Confocal Imaging

L929 cells in which endogenous STING was replaced with WT or mutated STING-Flag were plated onto cover slips in 24-well plates. On the next day, the cells were transfected with ISD for indicated times, and then washed with PBS and fixed in 3.7% formaldehyde in PBS for 15 min. Cells were permeabilized and blocked for 30 min at room temperature in a staining buffer containing Triton X-100 (0.2%) and BSA (3%), and then incubated with an antibody against Flag, IRF3, TBK1 or HA in the staining buffer for 1 hour. After washing three times in the staining buffer, cells were incubated with anti-mouse Texas Red or anti-rabbit FITC for 1 hour. The cover slips, which were washed extensively, were dipped once in water and mounted onto slides using mounting media (VectaShield with DAPI; Vector Laboratories). Imaging of the cells was carried out using Zeiss LSM510 META laser scanning confocal microscopy (Carl Zeiss MicroImaging, Inc).

SUPPLEMENTARY MATERIALS

Fig. S1. STING-dependent IRF3 Activation in L929 Cells and in Cell Free Assay

Fig. S2. Characterization of the Cell Free Assay of IRF3 activation by Recombinant STING Proteins.

Fig. S3. NEMO is Dispensable for IRF3 Activation by STING.

Fig. S4. Confocal Fluorescent Microscopy of STING, IRF3 and TBK1 in L929 cells

Fig. S5. Ser^{366} and Leu^{374} of STING Are Required for the Phosphorylation of IRF3 but not TBK1 in Cell Free Assays.

Fig. S6. S366A and L374A STING Mutants Still Form Aggregates

Fig. S7. STING is Phosphorylated by TBK1 upon ISD Stimulation

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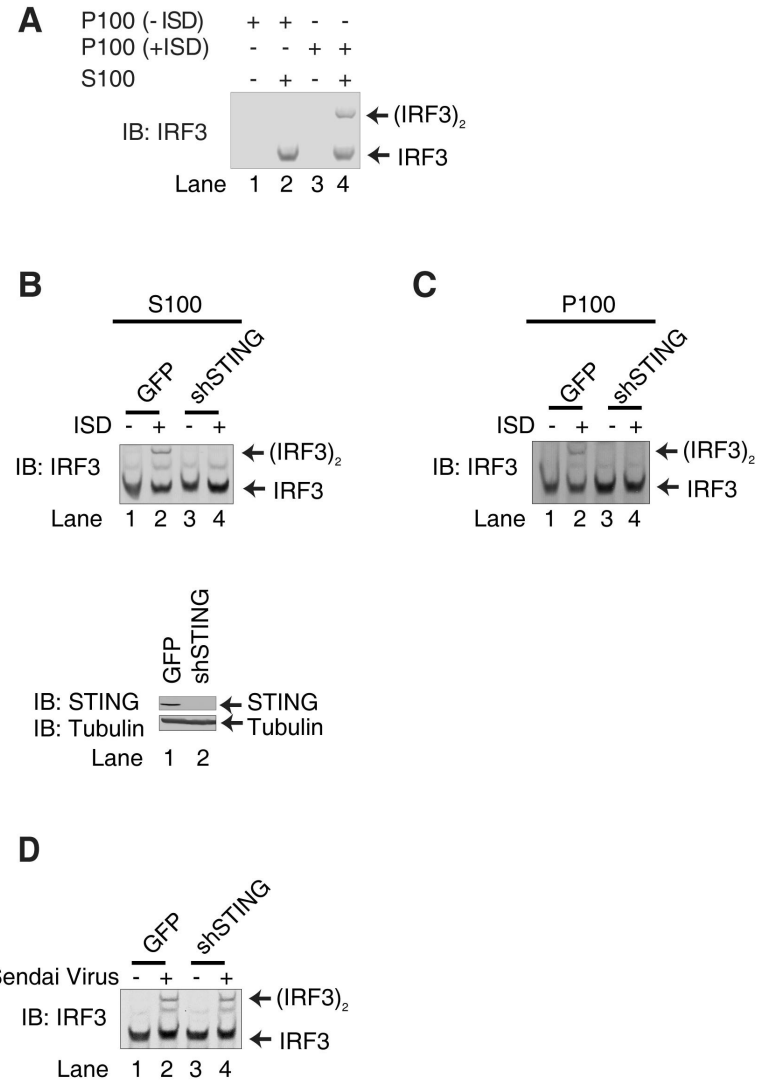

Fig. S1. STING-dependent IRF3 Activation in L929 Cells and in Cell Free Assay

A. The membrane fractions (P100) from ISD-transfected or mock-treated L929 cells were incubated with cytosolic extract (S100) from unstimulated cells in the presence of ATP. Dimerization of IRF3 was analyzed by native gel electrophoresis.

