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STING is a direct innate immune sensor of cyclic-di-GMP

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Abstract

The innate immune system detects infection by employing germline-encoded receptors specific for conserved microbial molecules. Recognition of microbial ligands leads to the production of cytokines, such as type I interferons (IFN), that are essential for successful pathogen elimination. Cytosolic detection of pathogen-derived DNA is one major mechanism of IFN induction[1,2], and requires signaling via Tank Binding Kinase 1 (TBK1), and its downstream transcription factor, Interferon Regulatory Factor 3 (IRF3). In addition, a transmembrane protein called STING (STimulator of INterferon Genes; also called MITA, ERIS, MPYS, TMEM173) functions as an essential signaling adaptor linking cytosolic detection of DNA to the TBK1/IRF3 signaling axis[3–7]. Recently, unique nucleic acids called cyclic dinucleotides, which function as conserved signaling molecules in bacteria[8], were also shown to induce a STING-dependent type I interferon response[9–12]. However, a mammalian sensor of cyclic dinucleotides has not been identified. Here we report evidence that STING itself is an innate immune sensor of cyclic dinucleotides. We demonstrate that STING binds directly to radiolabelled cyclic diguanylate monophosphate (c-di-GMP) and that this binding is competed by unlabelled cyclic dinucleotides but not by other nucleotides or nucleic acids. Furthermore, we identify mutations in STING that selectively affect

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Author Contributions: D.L.B. performed the luciferase assays, qRT-PCR, generated c-di-GMP[32], purified recombinant STING, performed c-di-GMP binding assays, and transduced *goldenticket* bone marrow macrophages. K.M.M. generated truncation mutations and performed luciferase assays. K.S-T. generated point mutants and performed luciferase assays. D.L.B., K.M.M and R.E.V. participated in study design and data analysis. D.L.B. and R.E.V. wrote the paper. B.E. contributed to protein purification methods. J.S.I. contributed to the design of ITC and equilibrium dialysis experiments and analysis of binding data. M.H. and Y.H. synthesized c-di-GMP.

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Statistical Analysis: Statistical differences were calculated with an unpaired two-tailed Student's t test using GraphPad Prism 5.0b.

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the response to cyclic dinucleotides without affecting the response to DNA. Thus, STING appears to function as a direct sensor of cyclic dinucleotides, in addition to its established role as a signaling adaptor in the interferon response to cytosolic DNA. Cyclic dinucleotides have shown promise as novel vaccine adjuvants and immunotherapeutics[9,13]. Our results provide insight into the mechanism by which cyclic dinucleotides are sensed by the innate immune system.

Although nucleotides are critical signaling molecules in all domains of life, cyclic dinucleotides appear to be produced solely by bacteria and archaea. For example, c-di-GMP is a ubiquitous second messenger that regulates biofilm formation, motility, and virulence in diverse bacterial species[8]. Recently, cyclic diadenylate monophosphate (c-di-AMP) was discovered as a bacterial regulatory molecule[14], although its role remains to be fully characterized. Since they are unique to microbes, cyclic dinucleotides serve as appropriate targets for immune recognition[15]. Indeed, induction of IFN by *Listeria monocytogenes* depends on bacterial secretion of cyclic-di-AMP[12]. However, it remains unclear how cyclic dinucleotides are sensed in mammalian cells.

To address the mechanism by which mammalian cells sense cyclic dinucleotides, we first confirmed that cyclic dinucleotides are detected in the host cell cytosol[10] by expressing RocR, a c-di-GMP-specific phosphodiesterase from *Pseudomonas aeruginosa*, in the cytosol of macrophages. In these cells, IFN induction by c-di-GMP (but not other stimuli) is reduced 10-fold compared to vector transduced cells (Fig. 1a), confirming that the cytosolic presence of c-di-GMP is important to induce IFN.

