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STING Recognition of Cytoplasmic DNA Instigates Cellular Defense

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SUMMARY

How the cell recognizes cytosolic DNA including DNA-based microbes to trigger host-defense-related gene activation remains to be fully resolved. Here, we demonstrate that STING (stimulator of interferon genes), an endoplasmic reticulum translocon-associated transmembrane protein, acts to detect cytoplasmic DNA species. STING homodimers were able to complex with self- (apoptotic, necrotic) or pathogen-related ssDNA and dsDNA and were indispensible for HSV-1-mediated transcriptional activation of a wide array of innate immune and proinflammatory genes in addition to type I IFN. Our data indicate that STING instigates cytoplasmic DNA-mediated cellular defense gene transcription and facilitates adoptive responses that are required for protection of the host. In contrast, chronic STING activation may manifest inflammatory responses and possibly autoimmune disease triggered by self-DNA.

INTRODUCTION

Potent activators of cellular innate responses are known to include microbial nucleic acid, derived from the genomes of viruses as well as bacteria (Kumar et al., 2011; Schenten and Medzhitov, 2011). For example, RNA viruses can trigger the production of innate immune genes, such as type I interferon (IFN), through their nucleic acid being recognized by the cytoplasmic RNA sensors retinoic acid-inducible gene-I (RIG-I) and melanoma differentiation antigen 5 (MDA5) (Yoneyama and Fujita, 2010). In addition, members of the Toll-like receptor (TLR) family, such as TLR3 and TLR7, have similarly evolved to be able to recognize viral RNA and to initiate the production of type I IFN. While the cellular molecular mechanisms responsible for sensing viral RNA have become clarified, less is known relating to how the cell senses microbial DNA species to trigger host defense associated gene regulation. It is established that TLR9 recognizes pathogen derived CpG DNA to trigger innate immune signaling predominantly in plasmacytoid dendritic cells (pDCs) (Hemmi et al., 2000). Moreover, absent in melanoma 2 (AIM2), a HIN-200 domain-containing protein, is known to be able to recognize cytoplasmic DNA species and trigger inflammasome-dependent interleukin-β synthesis (Alnemri, 2010; Schroder and Tschopp, 2010). However, we recently reported the isolation of a transmembrane component of the endoplasmic reticulum (ER) referred to as STING (stimulator of interferon Genes), which we demonstrated was essential for the production of type I IFN in fibroblasts, macrophages, and dendritic cells (DCs) in response to cytoplasmic double-stranded DNA (dsDNA) and select DNA viruses and intracellular bacteria, although the mechanisms remained to be fully elucidated (Ishikawa and Barber, 2008; Ishikawa et al., 2009). Here, we report that STING accomplishes these events by associating with aberrant cytoplasmic DNA species, including self-ssDNA (single-stranded DNA) and dsDNA, to trigger host defense related gene transcription. Our data indicate that STING is essential for innate responses triggered by intracellular DNA pathogens, while chronic activation may contribute toward DNA activated inflammatory disease.

RESULTS

STING Triggers the Expression of Multiple Primary Innate Immune and Proinflammatory Genes in Response to Intracellular ssDNA and dsDNA

The minimum size of dsDNA optimally required to activate STING-dependent type I IFN signaling in murine cells was noted to be approximately 45 bp (Ishikawa and Barber, 2008; Ishikawa et al., 2009; Stetson and Medzhitov, 2006). In normal human cells (hTERT-BJ1), however, we observed that dsDNA of approximately 90 bp (referred to herein as dsDNA90) was more efficient at activating type I IFN after in vitro transfection, although smaller sizes also remained capable of facilitating these events to a lesser degree (Figure 1A). Using RNA interference (RNAi) knockdown procedures, we additionally confirmed that STING (also referred to as MPYS/ MITA) (Jin et al., 2008; Zhong et al., 2008) is indeed essential for the production of type I IFN in hTERT-BJ1 cells (Figure 1B). Further analysis with microarray procedures used to measure messenger RNA (mRNA) expression confirmed that cytoplasmic dsDNA can induce a wide array of innate immune genes, in addition to type I IFN, in hTERT-BJ1s (Figure S1A available online). The induction of these innate genes which included members of the IFIT family appeared to be STING dependent since RNAi

