Moreover, we tested whether proximity of the IRF-3 binding pLxIS motif (p, hydrophilic residue; x, any residue; S, phosphorylation site) and the TBK1 binding PLPLRT/SD motif is necessary for STING-mediated signalling. We observed that insertion of six residues (GSGSSG) between these two motifs does not affect activation of the IFN3 reporter (Fig. 2e). In addition, we tested whether the two motifs on separate STING molecules could support signalling. We co-transfected the cells with the Ser366Ala and Leu374Ala mutants and observed that together, these two mutants support activation of the IFN3 reporter (Fig. 2f). Western blot showed that TBK1 and IRF-3 are activated in cells co-transfected with the Ser366Ala and Leu374Ala mutants, but not in cells transfected with either the Leu374Ala or the Ser366Ala mutant (Fig. 2g).

To decipher the molecular basis of TBK1 recruitment by STING, we determined the crystal structures of TBK1 bound to the STING CTD and CTD (Fig. 3, Extended Data Fig. 4, Extended Data Table 3). The structure of TBK1 bound to the STING CTD showed that a TBK1 dimer binds to two peptides from the STING CTD (Fig. 3a). Each STING peptide interacts with two TBK1 molecules simultaneously, forming a 2:2 complex (Fig. 3a). The STING-binding sites are located at the interface between the N-lobe of the kinase domain and the scaffold and dimerization domain of two TBK1 molecules (Fig. 3a, b). The STING CTD adopts an extended random coil structure that binds
TKB1 mainly through hydrophobic interactions and hydrogen bonds via the PLPLRT/SD motif (Fig. 3c). The main-chain carbonyl group of Lys370, the amide group of Leu372, and the carbonyl group of Pro373 interact with the side chains of Lys384, Gln581 and Tyr577 by hydrogen bonds (Fig. 3c). The side chain of Leu374 reaches into a hydrophobic pocket defined by residues Leu29, Arg27, Lys329, Asn578, Gln581, Ile582 and Phe585, anchoring the PLPLRT/SD motif to its binding groove (Fig. 3c). In addition, the amide group of Leu374 also forms a hydrogen bond with the carbonyl of Lys29. Arg375 forms two hydrogen bonds and Phe585, anchoring the PLPLRT/SD motif to its binding groove via the PLPLRT/SD motif (Fig. 3c). The main-chain carbonyl group of TBK1 mainly through hydrophobic interactions and hydrogen bonds between TBK1 and phosphorylated STING (Fig. 4a, Extended Data Fig. 5a–g). These studies showed that mutations Leu8Ala, Tyr577Ala, Asn578Ala and Ile582Ala abolish the interactions between TBK1 and phosphorylated STING (Fig. 4a, Extended Data Fig. 5a–d, f). In addition, mutations Gln581Ala and Lys584Ala also reduce the binding affinity by almost 20-fold (Fig. 4a, Extended Data Fig. 5e, g). By contrast, mutations Pro404Ala and Phe585Ala do not affect binding (Fig. 4a, Extended Data Fig. 5c, d). Similar results were obtained using mouse TBK1 for the binding studies (Fig. 4a). Next, we tested how these mutations of TBK1 affect STING-mediated signalling. We generated TBK1-knockout HEK293T cells, which were deficient in STING-mediated signalling, but were responsive after TBK1 transfection (Fig. 4b, Extended Data Fig. 5b–j). The Pro404Ala and Phe585Ala mutations only have minor effects on signalling (Fig. 4b). By contrast, mutations Arg27Ala, Lys29Ala, Ile582Ala and Lys584Ala reduce the signals by about 40% (Fig. 4b). Mutations Leu8Ala and Tyr577Ala reduce the signals by over 60% (Fig. 4b). Mutations Asn578Ala and Gln581Ala reduce the signals to a similar level to that of the kinase inactive Ser172Ala mutant (Fig. 4b). Western blot showed that mutations Leu8Ala, Arg27Ala, Tyr577Ala, Asn578Ala, Gln581Ala and Ile582Ala markedly reduced the phosphorylation of TBK1, STING and IRF-3 (Fig. 4c). Although mutations Lys29Ala and Lys584Ala also reduced TBK1 and STING phosphorylation, they only have minor effects on IRF-3 phosphorylation (Fig. 4c). By contrast, mutations Pro404Ala and Phe585Ala only have minor effects on the phosphorylation of TBK1, STING and IRF-3 (Fig. 4c). Moreover, immunoprecipitation confirmed that these residues are crucial in the recruitment of TBK1 by STING in cells (Extended Data Fig. 5k).

Although the CTT of STING can mediate activation of IRF-3 in an in vitro reconstitution system, expression of the CTD of STING alone is not sufficient to activate STING-mediated signalling in cells. To investigate how the transmembrane domain mediates activation of STING, we expressed full-length human STING in Freestyle 293F cells. Notably, in the absence of cGAMP, full-length STING aggregates.
Mutations at the TBK1–STING interface affect STING binding and signalling. a, Binding affinities (K_d) of TBK1 mutants to the phosphorylated human STING CTD determined by SPR. ND, no data. b, IFNβ luciferase reporter assays using TBK1-knockout HEK293T cells transfected with TBK1 mutants. The cells were transfected with pcDNA3.1-hSTING plasmids and/or pcDNA3.1-hTBK1 plasmids and stimulated with cGAMP. The kinase-inactive S172A mutant of TBK1 was used as a negative control. Data are mean ± s.e.m. and representative of three independent experiments. Each dot represents a technical replicate (n = 3). *P < 0.05, **P < 0.01, ***P < 0.001, two-tailed Student’s t-test. NS, not significant. c, Western blot showing the phosphorylation of TBK1, STING and IRF-3 in TBK1-knockout cells transfected with TBK1 mutants. The cells were transfected with pcDNA3.1-hSTING plasmids plus pcDNA3.1-hTBK1 plasmids and stimulated with cGAMP. Data are representative of three independent experiments.