To identify candidate cyclic dinucleotide sensors, we sought to identify molecules that could reconstitute the IFN response to cyclic dinucleotides in HEK293T cells, which do not respond to c-di-GMP[10]. Since STING is essential for the IFN response to cyclic dinucleotides[11], and STING expression is low/undetectable in HEK293T cells (Supplementary Fig. 1, data not shown), we first expressed STING in HEK293T cells. Overexpression of STING spontaneously induces an IFN reporter[3,6,7], so we transfected a low amount of STING that by itself was insufficient to induce IFN. To our surprise, low levels of STING were sufficient to reconstitute responsiveness of 293T cells to c-di-GMP (Fig. 1b) and c-di-AMP (Fig. 1c). By contrast, the non-functional *goldenticket* (*gt*) allele of STING (I199N)[11] did not restore responsiveness to c-di-GMP (Fig. 1b). Interestingly, expression of wild-type STING did not confer responsiveness of 293T cells to double-stranded (ds) DNA oligonucleotides (e.g., vaccinia virus (VV) 70mer or interferon stimulatory DNA (ISD)) that were previously shown to induce type I IFN in macrophages via STING[4,16] (Fig. 1b, Supplementary Fig. 2a). By contrast, induction of IFN by poly(dAT:dTA) DNA was identical in cells transfected with wild-type or *gt Sting*, demonstrating that the Pol III DNA-sensing pathway[17,18] is intact in these cells and is not responsible for detection of c-di-GMP (Fig. 1d). As a positive control, *Myd88*$^{-/-}$*Trif*$^{-/-}$immortalized macrophages, which express STING, responded similarly to c-di-GMP, poly(dAT:dTA), VV 70mer and ISD (Fig. 1e, Supplementary Fig. 2b). Our results demonstrate STING expression is sufficient to restore responsiveness of HEK293T cells to cyclic dinucleotides but not to DNA.

We next tested whether STING, or perhaps another protein in HEK293T cells, binds to c-di-GMP. We used an *in-vitro* ultraviolet (UV) crosslinking assay to identify putative sensor protein(s) in HEK293T cell lysates that interact directly with radiolabelled c-di-GMP (c-di-GMP[32)]. We expected to identify directly interacting proteins since only molecules within bond length proximity are efficiently crosslinked by UV[19]. We detected a prominent ~40 kDa radiolabelled protein, corresponding to the predicted molecular weight of monomeric STING, in lysates of cells transfected with STING-HA, but not in lysates of cells transfected with STING-HA I199N or vector only (Fig. 2a). The ~40 kDa band did not appear when the same lysates were crosslinked with GTP[32], implying that crosslinking to c-di-GMP[32] was specific (Fig. 2a). We also observed an ~80 kDa species that possibly corresponds to a previously reported STING dimer[6] (Fig. 2b). To test the hypothesis that STING crosslinks with c-di-GMP[32], we immunoprecipitated STING from transfected HEK293T cells, and performed the c-di-GMP[32] binding assay on the immunoprecipitate. Bands corresponding to the molecular weight of STING monomer and dimer were identified only in immunoprecipitates of lysates overexpressing STING and not in mock immunoprecipitates of lysates of vector-transfected cells (Fig. 2b). Thus, STING appears to bind c-di-GMP.

To confirm that binding of c-di-GMP[32] to STING is specific, we performed the c-di-GMP binding assay in the presence of unlabelled nucleotides. Unlabelled c-di-GMP and c-di-AMP specifically competed with c-di-GMP[32] for binding to STING (Fig. 2c, d). By contrast, GTP, other guanosine derivatives, or nucleic acids (including dsDNA), competed away non-specific binding (asterisks, Fig. 2c, d), but, under our specific assay conditions, could not compete efficiently with c-di-GMP[32] for binding to STING (arrows, Fig. 2c, d). Since the cell cytosol contains high (0.1–1mM) concentrations of GTP, a putative c-di-GMP sensor must exhibit a high degree of specificity for c-di-GMP over GTP. We found that c-di-GMP efficiently crosslinked to STING even in the presence of 1mM GTP (Fig. 2c).

Although these data imply that STING directly and specifically binds cyclic dinucleotides, they do not address whether other host proteins might also be required. STING is predicted to encode an N-terminal domain with multiple transmembrane segments, followed by a globular C-terminal domain (CTD). Since the CTD contains the amino acid substitution (I199N) that abolishes STING function in *goldenticket* mice[11], we hypothesized that the CTD might be involved in binding cyclic dinucleotides. Thus, we subjected purified recombinant His_6-tagged CTD of STING (amino acids 138–378) (Fig. 2e) to the c-di-GMP[32] binding assay. We found that the recombinant CTD of STING bound c-di-GMP[32], and that binding was specifically competed with cold c-di-GMP or c-di-AMP but not cold GTP or ATP (Fig. 2f). We used equilibrium dialysis to obtain an estimate of ~5μM for the affinity (K_d) of c-di-GMP binding to the STING CTD (Fig. 2g). In its native membrane-bound form, or in complex with other host factors, STING may exhibit a stronger affinity for c-di-GMP; nevertheless, a 5μM affinity is consistent with the dose response previously observed in macrophages[12]. Consistent with the ability of STING to dimerize[6], the binding data suggest a stoichiometry of one molecule of c-di-GMP per two molecules of STING.