B. Lentiviral knockdown of STING using short hairpin RNAs (shRNAs). L929 cells stably expressing the GFP control or shRNA against STING were transfected with ISD for 4 hours and the dimerization of endogenous IRF3 was analyzed by native gel electrophoresis. The knockdown of STING protein was confirmed by immunoblotting.

C. The membrane fractions (P100) from (A) were incubated with the cytosolic extract from untreated L929 (S100), and the dimerization of IRF3 was analyzed by native gel electrophoresis.

D. Similar to (B) except that cells were stimulated with Sendai virus for 16 hours.

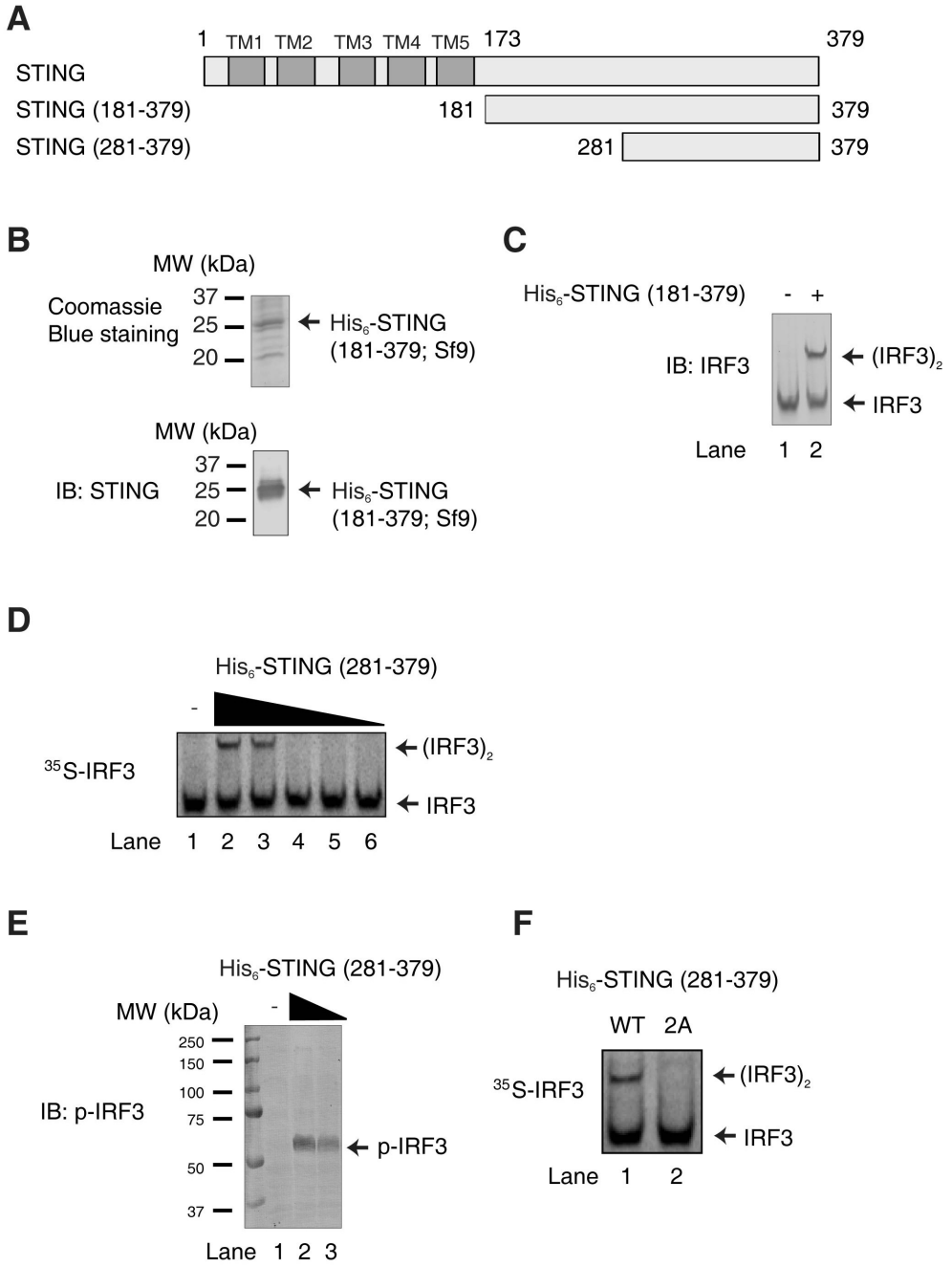

Fig. S2. Characterization of the Cell Free Assay of IRF3 activation by Recombinant STING Proteins.

A. Diagram of full-length and truncated STING.

B. His_6-STING (181-379) was expressed in and purified from Sf9 and then analyzed by Coomassie blue staining and immunoblotting.

C. His_6-STING (181-379) was incubated with HeLa S100 in the presence of ATP to measure IRF3 dimerization.