Fig. 4 | Mutations at the TBK1–STING interface affect STING binding and signalling. a, Binding affinities (K_d) of TBK1 mutants to the phosphorylated human STING CTD determined by SPR. ND, no data. b, IFNβ luciferase reporter assays using TBK1-knockout HEK293T cells transfected with TBK1 mutants. The cells were transfected with pcDNA3.1-hSTING plasmids and/or pcDNA3.1-hTBK1 plasmids and stimulated with cGAMP. The kinase-inactive S172A mutant of TBK1 was used as a negative control. Data are mean ± s.e.m. and representative of three independent experiments. Each dot represents a technical replicate (n = 3). *P < 0.05, **P < 0.01, ***P < 0.001, two-tailed Student’s t-test. NS, not significant. c, Western blot showing the phosphorylation of TBK1, STING and IRF-3 in TBK1-knockout cells transfected with TBK1 mutants. The cells were transfected with pcDNA3.1-hSTING plasmids plus pcDNA3.1-hTBK1 plasmids and stimulated with cGAMP. Data are representative of three independent experiments.

Fig. 3 | Crystal structures of STING in complex with TBK1. a, Ribbon representations of mouse TBK1 in complex with the human STING CTT. Left, side view into the active site of TBK1; right, bottom view of TBK1. The kinase domains (KD) are in yellow and cyan. The ubiquitin-like domains (ULD) are in pink and red. The scaffold and dimerization domains (SDD) are in green and slate. The CTT of STING is shown by the magenta ball-and-stick model. The black dashed lines indicate distances less than 3.5 Å.

b, The CTT of STING binds to the scaffold and dimerization domain of a TBK1 dimer. The kinase domain and the scaffold and dimerization domain are shown by the cyan and green surfaces, respectively. The C-terminal tail of STING is shown by the magenta ball-and-stick model. c, Interactions between the STING CTT and TBK1. The CTT of STING is shown by the magenta ball-and-stick model, and TBK1 is shown as ribbons. Residues of TBK1 involved in binding to STING are shown by the green and cyan ball-and-stick model. The black dashed lines indicate distances less than 3.5 Å.

d, Superposition of the structures of the human STING CTD (yellow ball-and-stick) bound to human TBK1, and the human STING CTT (pink ball-and-stick) bound to mouse TBK1. Mouse TBK1 is shown by the surface representation coloured according to surface electrostatic potential, with positively charged surface in blue and negatively charged surface in red.
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Correspondence and requests for materials should be addressed to P.L.

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METHODS

Mass spectrometry. We expressed a short peptide containing the 22 C-terminal residues human STING as SUMO-fusion. The peptide was purified and phosphorylated with TBK1. The phosphorylated peptide was analysed using a liquid chromatography electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) system, consisting of a Dionex Ultimate 3000 LC system (with an Acclaim PepMap 100 C18 column from Thermo) coupled to a Thermo Orbitrap Fusion mass spectrometer. Liquid chromatography system solvents were water with 0.1% formic acid (mobile phase A) and acetonitrile with 0.1% formic acid (mobile phase B). The peptides were eluted over a 60-min gradient from 2% B from 0 to 5 min, 2% to 45% from 5 to 37 min, 45% to 90% from 38 to 46 min, and down to 2% from 46 to 60 min at a flow rate of 0.4 μl min⁻¹. The mass spectrometry ion source was set to have a spray voltage of 2.3 kV, ion transfer tube temperature of 275 °C, the scan range was m/z 400–1,600 with a resolution of 120,000. MS/MS acquisition was performed with 3+ cycle time. The intensity threshold was set to 5,000; ions with charge states 2+ to 6+ were sequentially fragmented by electron transfer dissociation (ETD) with 100 ms ETD reaction time and 200,000 ETD reagent limits for peptide length searched range from 6 to 144, maximum ΔCn is set to 0.05. Maximum number of allowed missed cleavages is 2.

Cell culture. HEK293T cells were obtained from ATCC (CRL-3216), cultured in DMEM (1×) plus GlutaMAX medium ( Gibco) supplemented with 10% fetal bovine serum (Gibco), penicillin (100 U ml⁻¹) and streptomycin (100 μg ml⁻¹) at 37 °C in a humidified atmosphere with 5% CO₂. TBK1-knockout HEK293T cells were cultured under the same conditions. Freestyle 293F cells were obtained from Thermo Fisher Scientific (K9000-01) and cultured in Freestyle 293 Expression Medium (Thermo Fisher Scientific) supplemented with penicillin (50 U ml⁻¹) and streptomycin (50 μg ml⁻¹). The cells were cultured in a 37 °C incubator containing a humidified atmosphere of 8% CO₂ on an orbital shaker rotating at 130 r.p.m. All cell lines were kept at low passages to maintain their identity. The expression of STING and/or TBK1 in all cell lines was checked routinely to ensure they are the same as the original cell line. All cell lines tested negative for mycoplasma contamination.

IFN₃ luciferase reporter assays. The cDNA encoding human STING and TBK1 were cloned into a pCDNA3.1 (-) vector. All mutants were generated by site-directed mutagenesis and confirmed by DNA sequencing. HEK293T cells were seeded into a 12-well plate (Corning) at 4×10⁶ cells ml⁻¹ and induced with 1 μg ml⁻¹ biotin and cultured at 16 °C overnight. The SUMO fusion proteins and β-galactosidase were expressed in M9 medium supplemented with selenomethionine (Acros Organics) and purified as described for the wild type protein. Human and mouse TBK1 (residue 1–657) were expressed in sf9 insect cells after infection with recombinant baculovirus and purified as described previously59. In brief, the proteins were first purified using a nickel-NTA column. The SUMO tag was then cleaved using SUMO protease and removed using a nickel-NTA column. The proteins were further purified by gel filtration chromatography using Superdex75 or 200 (16/60 GL) columns (GE Healthcare). The Se-methionine substituted human STING was expressed in M9 medium supplemented with seleniummethione (Acros Organics) and purified as described for the wild type protein. Human and mouse TBK1 (residue 1–657) were expressed in s9 insect cells after infection with recombinant baculovirus and purified as described previously59. Biotin-labelled-Avi-His6-SUMO proteins and peptides were also expressed in BL21 (DE3) cells in M9 medium, with the exception of the biotin-labelled Avi-His6-SUMO-GBP nanobody, which was expressed in regular LB medium. The cells were co-transformed with the pET28(a) vector with a N-terminal Avi-His6-SUMO tag. For protein quantification, the mutation Val343Trp was introduced into human STING C-terminal tail (residue 342–379). All the proteins were expressed in BL21 (DE3) cells in M9 medium. The cDNA encoding human IRF-3 (residue 189–427) was cloned into another modified pET28(a) vector with a N-terminal His6-SUMO tag. IRF-3 was also expressed in BL21 (DE3) cells in regular LB medium (BD). Cells were harvested after induction with 0.5 mM IPTG at 30 °C. A biotinylated anti-β-galactosidase (1:500) was added into 1L FreeStyle 293-F cells. After 2 days, the cells were pelleted at 10,000 × g for 30 min, the supernatant was mixed with 2× Denaturing Sample Buffer (20 mM Tris pH 7.5, 150 mM NaCl, 1% SDS, 10% β-mercaptoethanol and 10 mM dithiothreitol (DTT)) for 5 min. The samples were analysed using 10% SDS–PAGE gel.