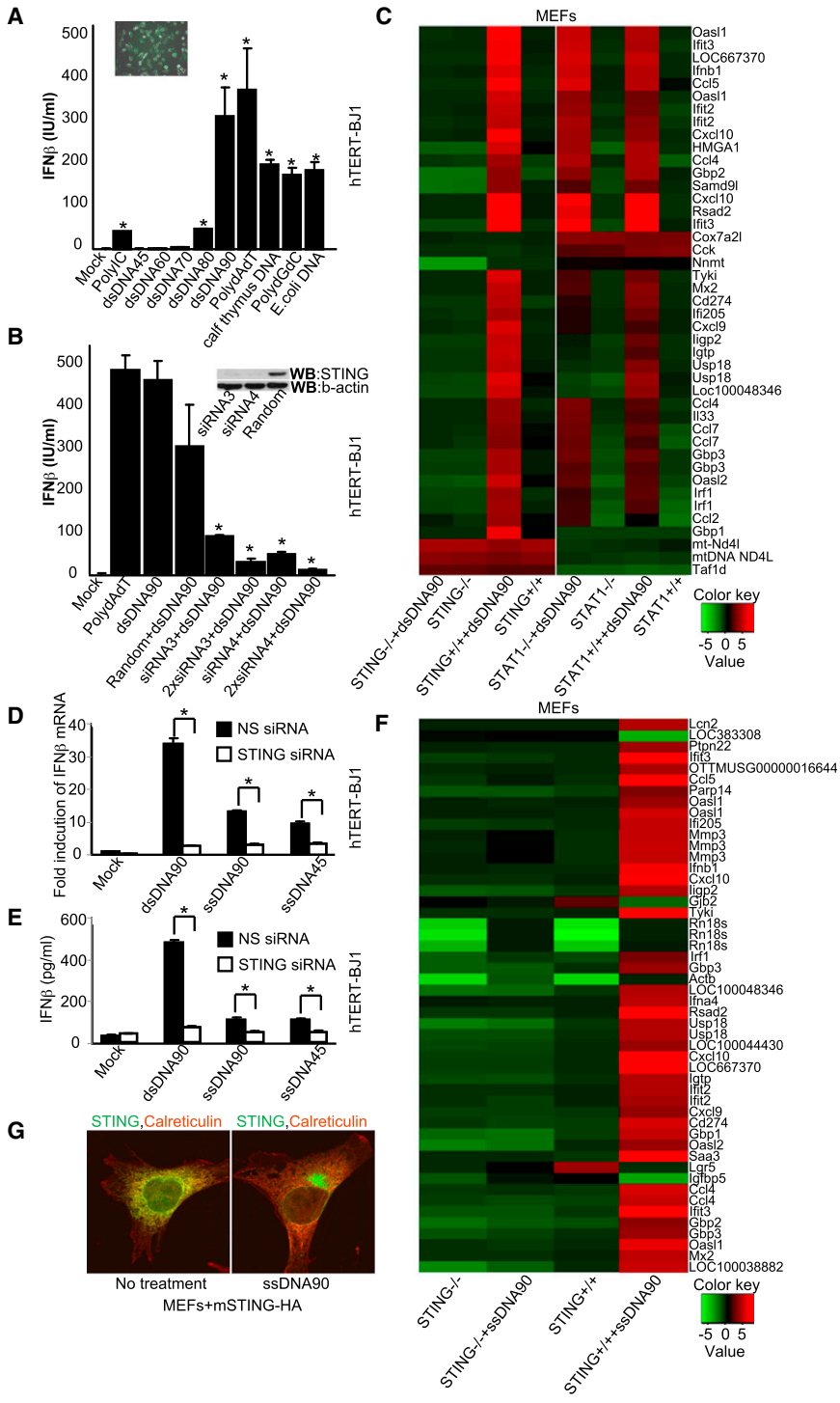

Figure 1. STING Controls Cytosolic ssDNA and dsDNA Innate Signaling

(A) Human telomerase fibroblasts (hTERT-BJ1) were transfected with various nucleotides (3 μg/ml) for 16 hr. Endogenous IFNβ levels were measured. hTERT-BJ1 cells were transfected with FITC-conjugated dsDNA90 and was examined by fluorescent microscopy to ensure efficient transfection.

(B) hTERT-BJ1 cells were transfected with mock, random, or two independent human STING siRNAs (siRNA 3 or 4) for 3 days followed by dsDNA90 transfection (3 μg/ml) for 16 hr. Endogenous IFNβ levels were measured. Silencing of hSTING protein was demonstrated by immunoblotting, with β-actin serving as a loading control.

(C) Primary *Sting*$^{+/+}$, *Sting*$^{-/-}$, *Stat1*$^{+/+}$, or *Stat1*$^{-/-}$ MEFs were transfected with or without dsDNA90 (3 μg/ml) for 3 hr. Total RNA was purified and examined for gene expression by Illumina Sentrix BeadChip Array (Mouse WG6 version 2). Most variable genes were selected. Rows represent individual genes; columns represent individual samples. Pseudocolors indicate transcript levels below, equal to, or above the mean (green, black, and red, respectively). The scale represents the intensity of gene expression (log10 scale ranges between −5 and 5).

(D) hTERT-BJ1 cells were treated with NS or STING siRNA. After 3 days, cells were treated with dsDNA90, ssDNA90, or ssDNA45 (3 μg/ml). IFNβ mRNA levels were measured by real-time RT-PCR after 16 hr.

(E) hTERT-BJ1 cells were treated with NS or STING siRNA. At 3 days, cells were treated with dsDNA90, ssDNA90 or ssDNA45 (3 μg/ml). IFNβ levels were measured after 16 hr.

(F) Primary *Sting*$^{+/+}$ or *Sting*$^{-/-}$ MEFs were transfected with or without ssDNA90 (3 μg/ml). After 3 hr, the same as (C).

(G) Primary MEFs with mSTING-HA were treated with or without ssDNA90 (3 μg/ml) for 3 hr and stained with anti-HA antibody (green) and calreticulin (red) as an ER marker.

*$p < 0.05$, Student's t test. Error bars indicate the SD. Data are representative of at least two independent experiments. See also Figure S1.

knockdown of STING in hTERT-BJ1s greatly eliminated their stimulation by cytoplasmic dsDNA (Figures S1B–S1F). That cytoplasmic dsDNA induced a variety of innate immune genes in a STING-dependent manner was confirmed with *Sting*$^{+/+}$ or *Sting*$^{-/-}$ murine embryonic fibroblasts (MEFs) (Figure 1C). To confirm that the induction of these mRNAs were STING-dependent genes (SDGs) and not stimulated through type-I-IFN-dependent autocrine or paracrine signaling, we similarly treated type I IFN signaling-defective *Stat1*$^{-/-}$ MEFs with dsDNA and verified that the production of the SDGs remained largely unaffected (Figure 1C). Real-time PCR analysis confirmed our array results (Figure S1G and data not shown). We noted that ssDNA of 45 nt (ssDNA45) weakly induced innate immune gene production in hTERT-BJ1s and less so in MEFs (Ishikawa and Barber, 2008; Ishikawa et al., 2009). However, transfected ssDNA comprising 90 nt (ssDNA90) was observed to more robustly activate an array of genes, including type I IFN in hTERT-BJ1s and MEFs (Figures 1D–1F, S1B, and S1G). We observed that STING probably