D. His_6-STING (281-379) was expressed and purified from *E. coli* as shown in Figure 2A (lane 1). Varying concentrations of this protein (0.125, 0.25, 0.5, 1.0 and 2.0 μM) were incubated with HeLa S100 and ^{35}S-IRF3 in the presence of ATP to measure IRF3 dimerization.

E. His_6-STING (281-379) (lane 2: 1.2 μM; lane 3: 0.6 μM) was incubated with HeLa S100 in the presence of ATP, and aliquots of the reaction mixtures were resolved by SDS-PAGE followed by immunoblotting with an antibody against phospho-IRF3 (p-IRF3 at Ser^{396}).

F. Similar to (D) except that wild-type (WT) ^{35}S-IRF3 or its mutant (S385A and S386A; denoted as 2A) was used.

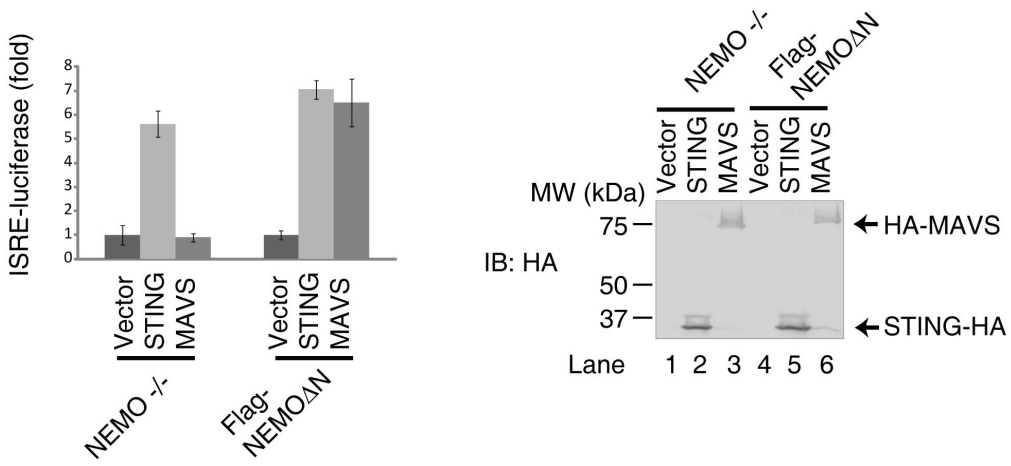

Fig. S3. NEMO is dispensable for IRF3 activation by STING.

MEFs lacking NEMO or reconstituted with Flag-NEMO ΔN were transiently transfected with expression vectors for STING-HA or HA-MAVS together with a ISRE-luciferase reporter (left panel). The luciferase activity was measured 48 hours after transfection. The error bars represent the variation ranges of duplicate experiments. The expression of STING and MAVS was confirmed by immunoblotting (right panel).

