EXTENDED EXPERIMENTAL PROCEDURES

Plasmid Construction

The human and murine STING sequences (reference sequences) were inserted into a modified pMAX-cloning (Amaxa, Cologne, Germany) by standard cloning techniques, in frame with a N-terminal FLAG-tag. Site-directed mutagenesis was performed by the Quikchange method (Agilent, Santa Clara, CA) using Pfu Ultra Hot Start DNA Polymerase (Agilent) or Phusion Polymerase (NEB, Ipswich, MA). Luciferase reporter constructs were as described (Gao et al., 2013). Constructs (Figure S7) were verified by restriction digest and Sanger sequencing (Seqlab, Göttingen, Germany).

Human STING mutants were assembled from C-terminal mutated constructs (140-C) and N-terminal reference sequence by Gibson Assembly (NEB) and confirmed by Sanger sequencing. STING^{THP1} and STING^{R232} cDNAs were cloned from THP1 cells and Peripheral Blood Mononuclear Cells of a voluntary human donor with informed consent, respectively.

Protein Expression and Purification

The sequences corresponding to residues aa 140–379 and 155–341 of hSTING^{H232}, hSTING^{R232} and hSTING$^{A230/R232}$ and residues 139–378 and 154–340 of mSting^{R231} were inserted into a modified pRSFDuet-1 vector (Novagen), in which the target protein was separated from the preceding His_6-SUMO tag by an ubiquitin-like protease (ULP1) cleavage site. The gene sequences were subsequently confirmed by sequencing. The fusion proteins were expressed in BL21 (DE3) RIL cell strain. The cells were grown at 37°C until OD600 reached approx. 0.6. The temperature was then shifted to 18°C and the cells were induced by addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) to the culture medium at a final concentration of 0.3 mM. After induction, the cells were grown overnight. The fusion proteins were purified over a Ni-NTA affinity column. The His_6-SUMO tag was removed by ULP1 cleavage during dialysis against buffer containing 40 mM Tris-HCl, 300 mM NaCl (pH 7.5). After dialysis, the His_6-SUMO tag was removed by Ni-NTA affinity column and the sample was further fractionated over a gel filtration 16/60 G200 Superdex column. The final sample of hSTING^{H232}, hSTING^{R232} and hSTING$^{A230/R232}$ and mSting^{R231} contain about 13 mg/ml protein, 20 mM Tris-HCl, 150 mM NaCl (pH 7.5). All the mutants were cloned and purified using the same protocol as used for preparation of the wild-type protein.

Crystallization

For crystallization of hSTING^{H232} (155-341) with c[G(2′,5′)pA(3′,5′)p] and c[G(2′,5′)pA(2′,5′)p], the protein was incubated with c[G(2′,5′)pA(3′,5′)p] (2 mM) or c[G(2′,5′)pA(2′,5′)p] (2 mM) and $MgCl_2$ (5 mM) for 0.5 hr at room temperature. The crystals were generated by sitting drop vapor diffusion method at 20°C, by mixing equal volume reservoir solution (for c[G(2′,5′)pA(3′,5′)p]: 0.01 M $NiCl_2$, 0.1 M Tris, 20% PEG2000 (pH 8.5); for c[G(2′,5′)pA(2′,5′)p]: 1.6 M NaH_2PO_4, 0.4 M Na_2HPO_4, 0.1 M phosphate-citrate [pH 4.2]) with the sample.

For crystallization of mSting^{R231} (154-340) with c[G(2′,5′)pA(3′,5′)p], c[G(3′,5′)pA(3′,5′)p] and DMXAA, the protein was incubated with c[G(2′,5′)pA(3′,5′)p] (2 mM) or c[G(3′,5′)pA(3′,5′)p] (2 mM) or DMXAA (4 mM) for 0.5 hr at room temperature. The crystals were generated by sitting drop vapor diffusion method at 20°C, by mixing equal volume reservoir solution (for c[G(2′,5′)pA(3′,5′)p]: 0.2 M di-ammonium tartrate, 20% PEG3350; for c[G(3′,5′)pA(3′,5′)p]: 0.2 M sodium formate, 20% PEG3350; for DMXAA: 1.6 M ammonium sulfate, 0.1 M Tris-HCl [pH 8.0]) with the samples.