In order to identify amino acids involved in c-di-GMP binding and/or IFN induction, we introduced point mutations into STING. Focusing on clusters of conserved and charged residues, we mutated a total of 67 amino acids, individually or in groups, and identified

mutants that fell in one of five categories (Fig. 3, Supplementary Table 1, Supplementary Fig. 3–4). Class I consists of mutations that abolish both binding and IFN induction (Fig. 3a–c, red, Supplementary Table 1). Class II mutants bind c-di-GMP but fail to induce IFN (Fig. 3c, purple). Class III comprises "hyperactive" mutants that spontaneously induce IFN at low levels of transfection (Fig. 3a–c, green, Supplementary Table 1). Class IV mutants induced IFN when overexpressed, but were not inducible in response to c-di-GMP (Fig. 3a–c, blue, Supplementary Table 1). Class V consists of mutants that had no effect on binding or IFN induction (Fig. 3c, yellow, Supplementary Table 1). Although mutation of STING can result in diverse phenotypes, a key finding is that all mutants that failed to bind c-di-GMP also lost the ability to induce IFN in response to c-di-GMP. Consistent with our observation that the CTD is sufficient to bind c-di-GMP (Fig. 2f), all mutations that affected c-di-GMP binding were located within the CTD.

DNA and cyclic dinucleotides induce indistinguishable transcriptional responses in macrophages[10] and STING appears essential for both responses[4,11]. However, in contrast to cyclic dinucleotides, we found STING expression is insufficient to restore responsiveness of HEK293T cells to DNA (Fig. 1). Moreover, our competition assays indicate that DNA does not compete with cyclic-di-GMP for binding to STING under the conditions tested (Fig. 2d). Thus, while our data indicate STING functions as a direct immunosensor of cyclic dinucleotides, additional host proteins appear likely to be involved in IFN induction by DNA. Indeed, two candidate DNA sensors, DAI and IFI16, have been identified[16,20], neither of which appear to be essential for the response to cyclic dinucleotides ([10]; unpublished data). To determine if responsiveness to cyclic dinucleotides and DNA are separable functions of STING, we sought to identify STING mutants that fail to respond to cyclic dinucleotides but still respond to DNA. We identified a STING mutant (R231A) that was unresponsive to c-di-GMP (Fig. 4a), though it still induced IFN when overexpressed (Fig. 4a) and still bound c-di-GMP (Fig. 4b). Interestingly, STING R231A was able to restore responsiveness of *goldenticket* bone marrow macrophages to DNA, but not to cyclic-di-GMP (Fig. 4c). Thus, cyclic dinucleotide sensing and DNA sensing can be uncoupled, suggesting that these two pathways are discrete but share STING as a common signaling molecule. It is unexpected that STING would function both as a direct immunosensor (of cyclic dinucleotides) and as a signaling adaptor (in the response to DNA). One possibility is that STING initially evolved as a cyclic dinucleotide sensor and was subsequently coopted for DNA sensing.

We previously used mouse mutagenesis to identify STING as an essential molecule in the *in vivo* IFN response to cyclic dinucleotides[11]. The requirement for STING can now be rationalized by our proposal that STING functions as a direct sensor of cyclic dinucleotides. Interestingly, STING does not share homology with any known immunosensor, and therefore appears to represent a novel category of microbial detector. Although a BLAST search of the mouse proteome for homologs of the *Listeria* diadenylate cyclase (lmo2120; DacA) identifies STING as the top hit, the homology is limited to a short region of the STING CTD (amino acids 311–358). STING does not appear to exhibit homology to PilZ-domain proteins that function as c-di-GMP receptors in bacteria[8]. Structural studies are required to determine if STING resembles any known proteins in mammals or bacteria.