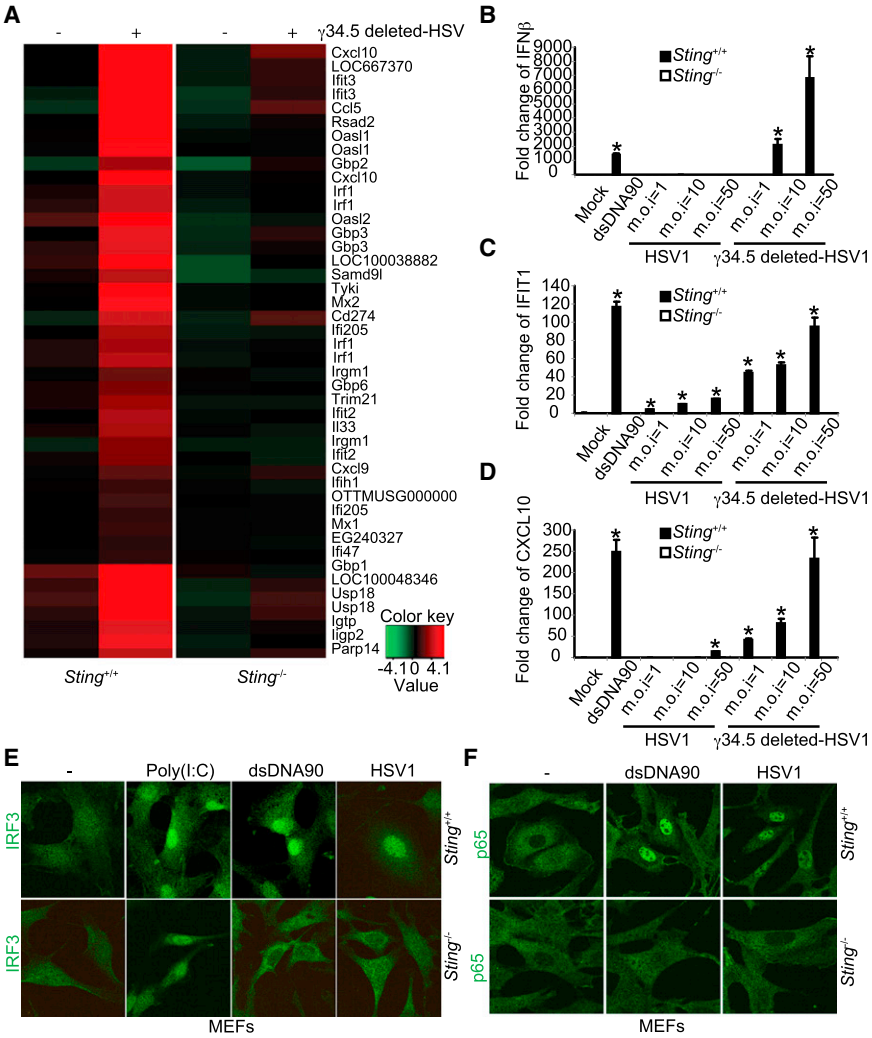

Figure 2. STING Is Essential for HSV1-Mediated Innate Immune Signaling

(A) MEFs were infected with γ34.5 deleted-HSV1 (multiplicity of infection = 1) for 3 hr. Total RNA was purified and examined for gene expression with Illumina Sentrix BeadChip Array (Mouse WG6 version2). Most variable genes were selected. Rows represent individual genes; columns represent individual samples. Pseudocolors indicate transcript levels below, equal to, or above the mean (green, black, and red, respectively). The scale represents the intensity of gene expression (log10 scale ranges between −4.1 and 4.1).

(B–D) *Sting*$^{+/+}$ or *Sting*$^{-/-}$ MEFs were treated with or without dsDNA, HSV1, or γ34.5-deleted HSV1 for 3 hr. Total RNAs were purified and examined by real-time PCR for IFNβ (B), IFIT1(C), or CXCL10 (D). Error bars indicate the SD.

(E) *Sting*$^{+/+}$ or *Sting*$^{-/-}$ MEFs were treated with poly(I:C), dsDNA90, or HSV1, and cells were stained by anti-iRF3 antibody. PolyIC is RIG-I/MDA5 dependent and STING independent.

(F) *Sting*$^{+/+}$ or *Sting*$^{-/-}$ MEFs were treated with dsDNA90 or HSV1 and cells were stained by anit-p65 antibody at 3 hr later.

See also Figure S6.

resides as a homodimer in the ER of both human and murine cells and migrates from the ER to perinuclear regions in the presence of cytoplasmic ssDNA or dsDNA ligands or HSV1 infection to activate type I IFN-dependent transcription factors (Ishikawa et al., 2009) (Figures 1G and S1H–S1K). A defective HSV1 mutant lacking the γ34.5 gene, which is responsible for preventing translational inhibition mediated by the double-stranded dependent protein kinase (PKR), was similarly observed to activate innate immune gene production in a STING dependent manner (Figures 2A–2D and data not shown). The translocation of IRF3 and NF-κB into the nucleus was observed after HSV1 infection and shown to be dependent on STING (Figures 2E and 2F). Accordingly, it was confirmed that many of the SDGs contained IRF3/IRF7 and NF-κB binding sites in their promoter region (data not shown). Interestingly, cyclic di-GMP, reported to bind STING, did not robustly activate STING in primary human or mouse cells similar to DNA (Figure S6). Thus, cytoplasmic ssDNA or dsDNA that includes transfected plasmid DNA can potently induce the transcription of a wide array of innate immune related genes that is dependent on STING.

STING Complexes with Intracellular DNA

Given the importance of STING in regulating cytoplasmic DNA signaling events, it remained plausible that STING itself could associate with DNA species. To evaluate whether STING bound cytoplasmic DNA, we first transfected hTERT-BJ1s with biotinylated dsDNA90 and subsequently treated them with or without the irreversible protein cross-linker disuccinimidyl suberate (DSS). After cells lysis, extracts were mixed with streptavidin beads. This experiment indicated that STING could be pulled down in both DSS-untreated and -treated cells, likely as a dimer (Figure 3A). Similar results were obtained with hTERT-BJ1s transfected with biotinylated dsDNA90 and subsequently UV treated to crosslink DNA-associating proteins (Figure 3B). RNAi knockdown of STING in hTERT-BJ1s eliminated the observed binding and STING-DNA complexes were also only observed in *Sting*$^{+/+}$ MEFs but not *Sting*$^{-/-}$ MEFs (Figures 3C and S2A). Of interest is that we similarly observed that transfected STING could also bind to ssDNA (Figures S2B–S2D). It has previously been reported that IFI16, a member of the HIN-200 family of proteins, or helicase DDX41 might be involved with sensing cytoplasmic DNA to initiate type I IFN production through STING. However, knockdown of IFI16 or DDX41 in hTERT cells did not affect the binding of STING to DNA. Nor did loss of IFI16 or DDX41 appear to affect STING trafficking or dsDNA-mediated translocation of IRF3/NF-κB by dsDNA or HSV1, or baculovirus, which cannot encode any viral products in mammalian cells but can stimulate cytosolic DNA-mediated innate immune pathways (Figure S3).

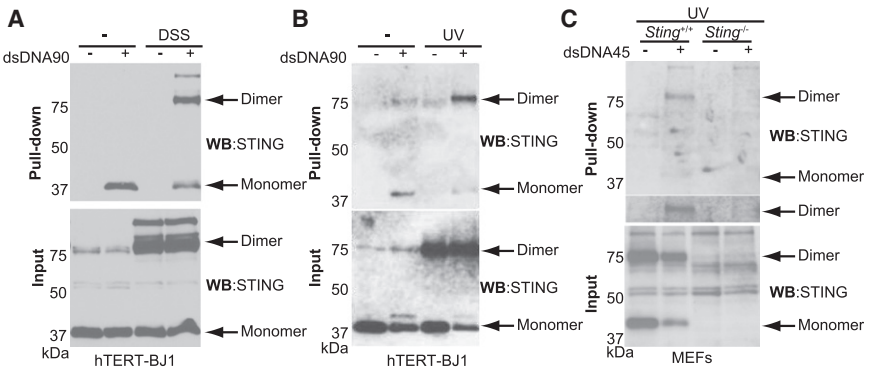

Figure 3. STING Binds to DNA In Vivo

(A) hTERT-BJ1 cells were transfected with biotin-conjugated dsDNA90 (3 μg/ml) for 6 hr and treated with DSS. Lysates were precipitated with streptavidin agarose beads and analyzed by immunoblotting with anti-STING antibody.

(B) hTERT-BJ1 cells were transfected with biotin-conjugated dsDNA90 (3 μg/ml) for 6 hr and treated with UV. Same as (A).

(C) *Sting*$^{+/+}$ or *Sting*$^{-/-}$ MEFs were transfected with biotin-conjugated dsDNA45 and crosslinked by UV. Lysates were precipitated by streptavidin agarose beads and analyzed by immunoblotting.

See also Figures S2 and S3.

However, loss of IFI16 did appear to influence the production of HSV1 confirming a role for this IFN-induced protein in antiviral activity (Figures S3B and S3C) (Gariano et al., 2012; Kerur et al., 2011; Unterholzner et al., 2010; Zhang et al., 2011).