Structure Determination

All the diffraction data sets (except mSting^{R231} with DMXAA) were collected at the Brookhaven National Laboratory, and were indexed, integrated and scaled using the HKL2000 program (Otwinowski and Minor, 1997). The data set for mSting^{R231} with DMXAA was collected at Argonne National Laboratory and was indexed, integrated and scaled using the RAPD online server. The structure of hSTING^{H232} with c[G(2′,5′)pA(3′,5′)p] or c[G(2′,5′)pA(2′,5′)p] was solved using molecular replacement method in PHASER (McCoy et al., 2007) using the complex structure of hSTING$^{A230/R232}$ and c[di-GMP] (PDB: 4F5D) as the search model. For mSting^{R231} with c[G(2′,5′)pA(3′,5′)p], c[G(3′,5′)pA(3′,5′)p] and DMXAA, the structure of mSting^{R231} and CMA (PDB: 4JC5) was used as the search model. For the cGAMP-STING binary structures, two STING molecules in the STING dimer have equal probability to bind with either G or A moiety of the cGAMP isomers. The electron density also clearly indicated that the cGAMP isomers adopt two alternative conformations. We therefore assigned two conformations with 0.5 occupancy for bound cGAMP isomers. The model building was conducted using the program COOT (Emsley et al., 2010) and structural refinement was conducted using the program REFMAC (Murshudov et al., 1997). The statistics of the data collection and refinement are shown in Tables S1 and S2.

Isothermal Titration Calorimetry Binding Assay

The dissociation constants (Kd) and thermodynamic parameters of binding reactions of hSTING^{H232} (aa 140–379), hSTING^{R232} (aa 140–379), hSTING$^{A230/R232}$ (aa 140–379), mSting^{R231} (aa 139–378), mSting^{A231} (aa 139–378) and hSTING^{H232} (aa 140–379) mutants with different cGAMP isomers were measured by isothermal titration calorimetry using a MicroCal ITC200 calorimeter at 25°C. First, wild-type and mutant protein samples were dialyzed overnight against working buffer (100 mM NaCl, 30 mM HEPES [pH 7.5]) at 4°C. Then, the protein samples were diluted with working buffer and the lyophilized cGAMP isomers were dissolved in working buffer. The titration was carried out with 16 successive injections of 2.4 μl cGAMP isomers, spaced 180 s apart, into the sample cell containing the protein solution. The data for mSting^{R231} (aa 139–378) and hSTING^{S162} (aa 140–379) mutants with DMXAA were collected using

the same protocol as used for STING with cGAMP isomers. The detailed concentration for different titrations was listed in Tables S3, S4 and S6. The data were fit using the program Origin 7.0 software.

We observe a mixture of exothermic and endothermic transitions for complex formation of hSTING/mSting complexes with c[G(3′,5′)pA(3′,5′)p] (green triangles, Figure 4, and Table S3), primarily endothermic transitions for complex formation of hSTING/mSting complexes with c[G(2′,5′)pA(2′,5′)p] (red circles, Figure 4, and Table S3), and exclusively endothermic transitions for hSTING/mSting complexes bound to c[G(2′,5′)pA(3′,5′)p] (black squares, Figure 4, and Table S3). Endothermic binding events are not uncommon and may reflect ligand-induced conformational changes in STING that yield significant solvent reorganization/displacement, often resulting in ''melting'' (an endothermic event) and release (an entropically favorable event) of ''restricted'' (bound) solvent (Table S3). The distinct thermodynamic outcome (exothermic versus endothermic) could possibly relate to very subtle structural differences or alternately to the use of distinct folding pathways with defined energy parameters to arrive at the same or a distinct endpoint.

Mice

Female C57B/6 mice between 6 and 10 weeks of age were purchased from the Jackson Laboratory and were used for the preparation of bone-marrow-derived macrophages. These mice were maintained in the animal facility at the Sloan-Kettering Cancer Institute. All procedures were performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institute of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments of Sloan-Kettering Cancer Institute (protocol number 96-04-017). *Irf3*$^{-/-}$ and *Sting*$^{Gt/Gt}$ mice were generated in the laboratories of Drs. Tadatsugu Taniguchi (University of Tokyo) and Russell Vance (University of California, Berkeley), respectively.

Generation of Bone-Marrow-Derived Macrophages

Bone marrow cells were collected and cultured in complete medium (CM) in the presence of 5% of supernatant of L929 mouse fibroblasts as conditioned medium providing macrophage colony-stimulating factor (M-CSF) for 7 days. CM is RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin, 0.1 mM essential and nonessential amino acids, 2 mM L-glutamine, 1 mM sodium pyruvate, and 10 mM HEPES-KOH buffer. Cells were fed at day 4 by replacing 50% of the old medium with fresh medium. Cells were plated into 6-well plate (1 million cells per well) at day 7, the day before stimulation.

THP1 Cell Culture

Human THP1 cells were cultured in RPMI1640 (Life Technologies) containing 10% FBS, 2 mM glutamine (Life Technologies), 1 mM sodium pyruvate (Life Technologies), and 100 U/ml penicillin/streptomycin (Life Technologies).

cGAMP Stimulation of Cells

For ELISA experiments, cells were plated at a density of $1x10^5$ per 96-well and stimulated either by incubation with cGAMP isomers at indicated concentrations for 18 hr, or by Digitonin permeabilization (30 min) at indicated concentrations as described (Woodward et al., 2010). Supernatants were taken after 18 hr. THP1 cells were plated at $8x10^4$ cells per 96-well and treated as described above. For RT-PCR analyses, $5x10^5$ THP1 cells were plated per 12-well dish and incubated overnight. 12 μM of the various cGAMP linkage isomers (synthesized by Roger Jones, Rutgers University) were added directly to the media and cells were harvested at indicated times.