Numerous studies have demonstrated that cyclic dinucleotides are potent immunostimulatory compounds that may be valuable as novel immunotherapeutics or adjuvants[9,13]. Therapeutic development of cyclic dinucleotides will be greatly facilitated by an improved understanding of the mechanism by which they are sensed. Furthermore, our finding that STING is a direct detector of cyclic dinucleotides provides insight into the fundamental mechanisms by which the innate immune system can detect bacterial infection.

Methods Summary

Transfections

Transfections were carried out using Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen). C-di-AMP was introduced into cells using digitonin permeabilization as previously described[12].

Recombinant STING

DNA encoding the carboxy terminal domain of mouse STING (nucleotides 414 - 1137) was cloned into pET28a for recombinant protein expression in *E. coli*.

UV Crosslinking

C-di-GMP[32] was enzymatically synthesized using recombinant WspR and used in a UV-crosslinking assay as described previously[21]. Briefly, 50 μg of HEK293T cell lysate at a final concentration of 2 μg/μl, or 1 μg of recombinant His_6-tagged STING, was incubated with 2 μCi of c-di-GMP[32] in binding buffer (20 mM Tris-HCl, pH 7.4, 200 mM NaCl, 1mM $MgCl_2$) for 15 minutes at room temperature. Reactions were UV irradiated at 254 nm and separated by SDS-PAGE.

Methods

Cell lines and animals

C57BL/6 *Myd88*$^{-/-}$*Trif*$^{-/-}$ knockout mice were obtained from G. Barton (University of California, Berkeley) and immortalized macrophages were generated as previously described[22]. Immortalized bone marrow macrophages were maintained in RPMI 1640 (Invitrogen) supplemented with 10% fetal bovine serum, penicillin-streptomycin, and glutamine. HEK293T cells were maintained in DMEM supplemented with 10% fetal bovine serum, penicillin-streptomycin, and glutamine. Animal use was approved by the Animal Care and Use Committee at UC Berkeley.

Plasmids

A construct encoding RocR (NP_252636) from *Pseudomonas aeruginosa* was a generous gift from Steve Lory[23]. *RocR* was cloned into the MSCV2.2 retroviral expression construct upstream of an IRES-GFP. MSCV-*rocR* was transduced into immortalized macrophages from *Myd88*$^{-/-}$*Trif*$^{-/-}$ mice and cells were sorted for GFP expression. Mouse *Sting* and the *goldenticket* (I199N) mutant allele of *Sting* were cloned into pcDNA3 with a carboxy terminal hemagglutinin (HA) tag as described previously[11]. DNA encoding the carboxy

terminal domain of mouse *Sting* (nucleotides 414 - 1137) was cloned into pET28a for recombinant protein expression in *E. coli*.

Site-directed mutagenesis

Mutations in *Sting* were generated using the QuikChange Site-directed mutagenesis kit (Stratagene) according to the manufacturer's guidelines.

Reagents

C-di-GMP was synthesized as described previously[24]. Purified C-di-AMP was the generous gift of Josh Woodward and Dan Portnoy (University of California, Berkeley). Poly(dAT:dTA), GTP, ATP, GMP, and guanosine were obtained from Sigma-Aldrich. Poly(I:C) was purchased from Invivogen. Guanosine-3',5'-bisdiphosphate (ppGpp) was obtained from Trilink. Sendai virus (SeV) was purchased from Charles River Laboratories. Theiler's virus (TMEV) strain GDVII was from M. Brahic and E. Freundt (Stanford). Single-stranded oligonucleotides corresponding to the Vaccinia Virus dsDNA 70-mer were purchased from Elim BIOPHARM and were annealed as described[2,16].

Cell stimulations

All transfections (excluding c-di-AMP) were carried out using Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen). Vaccinia virus 70mer was transfected at a final concentration of 0.5 μg/ml. C-di-GMP, poly(dAT:dTA), and poly(I:C) transfected at a final concentration of 4 μg/ml. C-di-AMP was used at a final concentration of 5.4 mM and stimulation was performed using digitonin permeabilization as previously described[12].

Luciferase Assay

HEK293T cells were plated in TC-treated 96 well plates at 0.5×10^6 cells/ml. The next day, cells were transfected as indicated along with IFNβ-firefly and TK-*Renilla* luciferase reporter constructs. Following stimulation for 6 hours with the indicated ligands, cells were lysed in passive lysis buffer (Promega) for 5 minutes at room temperature. Lysates were incubated with firefly luciferase substrate (Biosynth) and *Renilla* luciferase substrate coelenterazine (Biotium) and luminescence was measured on a SpectraMaxL (Molecular Devices). Relative IFNβ expression is calculated as firefly luminescence relative to *Renilla* luminescence.