Our analysis was complemented by transfection of 293T cells, which do not normally express STING with a variety of STING deletion constructs (Figure 4A). After 24 hr, cell lysates were mixed with biotinylated dsDNA90 agarose beads, and STING DNA binding was analyzed. This study complemented our above analysis and similarly indicated that STING was capable of binding dsDNA (Figure 4C). Further study indicated that the C-terminal regions of STING (aa 181–379) was sufficient for this association. In contrast, the N-terminal region of STING (aa 1–195) and three similarly HA-tagged controls (GFP, NFAR1, and IPS1) were not found to exhibit any binding to dsDNA90 (Figures 4C and 4D). The DNA binding exonuclease TREX1 served as a positive control (Figure 4C). A further series of extensive studies indicated that amino acid region 242–341 of STING was likely to be involved in binding dsDNA (Figure 4E). Competition experiments indicated that ssDNA and poly(dA:dT) could effectively compete with dsDNA90 for STING binding (Figure 4F). PolyIC did not bind to STING or prevent STING from associating with DNA (Figures S2E–S2G). All STING variants analyzed, but not the wild-type, lacked the ability to activate the type I IFN promoter when expressed in 293T cells, indicating the importance of STING's authentic conformation for effective function (Figure 4B). We were also able to rescue dsDNA-dependent signaling in a variety of cells after reconstitution of STING, including *Sting*$^{-/-}$ MEFs and 293T cells (Figures S4A–S4E).

To extend these studies further, we transfected dsDNA90 into hTERT-BJ1s or MEFs and treated them with formaldehyde to crosslink the cellular proteins to the nucleic acid. Subsequent chromatin immunoprecipitation (ChIP) analysis after STING pull-down further confirmed that transfected DNA can directly associate with STING as determined with dsDNA90-specific primers (Figures S5A and S5B). STING was also observed to bind to dsDNA in ELISA-based analysis (Figures S5C–S5E). Finally, we complemented our above analysis by designing biotinylated dsDNA (120-mers) representing the genomes of HSV1, cytomegalovirus (CMV), and adenovirus (ADV). Transfection of the 120-mers into MEF cells confirmed that they were potently able to induce the production of a variety of cytokines, such as type I IFN, CCL5, and tumor necrosis factor α (TNF-α)in a STING-dependent manner (Figures 5A–5C and S5F). Subsequently, these viral DNA's were incubated with 293T cell lysates previously transfected with STING and mixed with streptavidin beads. This approach indicated that these viral nucleic acids could also robustly bind to full-length STING, but not STING Δ242–290 or STING Δ291–341, indicating the importance of region 242–341 in nucleic acid association (Figures 5D and 5E). Collectively, our data demonstrate by multiple methods that STING is capable of associating with DNA.

STING Binds ssDNA and dsDNA without a Requirement for Accessory Molecules

To evaluate whether STING could associate with dsDNA alone or whether it requires accessory molecules, we extended our studies using in-vitro-transcribed STING and biotinylated dsDNA90 agarose beads. These experiments confirmed that the C-terminal STING (aa 181–379) could associate with dsDNA90 and suggested that this association did not likely require cofactors (Figures 5F–5H). As in our 293T experiments, region 242–341 was observed to be required for STING-dsDNA interaction. Given that we had observed that ssDNA could also trigger STING function and compete with dsDNA90, we next elaborated on whether ssDNA could comparably bind to STING. Our results indicated that ssDNA90 was indeed able to complex to the same regions of STING as dsDNA90 (Figure 5I). To further refine our studies, we generated point mutations focusing on conserved residues noted to be within the region of STING (aa 242–290) considered important for DNA binding (Figure 6A). STING variants with amino acids 245 or 268 changed to alanine (Y245A and L268A) were expressed in 293T cells and were not observed to be efficiently precipitated using biotinylated DNA. Such STING variants did not exhibit any ability to activate innate immune gene expression (Figures 6B and 6C). Similarly, STING K150R variant, reportedly deficient in lysine 63-linked ubiquitination via Trim56, was also capable of binding DNA (Figure S7). Further, reconstitution of these STING variants unto *Sting*$^{-/-}$ MEFs indicated that these variants did not traffic after transfection with dsDNA90. Larger STING variants (STINGΔ242–341, STINGΔ242–290, and STINGΔ291–341) similarly did not appear to exhibit function, indicating the importance of the cytoplasmic tail in the

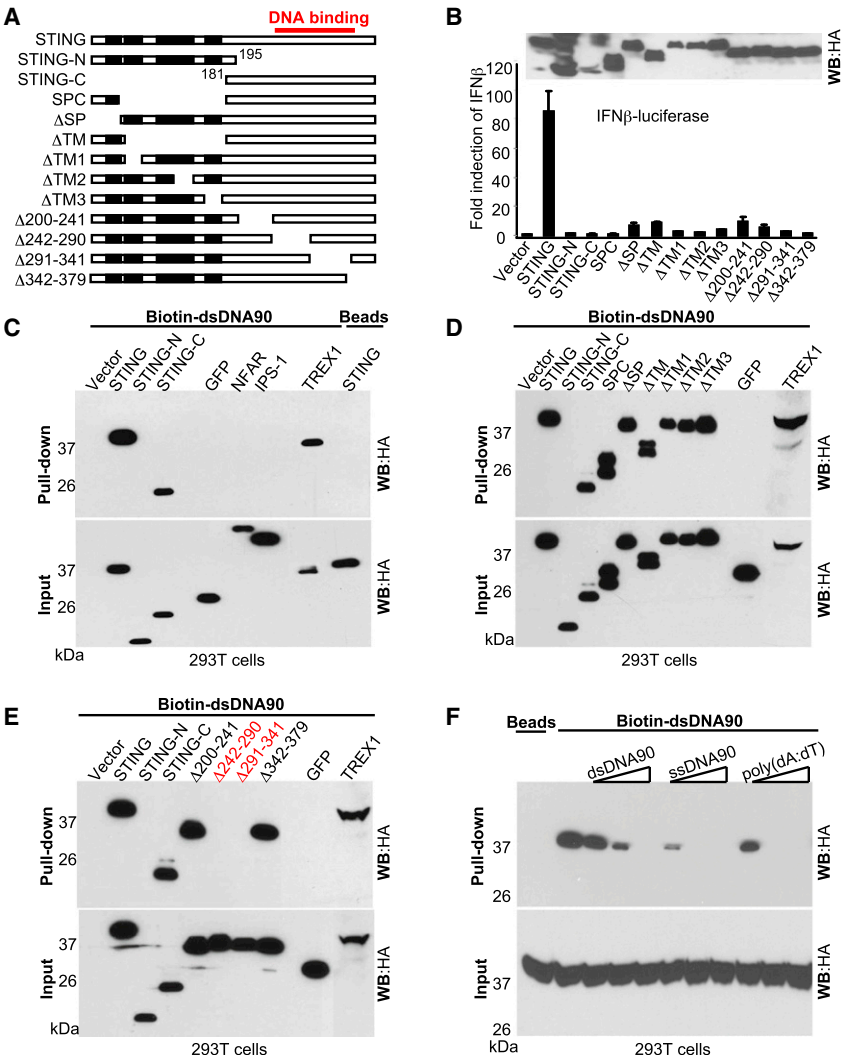

Figure 4. The C-Terminal Region of STING Binds DNA

(A) Schematic of STING variants.