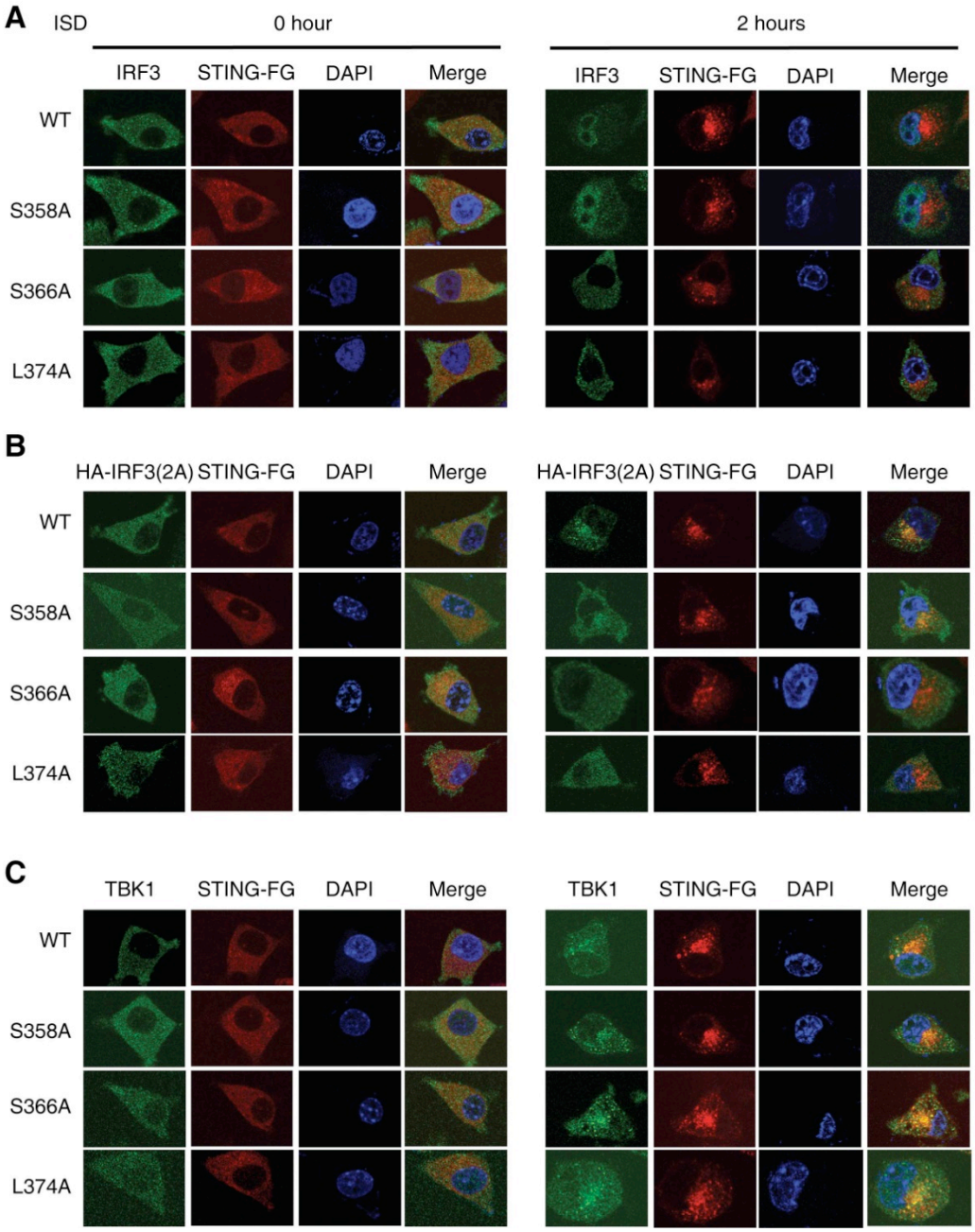

Fig. S4. Confocal Fluorescent Microscopy of STING, IRF3 and TBK1 in L929 cells

L929 cells in which endogenous STING was replaced with WT or mutated STING were transfected with ISD for 0 or 2 hours, then cells were stained with antibodies against Flag and IRF3 (A), HA (B) or TBK1 (C), followed by confocal fluorescence microscopy. STING proteins were stained with Texas Red, IRF3, HA-IRF3(2A) and TBK1 were stained with FITC (green), and the nuclei were stained with DAPI (blue). The images are representative of the cells under examination.