Quantitative PCR

Analysis of IFNβ expression in bone marrow macrophages was conducted as previously described[10].

Synthesis of c-di-GMP^{32}

Synthesis of c-di-GMP^{32} was carried as described[21]. Briefly, recombinant His_6-tagged WspR was incubated with GTP-alpha-P^{32} (3000 Ci/mmol, 10 μCi/μl, Amersham Biosciences) for 2 hours at room temperature, followed by heat inactivation of WspR at 95°C for 5 minutes. Residual GTP^{32} was removed by incubation with calf intestinal

phosphatase (New England Biolabs) for 10 minutes at 37°C. CIP was heat inactivated at 95°C for 5 minutes following by centrifugation at 16000 × *g* for 5 minutes. The GTP[32] used as a negative control was prepared identically except His_6-tagged WspR was omitted from the preparation. Radiolabelled nucleotides were quantified by separation by thin-layer chromatography on PEI-cellulose (Machery-Nagel) using 1.5 M KH_2PO_4, pH 3.65 (Supplementary Figure 5).

Preparation of HEK293T cell lysates and immunoprecipitations

HEK293T cells were plated at a density of 1×10^6 cells/well of a six well plate. The following day, cells were transfected with pcDNA3 or pcDNA3 expressing HA-tagged wild type or mutant STING using Lipofectamine 2000 (Invitrogen). The following day, cells were rinsed once with phosphate buffered saline followed by removal with PBS with EDTA (1 mM) into eppendorf tubes. Cells were pelleted briefly by centrifugation at 1000 × *g* at 4°C. The cell pellet was lysed in an equal volume of digitonin lysis buffer (0.5% digitonin, 20 mM Tris-HCl, pH 7.4, 150 mM NaCl) containing protease inhibitors (Roche) for 10 minutes on ice. Cell lysates were centrifuged at 10,000 × *g* at 4°C for 10 minutes. The protein concentration was measured in the resulting supernatant using the Bradford reagent (Bio-Rad). Cell lysates were subjected to a c-di-GMP binding assay (see below). Cell lysates were separated by SDS-PAGE, transferred to nitrocellulose and probed with rat anti-HA antibodies (Roche) to confirm STING-HA expression and mouse anti-β-actin (Santa Cruz Biotechnology). To immunoprecipitate HA-tagged STING, cell lysates were prepared similarly in digitonin lysis buffer and incubated with anti-HA conjugated agarose beads (Sigma) for 2 hours at 4°C. Washed beads were subjected to a c-di-GMP binding assay or separated by SDS-PAGE as described and stained with colloidal blue protein stain (Thermo).

C-di-GMP binding assay

The c-di-GMP binding assay was based on a method described previously[21]. Briefly, 50 μg of HEK293T cell lysate at a final concentration of 2μg/μl, or 1 μg of recombinant His_6-tagged STING, was incubated with 2 μCi of radiolabelled nucleotide in binding buffer (20 mM Tris-HCl, pH 7.4, 200 mM NaCl) for 15 minutes at room temperature. Reactions were irradiated at 254 nm for 20 minutes on ice at a 3 cm distance with a UVG-54 mineral light lamp (UVP). Immediately following crosslinking, the reaction was terminated by the addition of SDS sample buffer (40% glycerol, 8% SDS, 2% βME, 40 mM EDTA, 0.05% bromophenol blue, 250 mM Tris-HCl, pH 6.8) boiled for 5 minutes at 100°C and separated by SDS-PAGE. Gels were dried and exposed to a phosphor screen and visualized using a Typhoon Trio (GE Healthcare).

Protein purification

The construct expressing a constitutively active form of WspR (pQE-WspR*) was a generous gift from Steve Lory[23,25]. Purification of His_6-tagged WspR was carried out as described previously using Ni-NTA affinity chromatography (Qiagen)[26]. DNA encoding the carboxy terminal domain of mouse STING (nucleotides 414 - 1137) was cloned into pET28a

and purified by Ni-NTA affinity chromatography according to the manufacturer's instructions (Qiagen).