(B) 293T cells were transfected with IFNβ-luciferase and STING variants and luciferase activity were measured.

(C–E) 293T cells were transfected with the indicated plasmids. Cell lysates were precipitated with biotin-conjugated dsDNA90 agarose beads and analyzed by immunoblotting with anti-HA antibody.

(F) Full-length STING-HA was expressed in 293T cells, and lysates were incubated with biotin-conjugated dsDNA90 agarose beads in the presence of dsDNA90, ssDNA90, or Poly(dA:dT) and analyzed by immunoblotting with anti-HA antibody.

See also Figure S4.

interaction with DNA (Figure 6D). Thus, STING is likely able to interact with ssDNA species as well as dsDNA and STING variants unable to bind DNA exhibit little activity.

To confirm these observations, we purified full-length STING or GFP control protein from 293T cells and the C-terminal region of STING (181–379) from *E. coli* to greater than 95% homogeneity (Figures 7A, 7B, and S5G–S5I). Full-length STING purified from 293T cells via affinity chromatography was noted to bind to DNA under relatively high-salt and -detergent conditions (Figure 7C). However, we noted that full-length STING was insoluble after purification from *E. coli* unlike the carboxyl region of STING (aa 181–379), which remained soluble. We confirmed that the C-terminal region of STING, but not purified control GFP, could associate with biotinylated dsDNA90 or ssDNA90 (Figures S5G–S5I). To evaluate this interaction further, we used surface plasmon resonance (SPR) analysis to demonstrate that soluble purified STING (aa 181–379) bound to dsDNA90 with a calculated dissociation constant (K_D) of 13.7 μM as determined by this method (Figure S5I). To complement our analysis, we purified an extended version of the C-terminal region of STING (aa 152–379) from *E. coli*, which we found remained soluble throughout the purification process (Figures 7B and 7D). Coomassie gel analysis and size-exclusion chromatography again confirmed the high level of purity of STING and confirmed that STING exists as a dimer with a calculated molecular weight of approximately 53,000 Daltons, as previously reported and also indicated by our earlier studies (Figure 7D) (Huang et al., 2012; Ouyang et al., 2012; Shang et al., 2012; Shu et al., 2012; Yin et al., 2012). To complement our SPR studies, we used fluorescence anisotropy assays to start to determine the minimum size of DNA that could associate with STING. This approach confirmed our previous observations and demonstrated the binding of the C-terminal region of STING (aa 152–379) to dsDNA 30-mers or 18-mers with an affinity of K_D of 247 μM and 337 μM, respectively (Figures 7E and 7F). Collectively, these findings support the notion that STING can associate directly with DNA species.

DISCUSSION

Our data indicate that STING can control the activation of the transcription factors NF-κB and IRF3/IRF7 by cytoplasmic DNA species to initiate the production of a wide variety of primary innate immune genes that includes type I IFN, as well as members of the IFIT family and select chemokines. Studies here and elsewhere demonstrate that STING resides in the ER of the cell, almost certainly as a dimer (Ishikawa and Barber, 2008; Ishikawa et al., 2009). We have also previously provided evidence that STING forms part of the translocon complex. It is possible that endocytosed or phagocytosed viruses or even infected cell debris containing inappropriately digested viral or cellular DNA fuse with the ER, where STING is located. Our data demonstrate by multiple methods that STING can complex with both ssDNA and dsDNA, which would include plasmid-based DNA and gene therapy vectors. Indeed, the

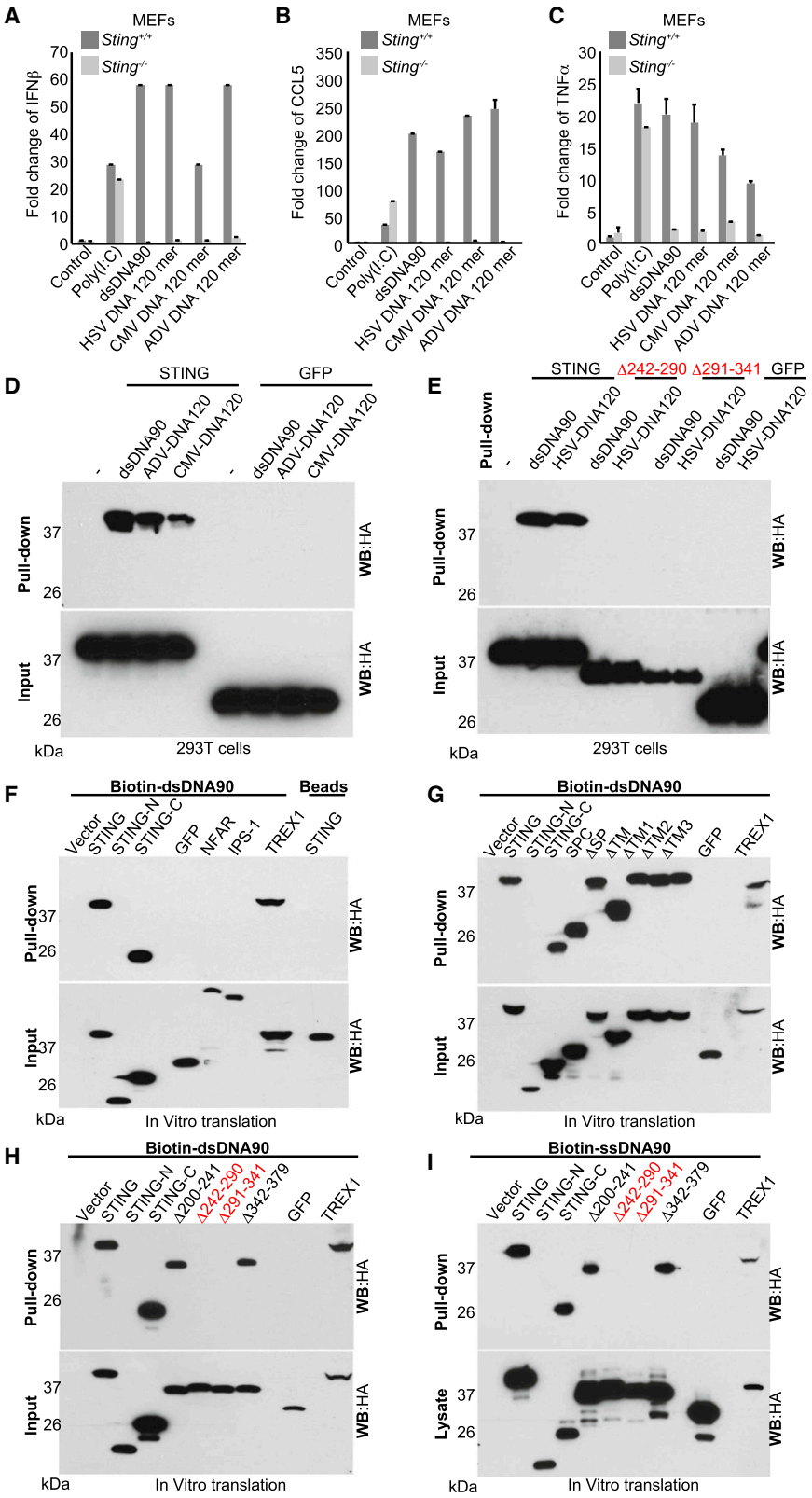

Figure 5. STING Binds to DNA In Vitro

(A–C) *Sting*$^{+/+}$ or *Sting*$^{-/-}$ MEFs were treated with poly(I:C), dsDNA90, HSV DNA 120-mer, CMV DNA 120-mer, or ADV DNA 120-mer for 3 hr. Total RNA was purified and examined by real-time PCR for gene expression of IFNβ (A), CCL5 (B) or TNF-α (C). Error bars indicate the SD.