RNA Isolation and Real-Time PCR

Total RNA was extracted from whole-cell lystates with an RNeasy Mini kit (QIAGEN) and reverse transcribed with the first Strand cDNA synthesis kit (Fermentas). Quantitative real-time PCR was performed in triplicate with the Applied Biosystem 7500 Real-Time PCR Detection System (Life Technologies) using Fast SYBR Green Master Mix and gene-specific primers. Relative expression was normalized to the levels of glyceraldehyde-3-phosphate dehydrogenase (GADPH).

RT-PCR Analysis

RNA was extracted and isolated from each sample using TRIzol (Life Technologies), per manufacturer's instructions. Superscript III RT-PCR Kit (Life Technologies) was used for generating cDNA libraries, using oligo-dT primers, per manufacturer's instructions. The following primers were used for PCR: IFNB1 (5′-GGACCATAGTCAGAGTGGAAATCCTAAG-3′, 5′-CACTTAAACAGCATCTGC TGGTTGAAG-3′), TUBA1B (5′-ACCTTAACCGCCTTATTAGCCA-3′, 5′-ACATTCAGGGCTCCATCAAATC-3′), CXCL10 (5′-GCTACC TACATACAATTCCAAACACATAC-3′, 5′-GTACTTAATTACATGTTATTCCATGTACACTGAAAAC-3′). PCR was accomplished using KOD Hot Start DNA polymerase (EMD Millipore) according to manufacturer's instructions. ImageJ (v1.47q) was used for quantitation of amplicons using TUBA1B for normalization.

Luciferase Assay

For cGAMP Luciferase Assays, $3x10^4$ HEK293T cells (Life Technologies, Carlsbad, CA) per 96-well were reverse-transfected with a mix of pGL3-IFNB1-Gluc (50 ng), pLenti-EF1-Fluc (10 ng), pMAX-Flag-STING (5 ng) and 35 ng empty plasmid (pMAX-cloning;

Amaxa, Cologne, Germany) using Trans-IT LT1 Reagent (MirusBio, Madison, WI). 12 hr after transfection, cGAMP isomers (synthesized by Roger Jones) and c[di-GMP] (Invivogen, San Diego, CA) were delivered with digitonin permeabilization as described (Woodward et al., 2010). DMXAA (Sigma) was diluted in fresh medium added to transfected cells. Luciferase expression was determined after 12 hr. For stimulation by cGAS, cells were transfected as described above, but instead of empty plasmid pLenti(p)-EF1-Flag-mm-cGAS (WT or nonfunctional E211A mutation) was used, and Luciferase expression was determined 30 hr after transfection. In this setting, expression plasmid served as cGAS stimulus at the same time. Cells were lysed in Passive Lysis Buffer. Firefly and gaussia luciferase activities were determined on an EnVision reader (Perkin Elmer, Waltham, MA) using their respective substrates (D-Luciferin and coelenterazine, PJK GmbH, Kleinblittersdorf, Germany) according to standard protocols. IFNB1-Gluc values were normalized to constitutive firefly luciferase values and fold induction was calculated in relation to control-plasmid pMAX-GFP.

ELISA

CXCL10 was measured using the BD Opteia human IP-10 ELISA Set according to manufacturer's recommendations. Murine IFN-α was determined by ELISA using antibody RMMA-1 for capture and rabbit-anti-murine IFN-α polyclonal antibody for detection (PBL Interferon Source, Piscataway, NJ). Dose-response curves were fitted and EC_{50} values were determined with Graphpad Prism (Graph Pad Software Inc., San Diego, CA).

Western Blot Analysis

BMDMs (1×10^6) were treated with cGAMP linkage isomers. At various times poststimulation, the medium was removed and cells were collected. Whole-cell lysates were prepared at 2, 4, and 8 hr after treatment. Equal amounts of proteins were subjected to SDS-PAGE and transferred to a nitrocellulose membrane. Phosphorylation of IRF3 was determined using a rabbit polyclonal antibody specific for phosphoserine-396 of IRF3 (Cell signaling). The level of IRF3 was determined by using a rabbit polyclonal antibody against IRF3. Phosphorylation of TBK1 was determined using a rabbit monoclonal antibody specific for phosphoserine-172 of TBK1 (Cell Signaling). The level of TBK1 was determined by using a rabbit monoclonal antibody against TBK1 (Cell Signaling). Anti-glyceraldehyde-3-phosphate dehydrogenase (GADPH) was used as loading controls.