Immunocytochemistry. HEK293T cells grown on poly-l-lysine coated glass coverslips in 12-well plates for 24 h and were transfected with equal amount of wild-type or mutant STING plasmids, respectively. The transfection mixture was combined plasmid DNA with dilution Lipofectamine 2000 in Opti-MEM medium (Gibco). After incubation for 24 h, the medium was replaced with fresh growth medium with or without 30 μg ml⁻¹ G4MCP. After incubation for 16 h, cells were washed with PBS, fixed with 4% paraformaldehyde in PBS for 10 min at room temperature and permeabilized with PBST (0.5% Triton X-100 in PBS). Cells were washed and blocked with 5% fetal bovine serum in PBST, followed by overnight incubation with primary antibodies, anti-STING (Cell Signaling, 13647, 1:200) and anti-TBK1 (GeneTex, GTX12116, 1:200). Cells were then washed three times by PBS and incubated with Alexa 488-conjugated donkey anti-rabbit IgG antibody (Jackson ImmunoResearch, 711-454-152, 1:1,000) and Alexa 555-conjugated donkey anti-Mouse IgG antibody (Thermo Fisher Scientific, A31570, 1:1,000) for 1 h at room temperature. The coverslips were then washed with PBS, mounted on slides with ProLong Gold antifade reagent with 4′,6-diamidino-2-phenylindole (DAPI) and imaged using a Nikon-Ti Fluorescence microscope.

Protein expression and purification. Constructs of human STING were cloned into a modified pET28(a) vector with an N-terminal Avi-His6-SUMO tag. For protein quantification, the mutation Val343Trp was introduced into human STING C-terminal tail (residue 342–379). All the proteins were expressed in BL21 (DE3) cells in M9 medium. The cDNA encoding human IRF-3 (residue 189–427) was cloned into another modified pET28(a) vector with a N-terminal His6-SUMO tag. IRF-3 was also expressed in BL21 (DE3) cells in regular LB medium (BD). Cells were harvested after induction with 0.5 mM IPTG at 30 °C. A biotinylated anti-β-galactosidase (1:500) was added into 1L FreeStyle 293-F cells. After 2 days, the cells were pelleted at 10,000 × g for 30 min, the supernatant was mixed with 2× Denaturing Sample Buffer (20 mM Tris pH 7.5, 150 mM NaCl, 1% SDS, 10% β-mercaptoethanol and 10 mM dithiothreitol (DTT)) for 5 min. The samples were analysed using 10% SDS–PAGE gel.

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with 500 μl streptavidin agarose beads (EMD Millipore), which have been coupled with biotin-labelled Avi-SUMO-GFP nanobody. After shaking at 4°C for another 2 h, the beads were pelleted and washed with pre-chilled washing buffer (20 mM Tris pH 7.5, 150 mM NaCl, 0.1% DDM). Finally, the beads were resuspended in 500 μl washing buffer and SUMO protease was added to cleave the target protein from the beads. The eluted protein was incubated with thrombin at 4°C overnight and further purified using a Superose 6 increase 10/300 GL column (GE Healthcare). All mutants of STING and TBK1 were generated using the QuickChange site-directed mutagenesis kit (Agilent) or a PCR-based technique with appropriate primers. The sequences of the plasmids were confirmed by plasmid DNA sequencing.

**Phosphorylation of STING.** Biotin-labelled Avi-His6-SUMO-STING CTD and CTT peptides were phosphorylated using glutathione S-transferase (GST)-tagged mTBK1 in a reaction buffer containing 20 mM HEPES, pH 7.5, 10 mM MgCl₂, 100 mM NaCl, 5 mM ATP, 0.1 mM Na₂VO₃, 5 mM NaF and 5 mM DTT. The target proteins were diluted to 1 mg ml⁻¹ in the reaction buffer and mixed with GST-mTBK1 in a 1:10 (v/w) ratio. After 24 h incubation at 27°C, the phosphorylated proteins were purified using a Superdex200 10/300 GL column (GE Healthcare) eluted with a buffer containing 20 mM Tris, 150 mM NaCl at pH 7.5.

**Electron microscopy.** Before cryo-EM imaging, the full-length STING sample in 0.1% DDM was exchanged into Amphilop A8-35. In brief, STING at approximately 1 mg ml⁻¹ was incubated for around 8 h with threefold excess by mass of A8-35. DDM was removed by incubation with Bio-Beads SM-2 (Bio-Rad) overnight at 15 mg Bio-Beads per millilitre of solution. The sample was filtered to remove Bio-Beads and purified using a Superose 6 increase 10/300 GL column eluted with a buffer containing 20 mM Tris, 150 mM NaCl and 5 μM GAMP at pH 7.5. For cryo-EM imaging, 3 μl of STING at a concentration of 0.6 mg ml⁻¹ stabilized with A8-35 was applied to glow-discharged C-flat holey carbon grids (1.2/1.3, 400 mesh). Grids were blotted for 8 s and plunge frozen in liquid ethane using a Vitrobot. Images were recorded by Latitude on a Titan Krios Transmission Electron Microscope operating at 300 kV. A Gatan K3 detector was used in counting mode at a nominal magnification of ×64,000 (yielding a pixel size of 1.42 Å). The dose rate on the camera was set to be 23.8 electrons per physical pixel per second. Exposure of 8 s was dose-fractionated into 84 movie frames, leading to a total accumulated guanine hydroxide per Å² on the specimen. Images were recorded with a defocus in the range from 1.0 to 4.0 μm. Movies were collected at 15° and 30° tilt to address the preferred orientation of particle distribution. A total of 3,956 movies were recorded. The cryo-EM images were subjected to MotionCor2 for whole-frame dose-weighted motion correction. Particles picking was performed automatically on the summed images in Gautomatch. A total of 880,703 particles were picked. Per-particle local CTFs were estimated by GCTF. Two rounds of 2D classification were then performed. After discarding bad class averages, 352,286 particles were re-centred and re-extracted to Relion 3D classification. Initial models for reconstruction were generated from scratch by the EMAN2 'e2initialmodel.py' program using selected unsupervised 2D averages of good quality based on visual comparison without applying any symmetry. A stack of 47,474 particles was selected to Relion 3D refinement using a 40 Å low-pass filter reconstruction from the initial model. The final 3D reconstruction of STING oligomer with GAMP has an overall resolution of 11.6 Å, using gold-standard Fourier shell correlation (FSC) = 0.143 criteria. The cryo-EM maps of STING oligomer was segmented using Segger. We took advantage of the availability of crystal structure of hSTING cytosolic region with cGAMP to guide the decision on the trimer representation. After applying the subpixel procedure for four steps to the original electron microscopy map, four regions were obtained. STING CTD structures were docked to the segmentation map using Chimera.