Equilibrium dialysis

The binding affinity of radioactive c-di-GMP was measured by equilibrium dialysis using a 96-well equilibrium dialyzer (Harvard Apparatus) with a 5,000 molecular weight cut-off membrane. One chamber contained 150 μL of 10 μM purified H_6-STING(138 – 378) in assay buffer (25 mM Tris-HCl pH 7.4, 100 mM NaCl, 1 mM $MgCl_2$, 10% glycerol) while the other was filled with 150 μL of c-di-GMP[32] in a range of concentrations from 40 nM to 160 μM. Equilibrium was reached after 48 hours at 25°C and three samples were drawn from each chamber and mixed with 2 mL of Econo-safe scintillation fluid. Samples were measured in an LS 6000 IC (Beckman). Data analysis was performed using GraphPad Prism software. K_d, B_{max} , and h (Hill slope) were generated using non-linear regression allowing for one site specific binding with a Hill slope.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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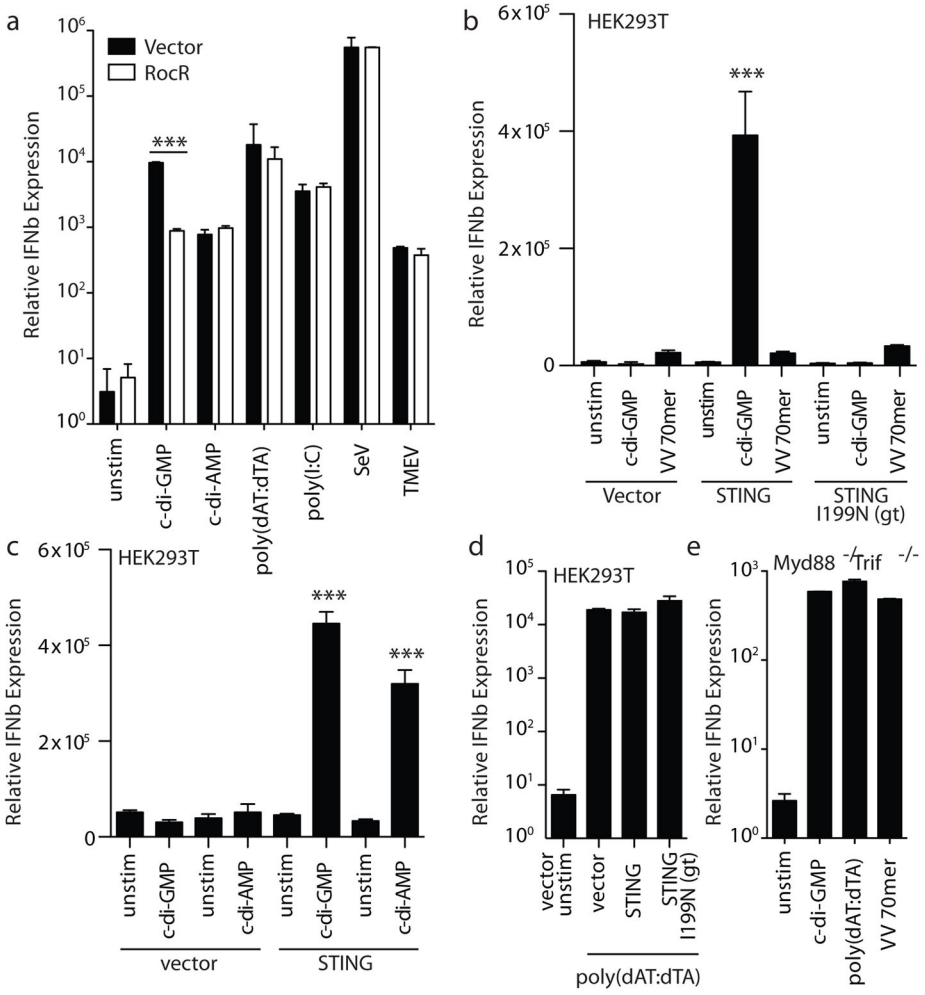