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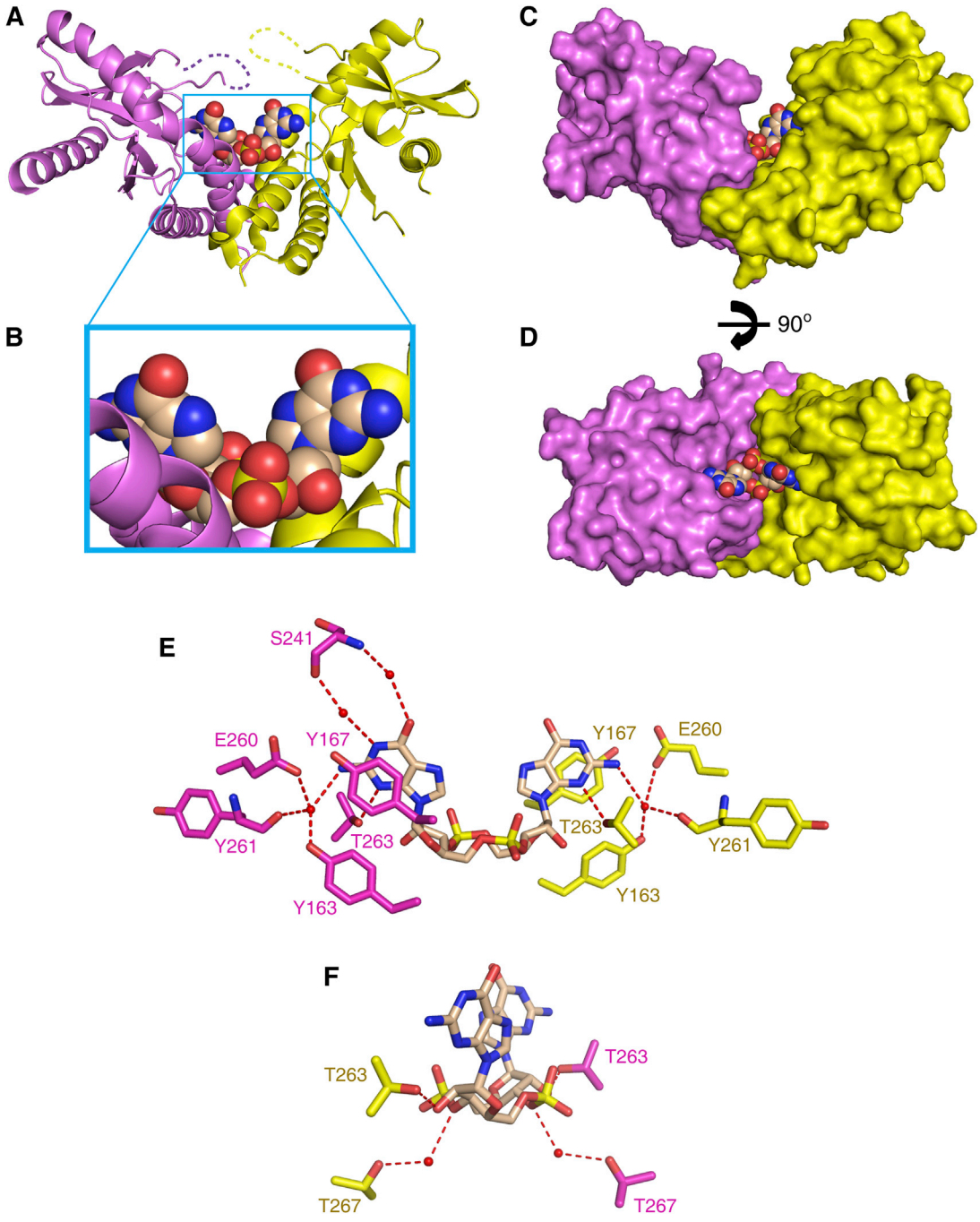

Figure S1. Surface Views of the Published Crystal Structure of c[di-GMP] Bound to hSTING^{H232} and Details of Intermolecular Contacts in the Complex, Related to Figure 1

(A) The published 2.15 Å crystal structure of c[di-GMP] bound to hSTING^{H232} (aa 139–379) (PDB: 4EF4). The representations and color codes are the same as used in Figure 1A. Note that the two loops that protrude over the binding pocket are disordered for about half their lengths as one proceeds toward the tips of these loops.

(B) An expanded view of the c[di-GMP] binding pocket in the complex.

(C) A surface representation of the published structure of the complex of c[di-GMP] bound to human STING^{H232} (aa 139–379) (PDB: 4EF4) with the same color-coding as in Figure 1A.

(D) The view in (C) rotated through 90°.