**Reporting summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this paper.

**Data availability**

Atomic coordinates and structure factors of human TBK1 in complex with the human STING CTD EMW mutant, and mouse TBK1 in complex with the human STING CTD have been deposited in the Protein Data Bank (PDB) with accession codes 6O8B and 6O8C, respectively.

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Extended Data Fig. 1 | Potential phosphorylation sites within the STING C-terminal tail. 
a. List of phosphorylated STING peptides (residues 358–379, Ser358Trp) identified by LC–MS/MS. Phosphorylated residues are underlined and shown in orange. b. Representative MS/MS spectra of phosphorylated STING peptides (residues 358–379, Ser358Trp). β and γ fragment ions are shown in red, and γ and z fragment ions are shown in blue. Residues phosphorylated are shown in orange. Data are representative of two independent experiments. c. STING- and cGAMP-dependent activation of the IFNβ luciferase reporter in HEK293T cells. The cells were transfected with indicated amounts of the pcDNA3.1-hSTING plasmid (STING WT) and stimulated with cGAMP. Data are mean ± s.e.m. and representative of three independent experiments. Each dot represents a technical replicate (n = 3). ***P < 0.001, two-tailed Student's t-test. d. Western blot of HEK293T cells transfected with the wild-type or ΔC9 mutant STING plasmid. Data are representative of three independent experiments.
Extended Data Fig. 2 | SPR binding studies of human STING with TBK1. 

**a** Domain organization of human STING and truncated forms of STING used in this study. 

**b** SDS–PAGE analyses of human STING (left) and human or mouse TBK1 (right) used in the SPR studies.  

**c**–**l** Top, SPR binding studies of human STING with human or mouse TBK1. Experiments without cGAMP in the running buffer are indicated. All others were conducted with 1 μM cGAMP. Bottom, the binding affinity (Kd) was determined by fitting the binding data to a one-site binding model. Data in **b**–**l** are representative of at least two independent experiments.

Human STING full length

| Domain | Start | End |
|--------|-------|-----|
| Transmembrane Domain | 21 | 136155 |
| C-terminal domain CTD | 155-379 |
| C-terminal domain ΔC9 | 155-370 |
| cGAMP-binding domain CBD | 341-379 |
| C-terminal tail CTT | 342-379 |
| STING 366-379 |

Extended Data Fig. 2 | SPR binding studies of human STING with TBK1. 

| Domain | Start | End |
|--------|-------|-----|
| Transmembrane Domain | 21 | 136155 |
| C-terminal domain CTD | 155-379 |
| C-terminal domain ΔC9 | 155-370 |
| cGAMP-binding domain CBD | 341-379 |
| C-terminal tail CTT | 342-379 |
| STING 366-379 |
Extended Data Fig. 3 | See next page for caption.
**Extended Data Fig. 3 | Binding studies of human STING mutants with TBK1.** a, SDS–PAGE analysis of human STING mutants and human IRF-3 used in the SPR binding studies. b–l, Top, SPR binding studies of human STING mutants (residues 155–379) with human TBK1 in the presence of 1 μM cGAMP. Bottom, the binding affinity (K_d) was determined by fitting the binding data to a one-site binding model. m, SPR binding study of the L374A STING mutant with IRF-3. n, The CTT of STING contains a highly conserved PLPLRT/SD motif. The sequence logo of STING is generated by WebLogo based on the sequence alignment of STING from mammals. The frequency of occurrence of an amino acid is indicated underneath the sequence. The PLPLRT/SD motif is downstream of the pLxIS motif that is involved in IRF-3 binding. o, SPR binding study of the high-affinity phosphomimetic EMW mutant of STING with human TBK1. p, IFN-β luciferase reporter assays of the S366A and L374A STING mutants. For each assay, HEK293T cells were transfected with pcDNA3.1-hSTING variants and stimulated with cGAMP. q, Time course IFN-β luciferase reporter assays of HEK293T cells transfected with wild-type and T376A mutant STING. The cells were stimulated with cGAMP. r, Western blot showing the phosphorylation of STING, TBK1 and IRF-3 in HEK293T cells transfected with wild-type or mutant (S366A or L374A) STING. Data in b–m, o and r are representative of at least two independent experiments. Data in p and q are mean ± s.e.m. and representative of three independent experiments. Each dot represents a technical replicate (n = 3 in p and n = 6 in q). **P < 0.01, ***P < 0.001, two-tailed Student’s t-test. NS, not significant.
Extended Data Fig. 4 | Crystal structures of STING in complex with TBK1. a, Ribbon representation of the structure of human TBK1 bound to the human STING CTD EMW mutant (residue 155–379, T376E, F378M and S379W). The kinase domains (KD) are in yellow and cyan, the ubiquitin-like domains (ULD) are in pink and red, the scaffold and dimerization domains (SDD) are in green and slate. The STING CTDs are shown by the blue and magenta ball-and-stick models. The TBK1 inhibitor BX795 is shown by the orange stick models. b, SDS–PAGE analysis of crystals of human TBK1 in complex with the human STING CTD EMW mutant. The data are representative of two independent experiments. c, Difference map of human STING CTT bound to mouse TBK1 contoured at 2.5σ. The σA-weighted Fobs − Fcalc map was calculated with STING CTT omitted from the model. The CTT of STING is shown by the purple stick model. The TBK1 dimer is shown by the ribbons coloured green and cyan. d, Difference map of human STING CTD bound to human TBK1 contoured at 2.5σ. The σA-weighted Fobs − Fcalc map was calculated with STING CTD omitted from the model. The CTT of STING is shown by the purple stick model. The TBK1 dimer is shown by the ribbons coloured green and cyan. e, Anomalous difference maps of Se-Met derivative of the human STING CTT EMW mutant bound to human TBK1. The blue map was calculated with model phases (φc) and the magenta map was calculated with experimental phases after density modification (φdm). The STING peptide is shown by the magenta ribbon and TBK1 shown by the green and cyan ribbons. f, Superposition of the structures of the human STING CTD EMW mutant (magenta) and human STING CTT (yellow) bound to human and mouse TBK1. Mouse TBK1 is shown by the green and cyan cartoon representation. Residues Glu376, Met378 and Trp379 from the STING CTD EMW mutant are shown by the magenta ball-and-stick models. Residues Thr376, Phe378 and Ser379 from the STING CTT are shown as the yellow ball-and-stick models.
Extended Data Fig. 5 | Binding studies of human STING with human TBK1 mutants and characterization of TBK1-knockout HEK293T cells.  