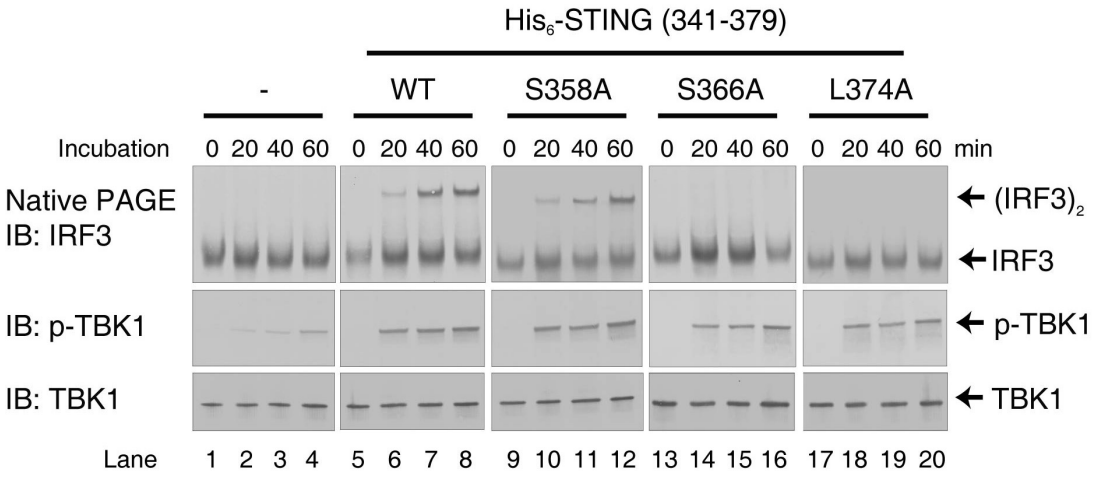

Fig. S5. Ser^{366} and Leu^{374} of STING Are Required for the Phosphorylation of IRF3 but not TBK1 in Cell Free Assays.

HeLa S100 was incubated with 1 μM of wild type or mutant (S358A, S366A and L374A) His$_6$-STING (341-379) in the presence of ATP for the indicated lengths of time. Aliquots of the reaction mixtures were immunoblotted for IRF3 after native PAGE or with antibodies against TBK1 or phospho-TBK1 (p-TBK1 at Ser^{172}) after SDS-PAGE.