(E and F) Intermolecular contacts in the complex of c[di-GMP] bound to hSTING^{H232}. The bound cyclic c[di-GMP] is shown in biscuit color, with individual STING subunits in the symmetrical dimer shown in magenta and yellow. The intermolecular contacts to the base edges of the ligand by the magenta and yellow subunits of STING are shown in (E), whereas the intermolecular contacts to the backbone phosphates of the ligand by STING are shown in (F).

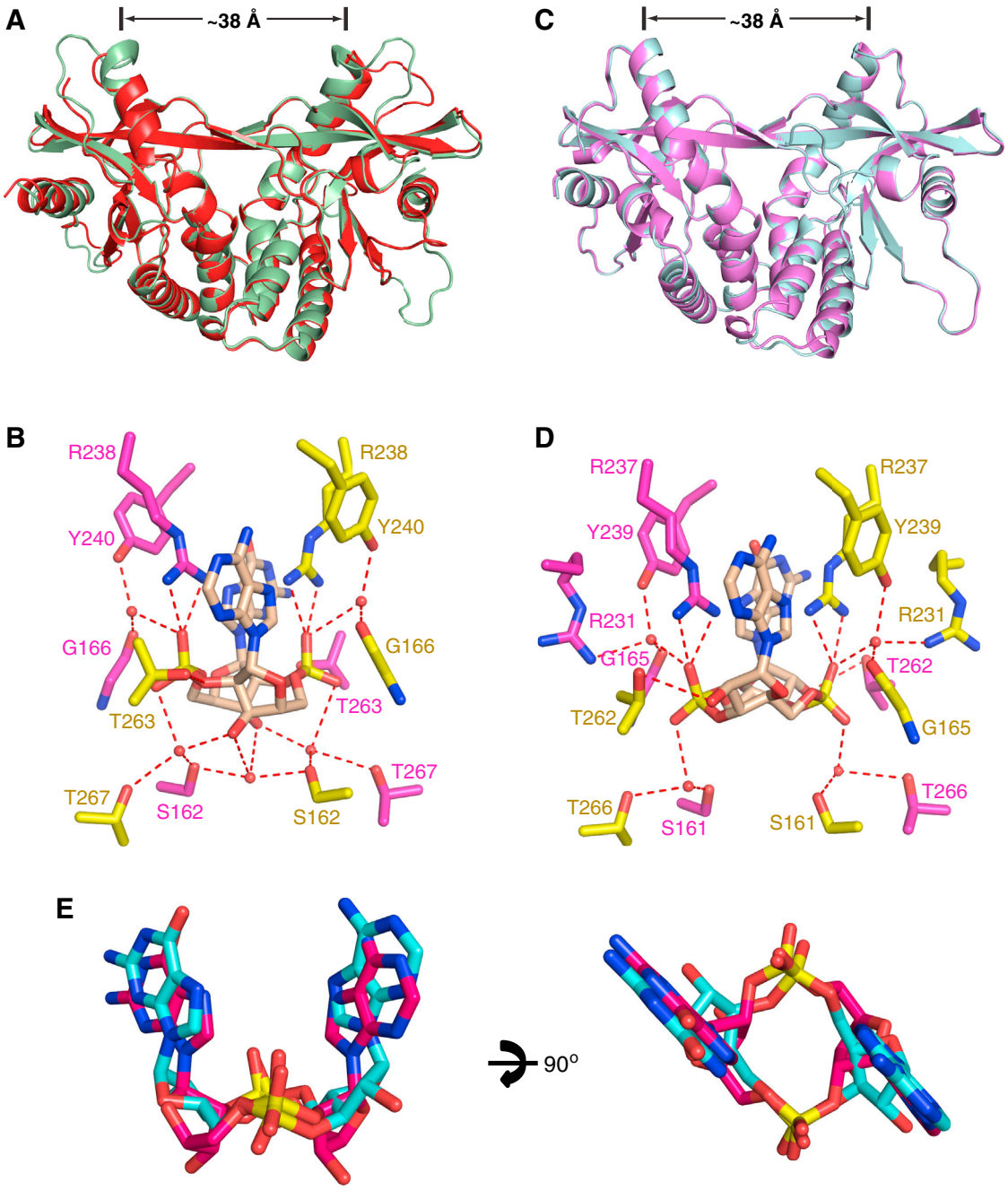

Figure S2. Details of c[G(2′,5′)pA(2′,5′)p]-hSTING^{H232} and c[G(3′,5′)pA(3′,5′)p]-mSting^{R231} Complexes, Related to Figures 1 and 3

(A) Superposition of the c[G(2′,5′)pA(2′,5′)p] (both subunits in red) and c[G(2′,5′)pA(3′,5′)p] (both subunits in green) bound structures of hSTING^{H232} (aa 155–341).