(D and E) 293T cells were transfected with indicated plasmids. Cell lysates were precipitated with biotin-dsDNA90, biotin-ADV DNA 120-mer, biotin-CMV DNA 120-mer (D), or biotin-HSV DNA 120-mer (E) agarose beads and analyzed by immunoblotting using anti-HA antibody.

(F–H) In vitro translation products were incubated with biotin-dsDNA90 agarose beads and analyzed by immunoblotting with anti-HA antibody.

(I) In vitro translation products were incubated with biotin-ssDNA90 agarose beads and analyzed by immunoblotting with anti-HA antibody.

See also Figure S5.

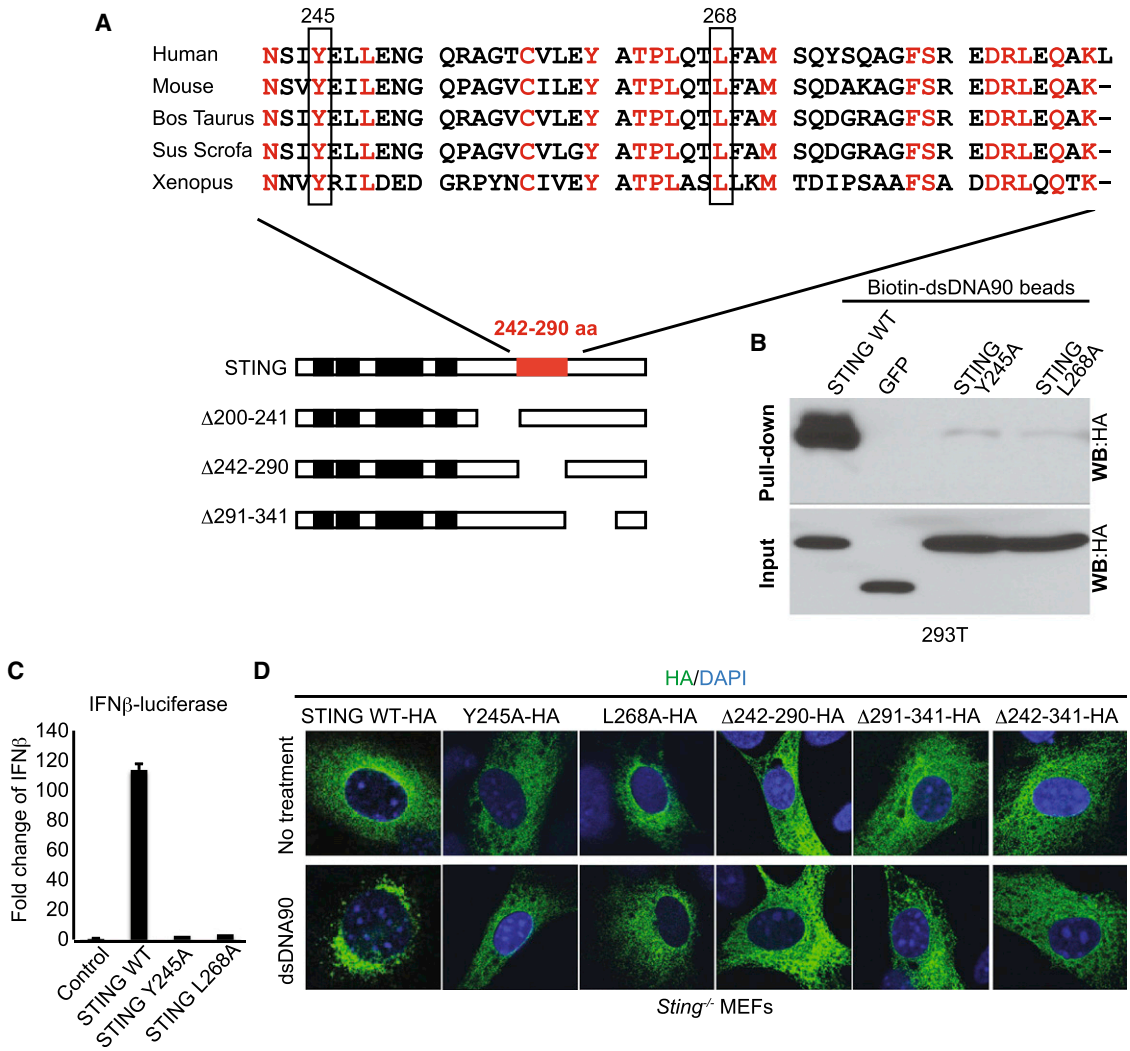

Figure 6. Y245 and L268 Are Important for STING Binding DNA

(A) Alignment of amino acid sequence of STING in 242–290 aa.

(B) 293T cells were transfected with STING WT-HA, GFP-HA, STING Y245A-HA, or STING L268A-HA for 24 hr. Cell lysates were precipitated with biotin-dsDNA90 beads and analyzed by immunoblotting with anti HA antibody.

(C) 293T cells were transfected with IFNβ-luciferase plasmid and plasmid encoding STING or mutants. After 24 hr, luciferase activity was measured. Error bars indicate the SD.

(D) *Sting*$^{-/-}$ MEFs were transfected with plasmid encoding STING or mutants with an Amaxa nucleofector apparatus (program A-023) with Amaxa MEF nucleofector kit 1 according to the manufacturer' s instructions. After 24 hr, the cells were treated with dsDNA90 for 6 hr and then stained with anti-HA (green) antibody and DAPI (blue).

See also Figure S7.

transfection of plasmid DNA or introduction of gene therapy vectors in the cell triggers the production of a wide variety of primary innate immune genes that is dependent on STING. Our studies indicate that the association of STING with aberrant cytoplasmic DNA, induces STING trafficking through the Golgi, in perhaps preautophagosome complexes to associate with transcription factors responsible for activation of innate immune responses (Figure S4H) (Ishikawa et al., 2009). While the mechanisms controlling these events remain unclear, STING has been shown to traffic with autophagosome markers ATG9 after the recognition of DNA species (Saitoh et al., 2009; Watson et al., 2012). Moreover, STING appears essential for escorting TBK1 to endosomal compartments for activation of IRF3/IRF7 (Ishikawa et al., 2009). After delivery of TBK1, STING is degraded to presumably avoid the sustained production of innate immune related proinflammatory genes that could have deleterious effects upon the host (Ahn et al., 2012).