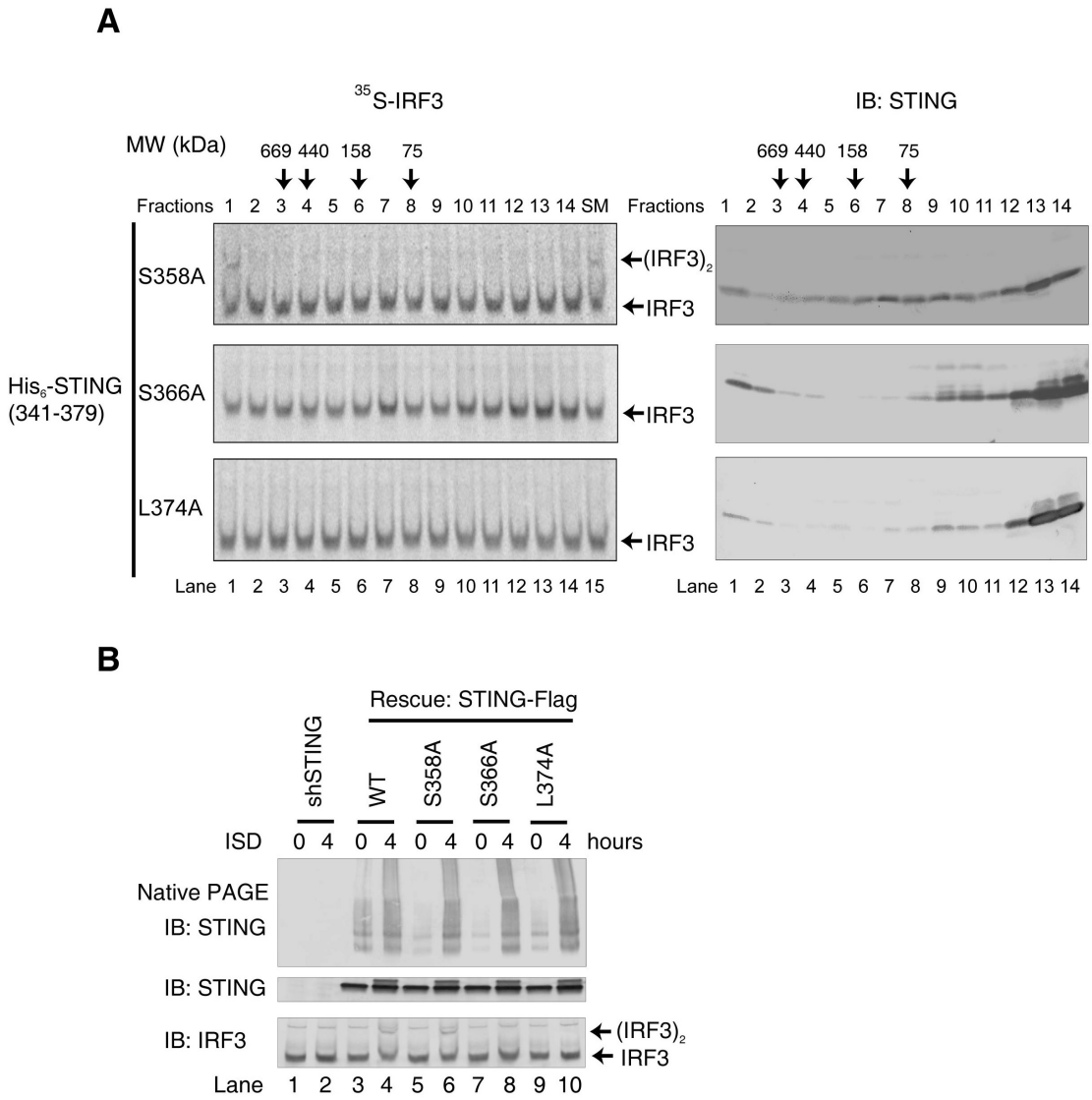

Fig. S6. S366A and L374A STING Mutants Still Form Aggregates

A. His_6-STING (341-379) proteins were fractionated by gel filtration on Superdex-200. Each fraction was analyzed by IRF3 dimerization assay and by immunoblotting as described in Figure 2D. SM: starting material.

B. L929 cells in which endogenous STING was replaced with WT or mutated STING were transfected with ISD for 4 hours and then membrane fractions (P100) were analyzed by native-PAGE or SDS-PAGE using a STING antibody. Cytosolic supernatant (S100) was analyzed immunoblotting with an IRF3 antibody after native PAGE.