(B) Details of the hydrogen-bonding interactions in the structure of the c[G(2′,5′)pA(2′,5′)p]-hSTING^{H232} complex. Note the water-mediated hydrogen bonds between the 3′-OH groups and side chains of S162 and T267.

(C) Superposition of the c[G(3′,5′)pA(3′,5′)p] (both subunits in cyan) and c[G(2′,5′)pA(3′,5′)p] (both subunits in magenta) bound structures of mSting^{R231} (aa 154–340).

(D) Details of the hydrogen-bonding interactions in the structure of the c[G(3′,5′)pA(3′,5′)p]-mSting^{R231} complex. Note the direct hydrogen bonds between the 2′-OH groups and the side chains of T262.

(E) Superposition of the c[G(2′,5′)pA(2′,5′)p] in its complex with hSTING^{H232} in red and c[G(3′,5′)pA(3′,5′)p] in its complex with mSTING^{R231} in cyan. The c[G(2′,5′)pA(2′,5′)p] is positioned deeper in the binding pocket than is its c[G(3′,5′)pA(3′,5′)p] counterpart.

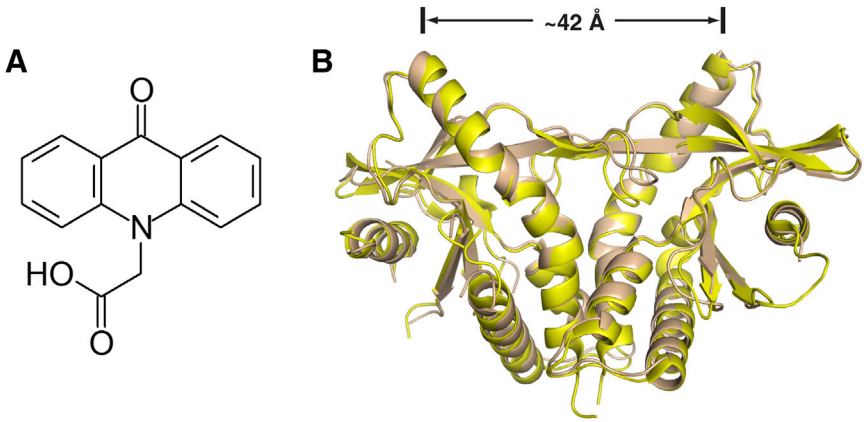

Figure S3. Crystal Structure of CMA Ligand Bound to mSting^{R231}, Related to Figure 3

(A) Chemical formula of 10-carboxymethyl-9-acridine (CMA).

(B) Superposition of the 2.90 Å DMXAA-bound structure of mSting^{R231} (aa 154–340) with both subunits in biscuit and 2.75 Å CMA-bound structure of mSting^{R231} (aa 149–348) with both subunits in yellow (PDB: 4JC5).

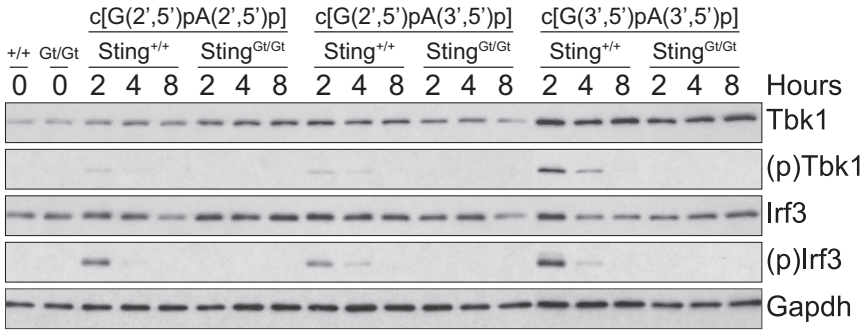

Figure S4. cGAMP Stimulation of BMDMs from Wild-Type and Mutant Sting Mice, Relates to Figures 6A and 6B

BMDMs from wild-type mice ($Sting^{+/+}$) and the N-ethyl-N-nitrosourea (ENU)-induced *Goldenticket* (*Gt*) mutant mice ($Sting^{Gt/Gt}$) were generated. Cells (1×10^6) were treated with cGAMP linkage isomers, c[G(2′,5′)pA(2′,5′)p], c[G(2′,5′)pA(3′,5′)p] and c[G(3′,5′)pA(3′,5′)p] at a final concentration of 15 μM. As before, cGAMP linkage isomers were provided by addition into media. Cells were collected at 2, 4, and 8 hr posttreatment. Mock treatment controls were included (0 h). Western blot analysis was performed with anti-phosphoserine-396 of IRF3 or anti-iRF3, anti-phosphoserine-172 of TBK1 and anti-TBK1. Glyceraldehyde 3-phosphate dehydrogenase (Gapdh) was used as a loading control.