STING has been shown to play a key role in activating innate immune signaling in response to a variety of DNA pathogens, including parasites and bacteria (Lippmann et al., 2011; Nazmi et al., 2012; Sharma et al., 2011). In addition, STING appears

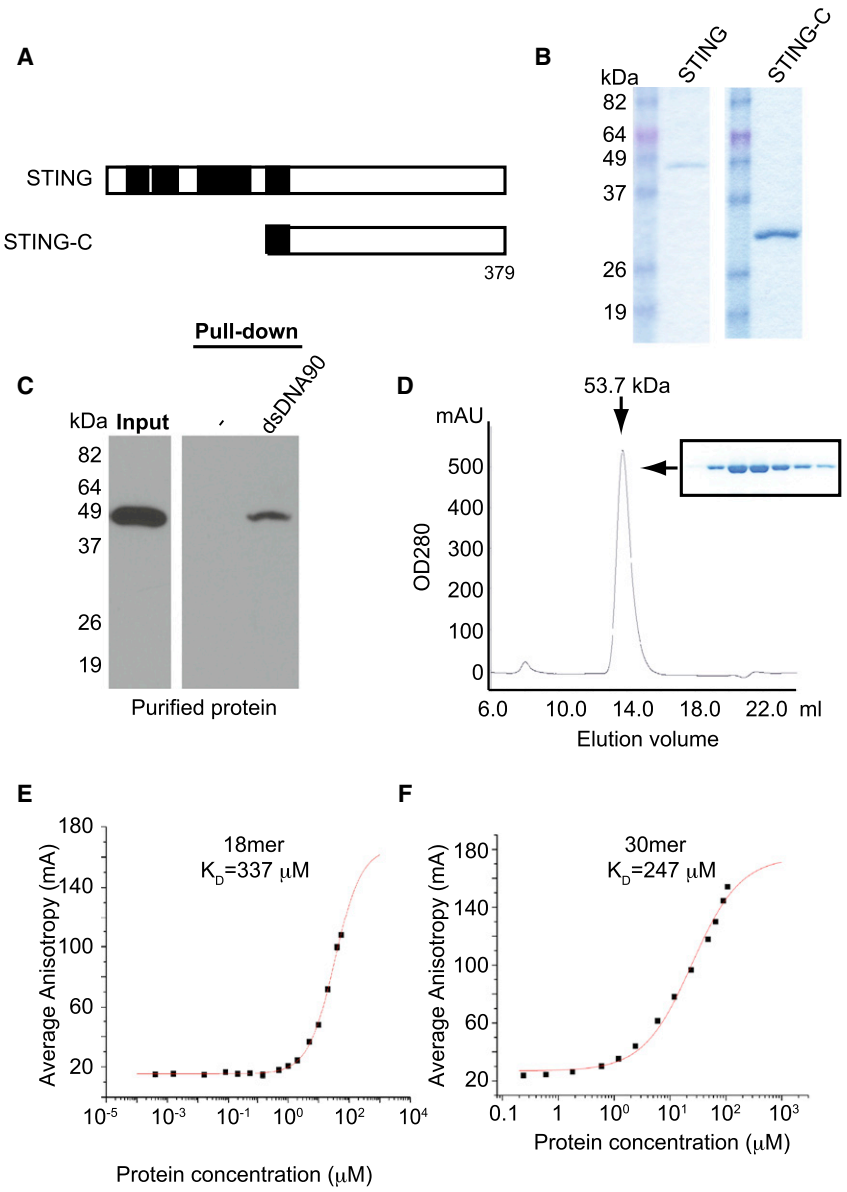

Figure 7. STING-DNA Binding Analysis

(A) Schematic of STING variants.

(B) Coomassie brilliant blue staining of purified hSTING-HA protein from 293T cells or purified hSTING-C-6xHis (aa 152–379) protein from *E. coli*.

(C) Purified hSTING-HA proteins were precipitated with biotin-conjugated dsDNA90 and analyzed by immunoblotting with STING antibody.

(D) hSTING-C was highly purified by N-affinity chromatography and subjected to Superdex 200 GL 10/30 size-exclusion column. Our analysis indicated that purified hSTING-C has a molecular weight of 53.7 kDa, which is consistent with that predicted size of a dimer.

(E and F) Direct binding of purified hSTING-C with 18-mer (E) or 30-mer (F) FAM-labeled dsDNA oligonucleotide was determined with fluorescence polarization (FP) assays. The raw data of average fluorescence anisotropy is shown in a sigmoidal binding curve, with a calculated dissociation constant (*KD*) of 337 ± 26 μM and 247 ± 38 μM, respectively.

See also Figure S5.

important for host defense against RNA viruses by mechanisms that remain to be clarified (Gall et al., 2012; Ishikawa and Barber, 2008; Ishikawa et al., 2009; Yan et al., 2010). For example, STING-deficient cells and mice are sensitive to RNA virus infection, but such viruses do not robustly stimulate STING trafficking or STING-dependent innate immune gene activation. Possibly, the mechanisms of STING activity against RNA viruses may involve the RIG-I pathway or other pathways. A growing number of reports indicate that STING may be commonly targeted for inactivation by a variety of pathogens, likely to thwart host defense countermeasures (Ishikawa et al., 2009; Sun et al., 2012).

STING may also be important for triggering inflammatory disease such as systemic lupus erythematosus, Aicardi-Goutieres syndrome, and/or polyarthritis (Ahn et al., 2012; Gall et al., 2012). Such disorders are characterized by the overproduction of cytokines such as type I IFN brought about by stimulation of host innate immune responses, speculatively by chronic infection or self nucleic acids from inappropriately apoptosed cells or even necrotic cells (Ahn et al., 2012; Nagata and Kawane, 2011). Indeed, our data confirm that STING is able to associate with self-apoptotic or -necrotic DNA, suggesting a putative role for this transmembrane protein in inflammatory disease (Figures S4F and S4G). Loss of STING rescues lethality observed in *DNase II*$^{-/-}$ or *TREX1*$^{-/-}$ mice, which are regarded as models for self-DNA-initiated inflammatory disease. Collectively, our data indicate that STING is critically important for early host defense and for initiating adaptive immune responses in response to DNA and perhaps RNA pathogens. However, chronic STING activation may lead to inflammatory disorders (Ahn et al., 2012).