a–g, SPR binding studies of the phosphorylated human STING CTD (residues 155–379) with human TBK1 mutants in the presence of 1 μM cGAMP. The binding affinity ($K_d$) was determined by fitting the binding data to a one-site binding model. SDS–PAGE analysis of proteins used in these studies is shown in the inset of panel a.  
h, Western blot characterization of TBK1-knockout HEK293T cell lines.  
i, IFNβ luciferase reporter assays using TBK1-knockout cells. For each assay, 0.2 ng pcDNA3.1-hSTING plasmids and/or 1.0 ng pcDNA3.1-hTBK1 plasmids were transfected into TBK1-knockout cells. Data are mean ± s.e.m. and representative of three independent experiments. Each dot represents a technical replicate ($n = 3$). ***$P < 0.001$, two-tailed Student’s t-test.  
j, Western blot showing the phosphorylation of TBK1, STING and IRF-3 in TBK1-knockout cells transfected with STING and TBK1 plasmids. TBK1-knockout cells were transfected with 0.2 ng pcDNA3.1-hSTING plasmids and/or 1.0 ng pcDNA3.1-hTBK1 plasmids and stimulated with cGAMP.  
k, Immunoprecipitation and immunoblot of Flag–STING and TBK1 in TBK1-knockout cells. The cells were transfected with Flag–STING and TBK1 mutants and stimulated with cGAMP. Flag–STING and TBK1 in the pull-downs and whole-cell lysates were analysed by immunoblotting. STING was visualized with Flag antibody. TBK1 in the pull-downs was detected with an antibody against TBK1. Data in a–h are representative of at least two independent experiments. Western blot data in j and k are representative of three independent experiments.
Extended Data Fig. 6 | See next page for caption.
Extended Data Fig. 6 | cGAMP binding induces the oligomerization of full-length STING. a, Gel-filtration chromatography analyses of full-length STING in the presence and absence of 1 μM cGAMP using a Superose 6 column. b, SDS–PAGE analyses of fractions containing full-length STING from gel filtration chromatography. c, Gel filtration chromatography analyses of full-length STING in 0.1% DDM or Amphipol A8-35 using a Superose 6 column in the presence of 1 μM cGAMP. d, SDS–PAGE analysis of cross-linked full-length STING. e, SEC-MALS analysis of full-length STING in 0.1% DDM and 1 μM cGAMP. f, Representative cryo-EM micrograph of full-length STING stabilized with Amphipol A8-35. g, Representative 2D averages of full-length STING particles in Amphipol A8-35. h–j, Three views of 12 Å resolution map of STING oligomers. Human STING CTD dimers bound to cGAMP were docked into the map. k, A list of human STING mutants in the transmembrane domain. l, Gel-filtration chromatography analyses of wild-type and mutants of full-length STING in the presence of 1 μM cGAMP using a Superose 6 column. m, SDS–PAGE analyses of fractions of wild-type STING and STING mutants purified by gel filtration chromatography using a superose 6 column. n, IFNβ luciferase reporter assays showing that the mutations in the N-terminal transmembrane domain affect STING-mediated signalling. Indicated amounts of pcDNA3.1-hSTING plasmids were transfected into HEK293T cells. Data are mean ± s.e.m. and representative of three independent experiments. Each dot represents a technical replicate (n = 3). ***P < 0.001, two-tailed Student’s t-test. NS, not significant. o, Western blot showing that mutations in the transmembrane domain of STING affect the phosphorylation of STING, TBK1 and IRF-3. HEK293T cells were transfected with indicated amounts of pcDNA3.1-hSTING plasmids and stimulated with cGAMP. p, Confocal microscopy images of HEK293T cells transfected with wild-type STING and STING mutants with or without cGAMP stimulation. Scale bars, 20 μm. Data in a–e, I and m are representative of at least two independent experiments. Data in f, g, o and p are representative of at least three independent experiments.
Extended Data Fig. 7 | Proposed model for the recruitment and activation of TBK1 and IRF-3 through the cGAS-STING pathway. (1) cGAS is activated by double-stranded DNA (dsDNA) in the cytosol and catalyses the synthesis of cGAMP from ATP and GTP. (2) cGAMP binding induces the oligomerization of STING at the ER or Golgi membranes. (3) TBK1 is recruited to the STING oligomers via its C-terminal PLPLRT/SD motif and activated by induced proximity in trans. Phosphorylation of STING by TBK1 increases the binding affinity between TBK1 and STING and facilitates further recruitment and activation of TBK1. (4) Activated TBK1 phosphorylates STING at the pLxIS motif, allowing it to recruit IRF-3 to the signalling complex. (5) The proximity of TBK1 and IRF-3 bound to adjacent STING molecules within the cGAMP-STING oligomer causes the phosphorylation of the pLxIS motif of IRF-3. (6) Phosphorylated IRF-3 dissociates from STING, oligomerizes, translocates to the nucleus, and initiates the transcription of IFNB gene.
## Extended Data Table 1 | Binding affinities of human STING mutants with TBK1