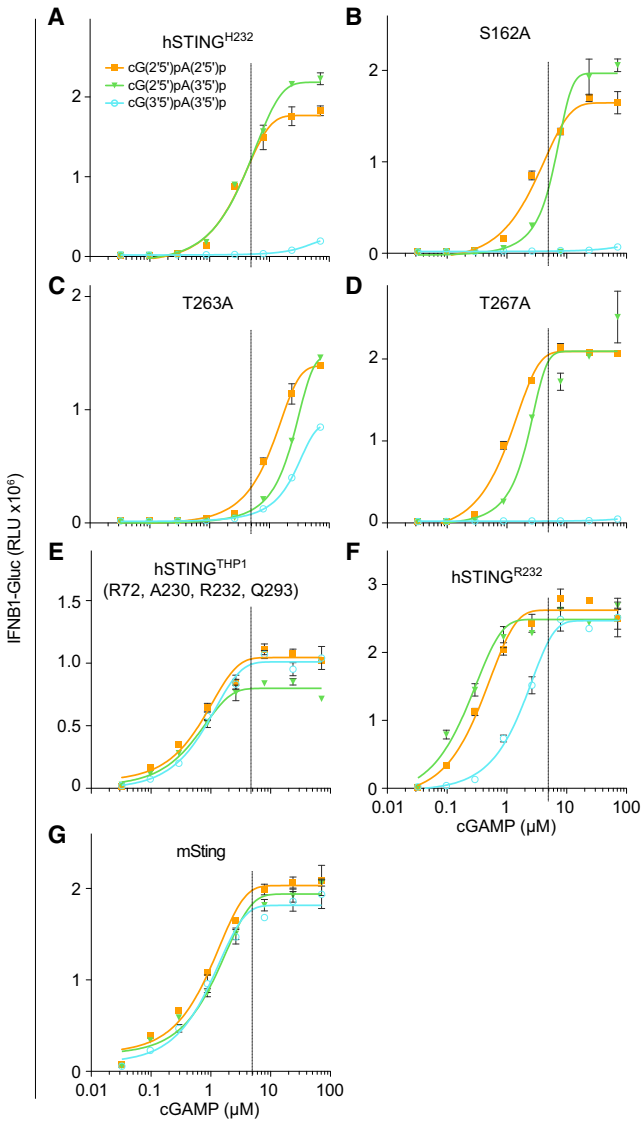

Figure S5. STING Point Mutants and Natural STING Variants Display Distinct Dose Responses to cGAMP Linkage Isomers, Related to Figure 7

(A-G) HEK293T cells were transfected with human (A–F) or murine (G) STING variants for 12 hr and cGAMP isomers delivered by Digitonin permeabilization (30 min) were titrated as indicated. Luciferase values were determined 12 hr after stimulation. The dotted line indicates the 5 μM cGAMP dose, whose corresponding values are that shown in Figure 7.

Data points were determined in triplicate and are depicted as the mean ± SEM.