Figure 1. STING is sufficient to restore responsiveness to cyclic dinucleotides

a, Immortalized *Myd88* $^{-/-}$*Trif* $^{-/-}$ macrophages were transduced with retrovirus expressing *RocR* and stimulated for 6h. SeV, Sendai Virus; TMEV, Theiler's Virus. IFN induction was measured by qRT-PCR and normalized to ribosomal protein 17 (*Rps17*). (**b–d**) HEK293T cells were transfected as indicated along with an IFN-luciferase reporter and luciferase activity was measured 6h following stimulation. VV 70-mer is a stimulatory dsDNA oligo derived from Vaccinia virus. ***, $P < 0.001$ **e,** *Myd88* $^{-/-}$*Trif* $^{-/-}$macrophages were stimulated for 6h and IFN induction was measured as in a. Data are mean ± s.d. (n = 3) and are representative of at least three independent experiments.

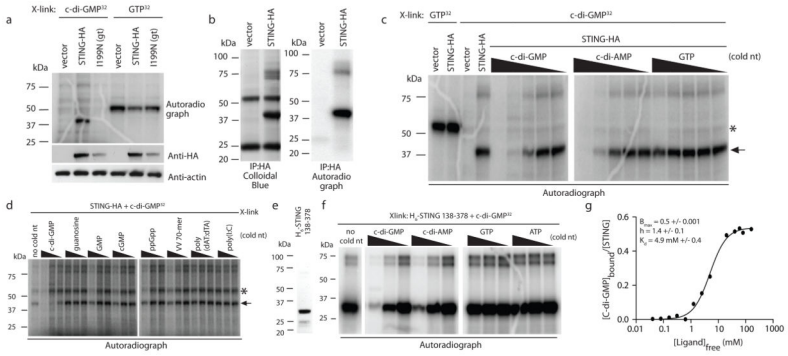

Figure 2. STING binds cyclic dinucleotides

a, HEK293T cells were transfected as indicated and cell lysates were subjected to an *in-vitro* UV-crosslinking assay with c-di-GMP^{32}. Samples were separated by SDS-PAGE and visualized by autoradiography or western blotting. **b,** HEK293T cells were transfected as in **a** and anti-HA immunoprecipitates were subjected to the c-di-GMP^{32} binding assay or stained with Colloidal Blue. **c** and **d,** HEK293T cells were transfected as in **a** and cell lysates were UV-crosslinked to c-di-GMP^{32} or GTP^{32} in the presence of cold competing nucleotides in 10-fold serial dilutions beginning at 1 mM, except for guanosine (0.1 mM), VV 70mer (500 μg/mL), poly(dAT:dTA) (50 μg/mL) and poly(I:C) (50 μg/mL). Arrow, STING; Asterisk, nonspecific bands. **e,** 1μg His_6-mSTING 138–378 was separated by SDS-PAGE and stained with coomassie. **f,** His_6-mSTING was analyzed as in **c. g**, C-di-GMP binding to purified His_6-mSTING (10μM) was measured by equilibrium dialysis. Data are representative of three independent experiments.