| Human STING (hSTING) | Human TBK1 1-467, S172A $K_a$ (μM) | Mouse TBK1 1-467, S172A $K_a$ (μM) | Mouse TBK1 1-467 $K_a$ (μM) |
|----------------------|-----------------------------------|-----------------------------------|-----------------------------|
| hSTING 155-379 (no cGAMP) | ~230 | ~260 |
| hSTING 155-379 | ~290 | ~170 |
| p-hSTING 155-379 | 11.0 | 9.1 | 13.3 |
| p-hSTING 155-379 (no cGAMP) | 20.0 | 12.5 |
| p-hSTING 155-341 | no binding | no binding |
| p-hSTING 155-370 | no binding | no binding |
| p-hSTING 342-379 | 16.6 | 15.3 |
| p-hSTING 342-370 | no binding | no binding |
| p-hSTING 355-379 | 22.6 | 25.1 |
| p-hSTING 366-379 | 35.6 | 30.8 |
| hSTING 306-379, F378W | ~190 |
| hSTING 155-379, T378E | ~62.0 |
| hSTING 306-379, T378E, F378W | ~50.0 |
| hSTING 306-379, T378E, F378W, S379E | ~127 |
| hSTING 306-379, T378E, F378W, S379K | 43.9 |
| hSTING 306-379, T378E, F378W, S379H | ~59.0 |
| hSTING 306-379, T378E, F378W, S379P | 27.1 |
| hSTING 306-379, T378E, F378W, S379W | 2.6 | 2.7 |
| hSTING 155-379, T378E, F378W, S379W | 1.4 | 1.2 |
Extended Data Table 2 | Sequences of STING C-terminal region from 60 mammals

| Species                          | NCBI Entry   | Sequence                  |
|---------------------------------|--------------|---------------------------|
| Acinonyx jubatus                | XP_014919539.1 | NLLISGMEQLPLRTLVDF        |
| Ailurus fulgens melanoleuca     | XP_002912620.1 | KLLISGLEGQLPLRTDVF       |
| Balaenoptera acutorostrata scammoni | XP_007172318.1 | ELLISGMEQPLPLASDFV        |
| Bison bison                     | XP_01084832.1  | ELLISGLEGQLPLRTDVF        |
| Bos taurus                      | NP_001035822.1 | ELLISGLEGQLPLRTDVF        |
| Callichoix jacchus              | XP_002744307.1 | ELLISGMEQPLPLRSDFV        |
| Camelus bactrianus              | NP_001306707.1 | ELLISGMEQPLPLRDVF         |
| Canis lupus familiaris          | XP_005617314.1 | NLIFISGLEGQLPLRDVF        |
| Capra hircus                    | NP_001306207.1 | ELLISGMEQPLPLRDVF         |
| Carlito synchita                | XP_008064767.1 | ELLISGMEQPLPLRDVF         |
| Castor canadensis               | XP_002023516.1 | ELLISGMEQPLPLRDVF         |
| Cavia porcellus                 | XP_003477199.1 | QLJ1SMEQPLPLRDVF          |
| Ceratotherium simum simum       | XP_014650944.1 | ELLISGTEQPLPLRDVF         |
| Chrysocloris asiatica           | XP_006866201.1 | RFLISDEEQPLPLRDVF         |
| CondyIIura cristata             | XP_012583163.1 | QLLISGDMLPLPLRDVF         |
| Dipodomys ordii                 | XP_012875724.1 | QLLISGDMLPLPLRDVF         |
| Echinosops telfair              | XP_004697079.1 | MFLISGDMLPLPLRDVF         |
| Enhydra lutris kenyoni          | XP_022349371.1 | KLLISGLEGQLPLRTDVF        |
| Equus caballus                  | XP_014709351.1 | QLJ1SMEQPLPLRDVF          |
| Equus caballus                  | XP_005599422.1 | QLJ1SMEQPLPLRDVF          |
| Erinaceus europaeus             | XP_007517598.2 | ELLISGMEQPLPLRDVF         |
| Felis catus                     | XP_023111467.1 | NLLISGMEQPLPLRDVF         |
| Gouilla gorilla                 | XP_004042660.1 | ELLISGMEQPLPLRDVF         |
| Heterocephalus glaber           | XP_021111568.1 | QLLISGDMLPLPLRDVF         |
| Homo sapiens                    | NP_938023.1   | ELLISGMEQPLPLRDVF         |
| Ictidomyys trigalmineatus       | XP_005327332.1 | KLLISGMEQPLPLRDVF         |
| Jaculus jaculus                 | XP_004652491.1 | KLLISGMEQPLPLRDVF         |
| Leptonychotes weddellii         | XP_006730795.1 | KLLISGLEGQLPLRDVF         |
| Lipotes vexillifer              | XP_007461503.1 | ELLISGMEQPLPLRDVF         |
| Loxodonta africana              | XP_003404845.1 | KLLISGMEQPLPLRDVF         |
| Macaca mulatta                  | XP_014996496.1 | ELLISGMEQPLPLRDVF         |
| Mandrillus leucocephalus        | XP_011852614.1 | ELLISGMEQPLPLRDVF         |
| Microcebus murinus              | XP_012604522.1 | ELLISGMEQPLPLRDVF         |
| Monodelphis domestica           | XP_016284133.1 | ELLISGMEQPLPLRDVF         |
| Mus musculus                    | NP_082537.1   | ELLISGMEQPLPLRDVF         |
| Mustela putorius furo           | XP_012907883.1 | KLLISGLEGQLPLRDVF         |
| Myotis lucifugus                | XP_006086577.1 | QLJ1SMEQPLPLRDVF          |
| Neomnachus schauinslandi        | XP_021557627.1 | KLLISGMEQPLPLRDVF         |
| Nomascus leucogenys             | XP_012360436.1 | ELLISGMEQPLPLRDVF         |
| Odocoileus rosmarus divergens   | XP_004397863.1 | ELLISGMEQPLPLRDVF         |
| Odocoileus virginianus texanus  | XP_020764082.1 | ELLISGMEQPLPLRDVF         |
| Orcinus roca                    | XP_004280346.1 | ELLISGMEQPLPLRDVF         |
| Orycteropus afer afer           | XP_007937166.1 | KPLISGLEGQLPLRTDVF        |
| Oryctolagus cuniculus           | XP_002710295.1 | QLJ1SMEQPLPLRDVF          |
| Otolymem gamettii               | XP_012663496.1 | KLLISGMEQPLPLRDVF         |
| Ovis aries                      | XP_004008906.1 | ELLISGMEQPLPLRDVF         |
| Pan troglodytes                  | XP_001135484.1 | ELLISGMEQPLPLRDVF         |
| Panthera tigris altaica         | XP_007077937.1 | NLLISGMEQPLPLRDVF         |
| Panthera hodgsonii              | XP_005971883.1 | ELLISGMEQPLPLRDVF         |
| Papio anubis                    | XP_003980232.1  | ELLISGMEQPLPLRDVF         |
| Pongo abelii                    | XP_002815998.1  | ELLISGMEQPLPLRDVF         |
| Propithecus coquereli           | XP_012501803.1 | ELLISGMEQPLPLRTDVF        |
| Pteropus vampyrus               | XP_011380568.1 | ELLISGMEQPLPLRTDVF        |
| Rattus norvegicus               | NP_001102592.1 | ELLISGMEQPLPLRTDVF        |
| Sarcophilus harrisii            | XP_003756672.1 | QLJ1SMEQPLLRTDGF          |
| Sus scrofa                      | NP_001136310.1 | ELLISGMEQPLPLRDVF         |
| Trichechus manatus latirostris  | XP_004381119.2 | KLLISGMEQPLPLRDVF         |
| Tursiops truncatus              | XP_019789073.1  | ELLISGMEQPLPLRDVF         |
| Ursus maritimus                 | XP_008689754.1  | KLLISGLEGQLPLRDVF         |
| Vicugna pacos                   | XP_015094987.1  | KLLISGMEQPLPLRDVF         |