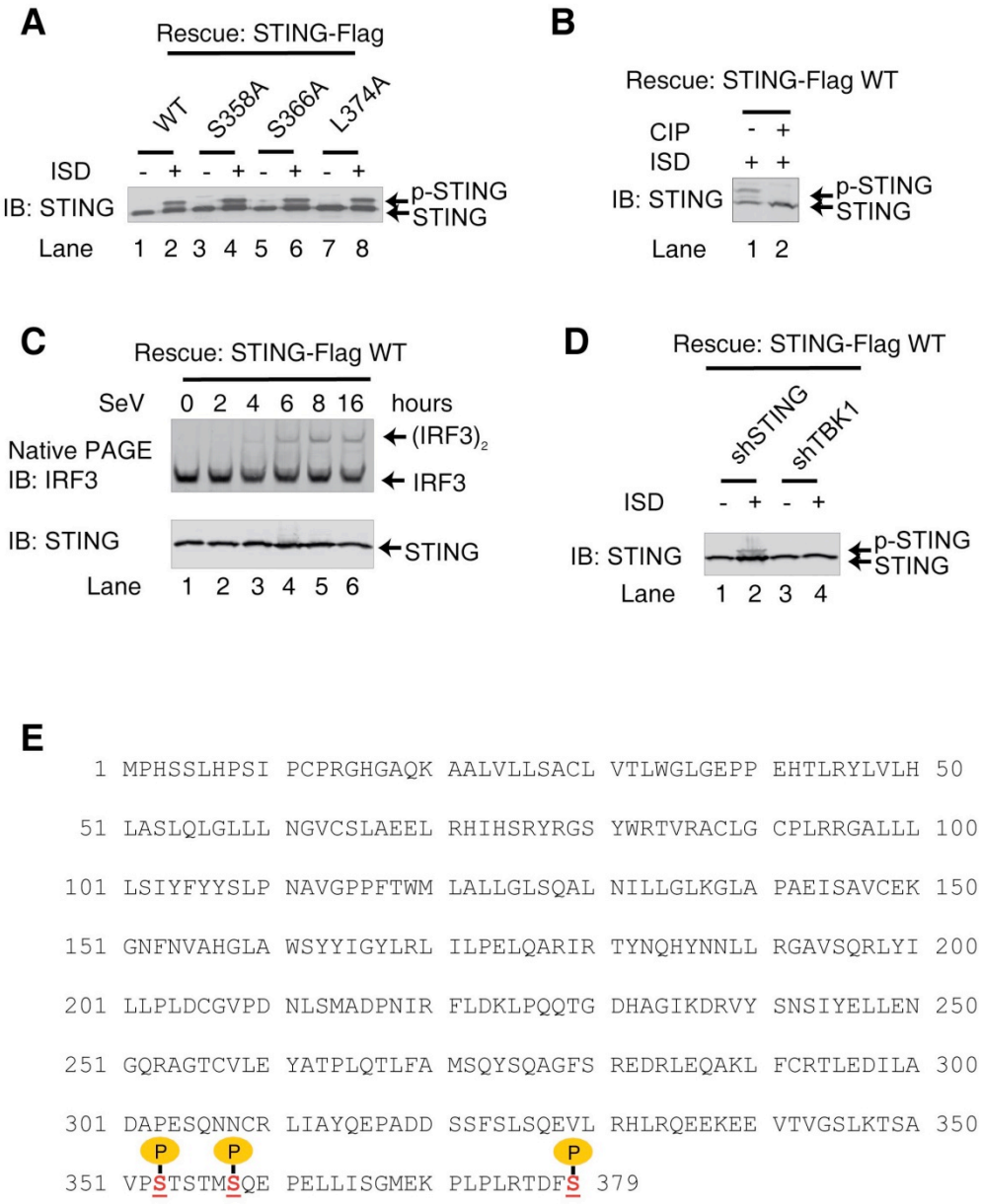

Fig. S7. STING is Phosphorylated by TBK1 upon ISD Stimulation

A. L929 cells in which endogenous STING was replaced with WT or mutated STING were stimulated with ISD for 4 hours, and then the membrane fraction (P100) was analyzed by immunoblotting with a STING antibody.

B. P100 from ISD-stimulated L929 cells expressing STING-Flag was treated with CIP and immunoblotted with a STING antibody.

C. Similar to (A), except that L929 cells expressing STING-Flag were infected with Sendai virus for the indicated time.

D. L929 cells stably expressing STING-Flag were depleted of endogenous STING or TBK1, and then stimulated with ISD for 4 hours. P100 from the cells was

immunoblotted with a STING antibody.

E. L929 cells in which endogenous STING was replaced with STING-Flag were transfected with ISD for 4 hours. P100 from the cells was solubilized with n-dodecyl beta-D-maltoside (DDM; 1%) and then STING-Flag was immunoprecipitated with anti-Flag agarose. Following elution with the Flag peptide, the STING protein was further purified using an anti-STING antibody and then resolved by SDS-PAGE and detected by silver staining. The band corresponding to phosphorylated STING was excised from the gel, trypsinized and analyzed by tandem mass spectrometry using LTQ-XL. The phosphorylation sites are highlighted in red.