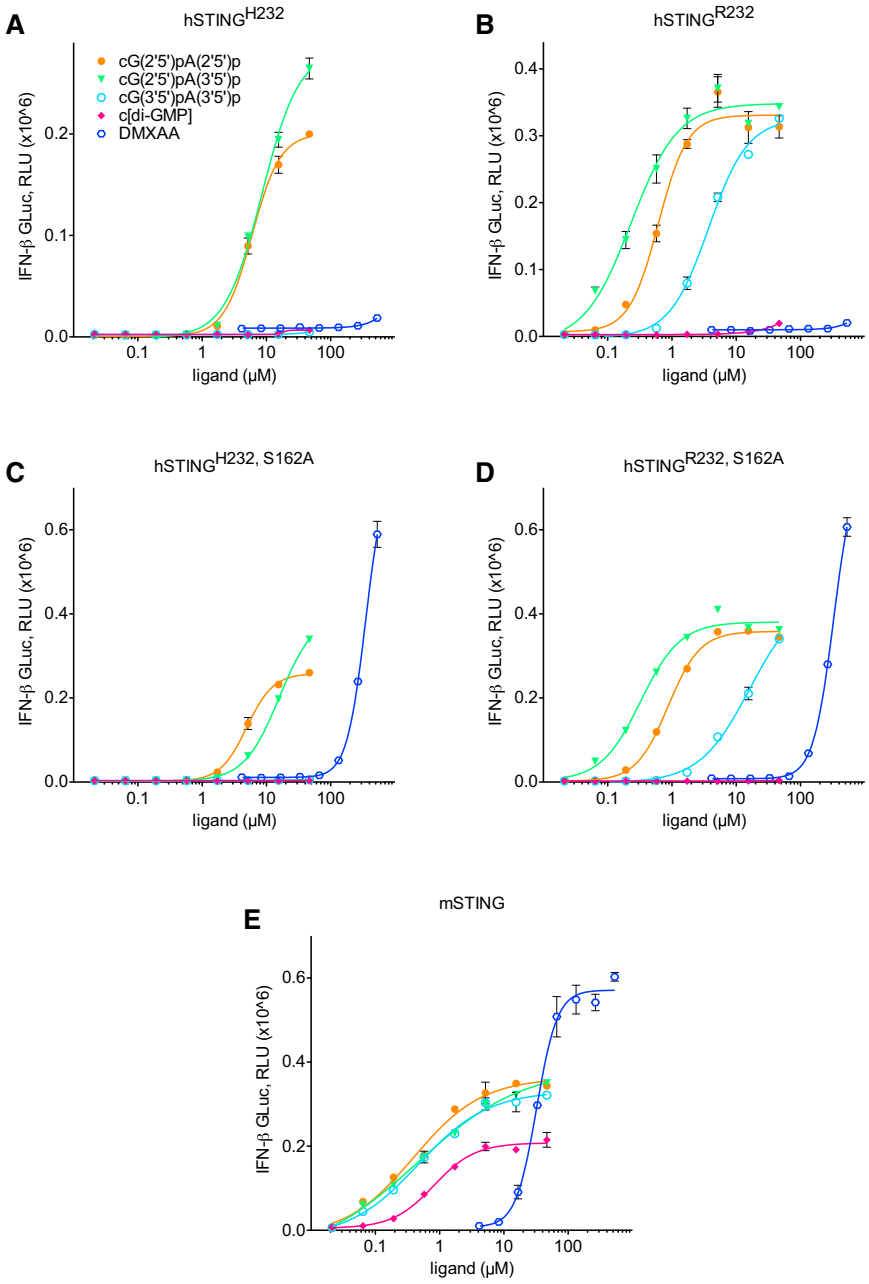

Figure S6. The S162A Point Mutation Acquires DMXAA Responsiveness in Both hSTING^{H232} and hSTING^{R232} Variants, Related to Figure 7

HEK293T cells were transfected with human STING variants and murine STING as indicated, and 12 hr later incubated with DMXAA without permeabilization or stimulated with c[di-GMP] and cGAMP isomers following digitonin permeabilization (30 min). Luciferase values were determined 12 hr after stimulation. Panels A and B show human STING^{H232} and STING^{R232}, (C and D) show the respective S162A mutants. In (E) responsiveness of murine Sting to DMXAA, c[di-GMP] and cGAMP isomers is shown for comparison.

Data points were determined in triplicate and are depicted as the mean ± SEM.

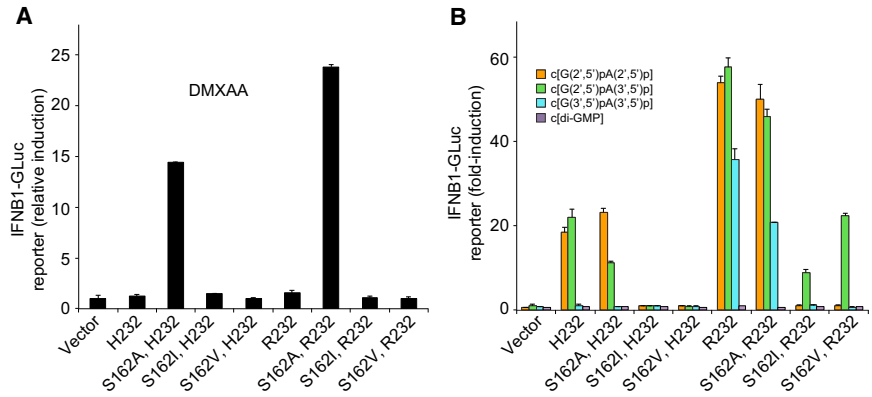

Figure S7. hSTING Position 162 Variants and their Sensitivity to Cyclic Dinucleaotides and DMXAA, Related to Figure 7

(A) Conversion of S162 to Ala in H232 or R232 hSTING background renders the protein sensitive to DMXAA stimulation. However, substitution by V162 or I162 does not yield similar results with DMXAA.

(B) Conversion of S162 to Ala in H232 or R232 hSTING background does not significantly affect the respective protein sensitivities to cGAMP linkage isomers or c-[di-GMP]. Substitution by V162 or I162 reduces/eliminates stimulation by any cyclic dinucleotide.

293T cells were transfected with reporter plasmids and hSTING variants as indicated. 12 hr after transfection, cells were stimulated by incubation with 75 μg/ml DMXAA (A) or Digitonin-permeabilized for 30 min in the presence of cyclic dinucleotides (5μM) as indicated (B). After another 12 hr, cells were lysed and luciferase assay was performed.

Data points were determined in triplicate and are depicted as the mean ± SEM.