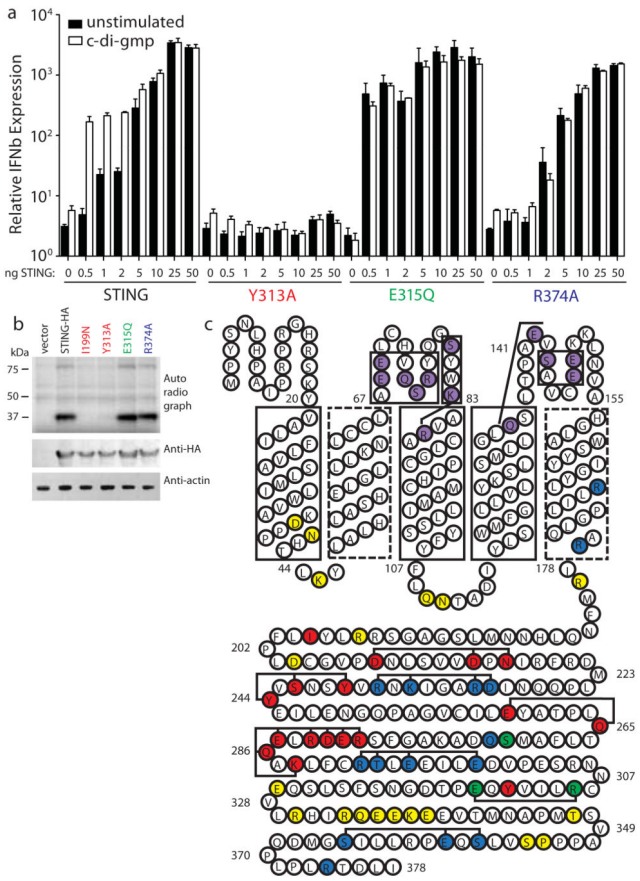

Figure 3. Mutational analysis of STING

a, HEK293T cells were transfected as indicated along with an IFN-luciferase reporter and luciferase activity was measured 6h following stimulation. **b,** HEK293T cells were transfected as in **a** except the lysates were subjected to the c-di-GMP[32] binding assay as in Fig. 2a. **c,** Organization of STING based on the membrane topology prediction programs SOSUI, TMHMM, HMMTOP and TMPRED. Strongly predicted transmembrane domains are boxed; weakly predicted transmembrane domains have dashed boxes. Colored residues indicate mutant classes (see text): Class I (red), Class II (purple), Class III (green), Class IV (blue) and Class V (yellow). Bracketed mutations were made in combination. Data are representative of at least three independent experiments. Data are mean ± s.d. (n = 3).

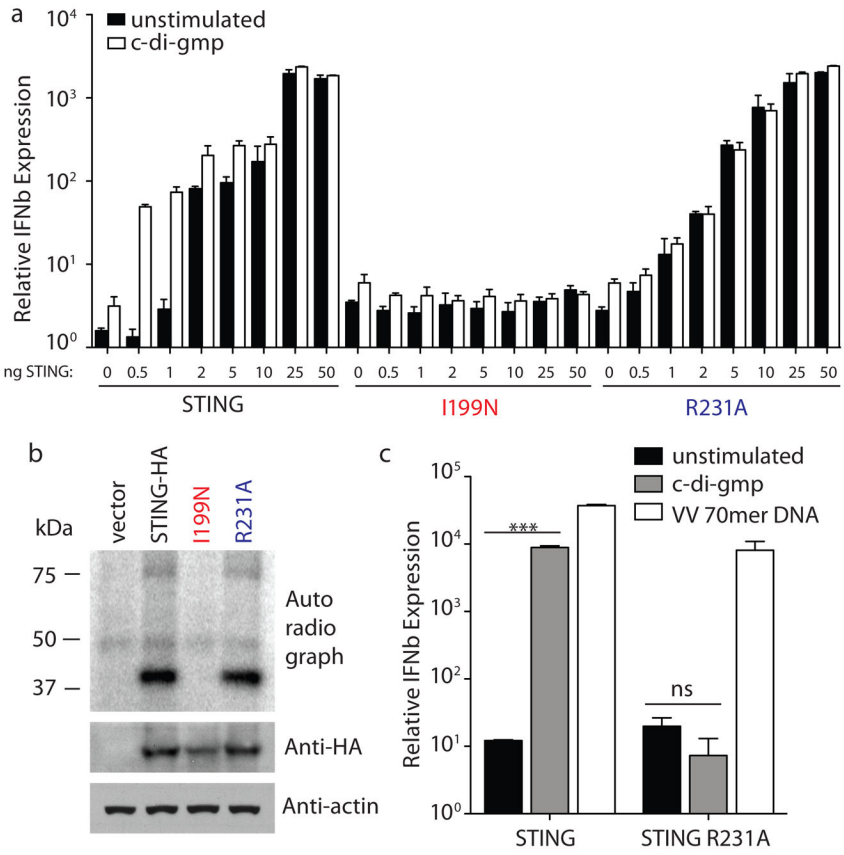

Figure 4. The IFN response to DNA and c-di-GMP can be uncoupled

a, HEK293T cells were transfected and IFN reporter activity was measured 6h following stimulation. **b,** HEK293T cells were transfected as in **a** except the lysates were subjected to the c-di-GMP[32] binding assay as in Fig. 2a. **c**, Bone marrow-derived macrophages from *Sting*-deficient *goldenticket* mice were transduced with the indicated constructs. IFN induction by transfected cyclic-di-GMP and VV 70-mer dsDNA was measured by qRT-PCR and normalized to ribosomal protein 17 (*Rps17*). ***, $P < 0.001$. ns, not significant, $P = 0.1205$) Data are representative of at least three independent experiments. Data are mean± s.d. (n = 3).