The conserved PxxIxI motif and PLPLRT/SD motif are highlighted in blue and red, respectively.
Extended Data Table 3 | Data collection and refinement statistics

|                      | hTBK1/hSTING (155-379, EMW) Complex | mTBK1/hSTING (WT, 342-379) Complex | hTBK1/Se-Met hSTING (EMW, 342-379) Complex |
|----------------------|-------------------------------------|-----------------------------------|------------------------------------------|
| **Data collection**  |                                     |                                   |                                          |
| Space group          | P6$_2$22                            | P6$_2$22                          | P6$_2$22                                  |
| Cell dimensions      |                                     |                                   |                                          |
| $a$, $b$, $c$ (Å)    | 250.69, 250.69, 239.24              | 249.51, 249.51, 243.78            | 250.57, 250.57, 236.83                    |
| $\alpha$, $\beta$, $\gamma$ (°) | 90.0, 90.0, 120.0 | 90.0, 90.0, 120.0 | 90.0, 90.0, 120.0 |
| Resolution (Å)       | 3.40 (3.49 to 3.40) *               | 3.17 (3.24 to 3.17)               | 3.40 (3.49 to 3.40)                      |
| $R_{merge}$          | 0.152 (2.0)                         | 0.184 (2.7)                       | 0.437 (7.2)                              |
| $I / \sigma I$       | 7.8 (1.0)                           | 13.8 (1.0)                        | 8.0 (0.7)                                |
| Completeness (%)     | 99.3 (99.5)                         | 100.0 (100.0)                     | 100.0 (100.0)                            |
| Redundancy           | 6.2 (5.9)                           | 12.3 (11.3)                       | 17.4 (17.4)                              |
| **Refinement**       |                                     |                                   |                                          |
| Resolution (Å)       | 3.40                                | 3.17                              |                                          |
| No. reflections      | 60195                               | 75886                             |                                          |
| $R_{work}$ / $R_{free}$ | 0.244/0.258                      | 0.214/0.230                      |                                          |
| No. atoms            |                                     |                                   |                                          |
| Protein              | 10829                               | 10538                             |                                          |
| Ligand/ion           | 68                                  | 68                                |                                          |
| Water                | 0                                   | 0                                 |                                          |
| $B$-factors          |                                     |                                   |                                          |
| Protein              | 137.6                               | 108.2                             |                                          |
| Ligand/ion           | 117.8                               | 90.6                              |                                          |
| Water                |                                     |                                   |                                          |
| R.m.s. deviations    |                                     |                                   |                                          |
| Bond lengths (Å)     | 0.002                               | 0.012                             |                                          |
| Bond angles (°)      | 0.577                               | 1.086                             |                                          |

One crystal was used to collect each of the datasets.

*Values in parentheses are for the highest-resolution shell.
Reporting Summary

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For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

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- The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement
- A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
- The statistical test(s) used AND whether they are one- or two-sided
- A description of all covariates tested
- A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
- A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
- For null hypothesis testing, the test statistic (e.g. F, t, r) with confidence intervals, effect sizes, degrees of freedom and P value noted

Give P values as exact values whenever suitable.

- For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
- For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
- Estimates of effect sizes (e.g. Cohen’s d, Pearson’s r), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

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Policy information about availability of computer code

Data collection

The CCP4 package (v. 7.0) including imosflm and Shelx, Biacore X100 control software (v. 2.0), Gen5 2.06 (BioTek Synergy HTX plate reader), Image Lab Touch Software (v. 2.3.0.07, Bio-rad)

Data analysis

Phenix (v. 1.12, 1.13) and the CCP4 (v. 7.0) package, Coot (v. 0.8.6), PyMol (v. 1.2), Biacore X100 evaluation software (v. 2.0), GraphPad Prism 8.0, Microsoft Excel 2016, Thermo Proteome Discoverer (v. 2.1.0.81) software platform including the SEQUEST, MotionCor2, Gautomatch (v. 0.56), GCTF (v. 1.18), EMAN2.2, Relion (v. 3.0), Segger (v. 1.9.2), Chimera (v. 1.13)

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors/reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.

Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

The atomic coordinates of the crystal structures have been deposited in the wwPDB with access codes: 6O8B and 6O8C
# Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

- [x] Life sciences
- [] Behavioural & social sciences
- [] Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see nature.com/documents/nr-reporting-summary-flat.pdf

## Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

| Sample size | No statistical methods were used to predetermine sample size. We conducted preliminary experiments to estimate variances in each assay and determined sufficient sample size. The sample size has been stated in the figure legends. |
|-------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Data exclusions | No data were excluded from the analyses. |
| Replication | All attempts at replication were successful. The number of the independent experiments has been stated in the figure legends. |
| Randomization | No randomization was used in this study. No animals or human research participants were involved in this study. For the cell based assays, the cells were transfected with indicated plasmids and treated with indicated amounts of cGAMP. |
| Blinding | No blinding was performed during data collection and/or analysis since all the structural and functional data were analyzed using the same methods. |

## Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

### Materials & experimental systems

| n/a | Involved in the study |
|-----|-----------------------|
| x   | Antibodies            |
| x   | Eukaryotic cell lines |
|     | Palaeontology         |
|     | Animals and other organisms |
| x   | Human research participants |
|     | Clinical data         |

### Methods

| n/a | Involved in the study |
|-----|-----------------------|
| x   | ChIP-seq              |
| x   | Flow cytometry        |
|     | MRI-based neuroimaging |

### Antibodies

| Antibodies used | TBK1 antibody (Cell signaling, Cat# 3013, Dilution 1:1000), TBK1 phospho-Ser172 antibody (Cell signaling, Cat# 5483, Dilution 1:1000), STING antibody (Cell signaling, Cat# 13647, Dilution 1:1000 or 1:200), STING phospho-Ser366 antibody (Cell signaling, Cat# 85735, Dilution 1:1000), IRF-3 antibody (Cell signaling, Cat# 4302, Dilution 1:1000), IRF-3 phospho-Ser386 antibody (Abcam, Cat# ab76493, Dilution 1:2500), Flag M2-Peroxidase antibody (Sigma, Cat# A8952, Dilution 1:2000), Actin antibody (Thermo fisher scientific, Cat# MA5-11869, Dilution 1:4000), TBK1 antibody (GeneTex, Cat# GTX12116, Dilution 1:200), Alexa 488-conjugated Donkey Anti-Rabbit IgG antibody (Jackson ImmunoResearch, Cat# 711-545-152, Dilution 1:1000), Alexa 555-conjugated Donkey Anti-Mouse IgG antibody (Thermo Fisher Scientific, Cat# A31570, Dilution 1:1000), Anti-rabbit IgG, HRP-linked Antibody (Cell Signaling, Cat# 7074, Dilution 1:1000), Anti-mouse IgG, HRP-linked Antibody (Cell Signaling, Cat# 7076, Dilution 1:1000) |

### Validation

All antibodies used in this study are commercial. Most of them were evaluated in Western Blots of cell lysates containing endogenous or overexpressed target proteins.

- TBK1 antibody (Cat# 3013), Species (human, mouse, rat monkey), Application (WB, IP). As noted on the manufacturer’s website, the citation is 59. https://www.cellsignal.com/products/primary-antibodies/tbk1-nak-antibody/3013
- TBK1 phospho-Ser172 antibody, Species (human, mouse), Application (WB, IP, IF, F). As noted on the manufacturer’s website, the citation is 112. https://www.cellsignal.com/products/primary-antibodies/phospho-tbk1-nak-ser172-d52c2-xp-rabbit-mab/5483
- STING antibody, Species (human, mouse), Application (WB, IP, IHC). As noted on the manufacturer’s website, the citation is 40. https://www.cellsignal.com/products/primary-antibodies/sting-d2p2f-rabbit-mab/13647
- STING phospho-Ser366 antibody, Species (human), Application (WB, IP). As noted on the manufacturer’s website, the citation is 12. https://www.cellsignal.com/products/primary-antibodies/phospho-sting-ser366-antibody/85735
- IRF-3 antibody, Species (human, mouse, rat, monkey), Application (WB, IP). As noted on the manufacturer’s website, the citation is 111. https://www.cellsignal.com/products/primary-antibodies/irf-3-d8369-rabbit-mab/4302
IRF-3 phospho-Ser386 antibody, Species (human), Application (WB, Dot blot). As noted on the manufacturer’s website, the citation is 54. https://www.abcam.com/irf3-phospho-s386-antibody-epr2346-ab76493.html
Flag M2-Peroxidase antibody, Application (WB, ICC, ELISA). As noted on the manufacturer’s website, the citation is 701. https://www.sigmaaldrich.com/catalog/product/sigma/a8592?lang=en&region=US
Actin antibody, Species (human, mouse, bovine, pig, dog, chicken, rabbit, rat, Protozoa), Application (WB, ICC, IF, IP, IHC). As noted on the manufacturer’s website, the citation is 293. https://www.thermofisher.com/antibody/product/Actin-Antibody-clone-ACTNOS-C4-Monoclonal/MA5-11869
TBK1 antibody (Cat# GTX12116), Species (human, mouse, rat, bovine, dog), Application (WB, IHC, ICC, IF). As noted on the manufacturer’s website, the product was used in publication: Ogasawara N et al. J Clin Biochem Nutr 2011; 48 (2):154-60. https://www.genetex.com/Product/Detail/NAK-antibody-108A429/GTX12116
Alexa 488-conjugated Donkey Anti-Rabbit IgG antibody, Species (Rabbit). As noted on the manufacturer’s website, the citation is 213. https://www.jacksonimmuno.com/catalog/products/711-545-152
Alexa 555-conjugated Donkey Anti-Mouse IgG antibody, Species (mouse), Application (IF, ICC, IHC). As noted on the manufacturer’s website, the citation is 23. https://www.thermofisher.com/antibody/product/Donkey-anti-Mouse-IgG-H-L-Highly-Cross-Adsorbed-Secondary-Antibody-Polyclonal/A-31570
Anti-rabbit IgG, HRP-linked Antibody, Species (Rabbit), Application (WB). As noted on the manufacturer’s website, the citation is 3422. https://www.cellsignal.com/products/secondary-antibodies/anti-rabbit-igg-hrp-linked-antibody/7074
Anti-mouse IgG, HRP-linked Antibody, Species (Mouse), Application (WB). As noted on the manufacturer’s website, the citation is 1820. https://www.cellsignal.com/products/secondary-antibodies/anti-mouse-igg-hrp-linked-antibody/7076

Eukaryotic cell lines

Policy information about cell lines

Cell line source(s) HEK293T cells were purchased from ATCC (Cat# CRL-3216). Freestyle 293F cells were purchased from Thermo Fisher Scientific (Cat# K9000-01)

Authentication All cell lines were kept at low passages in order to maintain their identity. The expression of STING and/or TBK1 in all cell lines were checked routinely to ensure they are the same as the original cell line.

Mycoplasma contamination All cell lines were tested negative for mycoplasma contamination

Commonly misidentified lines (See ICLAC register) No commonly misidentified cell lines were used in this work.