Aside from our findings that STING can complex DNA, it also been reported that cyclic di-GMP produced from bacteria can bind to STING (Burdette et al., 2011; Woodward et al., 2010). Thus, STING may be a sensor not only for pathogen derived ssDNA and dsDNA, but also cyclic di-GMP. However, we did not notice as robust STING trafficking or cyclic di-GMP-induced gene induction in mammalian cells compared to that invoked by cytoplasmic DNA, for reasons that remain unclear (Figure S6). Crystal structures indicate that cyclic di-GMP binds to STING within the region aa 242–290 (Huang et al., 2012; Ouyang et al., 2012; Shang et al., 2012; Shu et al., 2012; Yin et al., 2012). Our own data indicate that DNA similarly binds to these regions, although further crystallographic analysis will be required for a comparative analysis. We also noticed that region

291–341 was also important for STING binding and it is plausible that effective DNA association may require both of these regions for stabile interactions. We noted that although dsDNAs greater than 45 bp were required for full activation of STING in the cell, smaller pieces of DNA were able to associate with purified STING in vitro. Thus, it is possible that a certain number of STING molecules may be required to associate with DNA in the cell to initiate autophagosome-like innate signaling events. Plausibly, only longer pieces of dsDNA or ssDNA may be eligible to trigger innate immunity. Posttranslational modifications such as phosphorylation and ubiquitination likely regulate STING function, although we observed that TRIM56 was not required for STING to bind DNA (Figure S7) (Tsuchida et al., 2010). A number of alternate, putative DNA sensors have also been reported to regulate innate immune signaling such as IFI16 and DDX41 (Unterholzner et al., 2010; Zhang et al., 2011). However, our data indicate that STING alone is sufficient and necessary to bind DNA and facilitate cytoplasmic DNA-mediated innate immune signaling (Figures 7 and S3). Our data are in agreement with Brunette et al., who also showed that STING activity is independent of IFI16 (Brunette et al., 2012). Perhaps such molecules exhibit importance in cell types other than the ones portrayed here and the establishment of murine models that lack such genes may shed further light into their function. In summary, our data indicate that STING is essential for detecting aberrant cytoplasmic DNA species and for stimulating innate immune signaling events. However, chronic STING activation may be responsible for DNA-triggered inflammatory disease (Ahn et al., 2012). Understanding of STING function may conceivably lead to the development of potent adjuvants for vaccine development or conversely therapeutics that could control inflammation aggravated disease.

EXPERIMENTAL PROCEDURES

Cells and Viruses

293T cells were obtained from ATCC and were maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 10% fetal bovine serum (FBS; GEMINI Bio-Products). hTERT-BJ1 cells were purchased from Clontech and cultured in 4:1 ratio of DMEM:Medium 199 (Invitrogen and Sigma Aldrich, respectively) supplemented with 10% FBS, 1 mM sodium pyruvate, and 4 mM L-glutamine (Invitrogen). *Stat1*$^{+/+}$ and *Stat1*$^{-/-}$ MEFs were provided by David Levy.

Antibodies

Rabbit polyclonal antibody against STING was described previously (Ishikawa and Barber, 2008). Other antibodies were obtained from the indicated sources: HA (Sigma Aldrich), His (Sigma Aldrich), Flag (Sigma Aldrich), β-actin (Sigma Aldrich), IRF3 (Santa Cruz Biotechnology), p65 (Cell signaling), IFI16 (Santa Cruz Biotechnology), TRIM56 (Abcam), and DDX41 (Santa Cruz Biotechnology).

RNA Interference

hTERT-BJ1 cells were transfected with corresponding small interfering RNAs (siRNAs; Dharmacon) with Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's instruction. At 72 hr after siRNA transfection, cells were used for further experiments. Experiments were done in duplicate or triplicate using more than one RNAi to each gene.

Gene Array Analysis

Total RNA was isolated from cells with the RNeasy RNA extraction kit (QIAGEN). Total RNA was analyzed by Bioanalyzer RNA 6000 Nano (Agilent Technologies). Gene array analysis was examined by Illumina Sentrix BeadChip Array (Mouse WG6 version 2 for RNA extracted from MEFs, and Human HT-12_V4_BeadChip for RNA extracted from human cell lines; Affymetrix) at the Oncogenomics Core Facility, University of Miami. Microarray analysis was performed at the Center of Computational Science, University of Miami.

Real-Time PCR

RNA was converted into complementary DNA with the QuantiTect Reverse Transcription Kit (QIAGEN), and real-time PCR was performed with the TaqMan gene Expression Assay (Applied Biosystems).

Confocal Microscopy

Cells were fixed with 4% paraformaldehyde in DMEM for 15 min at 37°C and were permeabilized with 0.2% Triton X-100. Fixed and permeabilized cells were preincubated with 0.1% BSA in PBS and were then incubated with primary antibodies in 0.1% BSA in PBS. Cells were then incubated with fluorophore-conjugated secondary antibodies.

Virus Infection

MEFs cells were seeded in 24-well plates and grown to 70% confluence. After being washed with PBS, the cells were infected with HSV1 (KOS strain) or γ34.5 deleted-HSV1 at the indicated multiplicity of infection. The cells were incubated with virus for 1 hr at 37°C in serum-free DMEM (Invitrogen). The cells were washed with PBS twice, and complete medium was added to the cells.

Biotin dsDNA or ssDNA Agarose Beads

Streptavidin agarose beads (10 μl; Thermo Scientific) were incubated with 0.1 nmol biotin-conjugated dsDNA or ssDNA (Sigma Aldrich) in PBS at 4°C for 1 hr. After incubation, the agarose beads were washed three times and resuspended in lysis buffer.

DNA90 Sequences

An ssDNA sense strand containing a biotin label at the 5′ end (TACAGATCT ACTAGTGATCTATGACTGATCTGTACATGATCTACATACAGATCTACTAGTG ATCTATGACTGATCTGTACATGATCTACA) was annealed to ssDNA90 antisense, to create dsDNA90.

Reporter Analysis

293T cells were transiently transfected with 250 ng firefly luciferase reporter plasmid together with a total of 250 ng of various expression plasmids, empty plasmids, or 125 ng pRT-TK (internal control) with Lipofectamine2000 (Invitrogen) according to the manufacturer's instruction. Twenty-four hours later, the luciferase activity in the total cell lysate was measured.

In Vivo DNA Binding Analysis

For endogenous STING pull-down assay, hTERT-BJ1 cells were transfected with biotin-conjugated dsDNA90 or ssDNA90 with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. After 6 hr, cells were crosslinked with disuccinimidyl suberate (DSS, 2 mM; Thermo Scientific) or UV (UVC, 254 nm) with a Hoefer UV Crosslinker (Hoefer) for 3 min (12×10^4 μJ/cm^2). Cell lysis was performed with CHAPS buffer (10 mM CHAPS in PBS, 1 mM NaF, 1 mM Na_3VO_4, and protease inhibitors) for 1 hr at 4°C. Cell lysates were incubated with 10 μl streptavidin agarose beads (Thermo Scientific) at 4°C overnight. The complexes were washed five times with CHAPS lysis buffer. The agarose beads were boiled in SDS sample buffer. Images were obtained with Supersignal West Femto Maximum Sensitivity Substrate (Thermo Scientific). 293T cells were transfected with corresponding plasmids by calcium phosphate method. After 24 hr, cells were lysed in CHAPS lysis buffer (10 mM CHAPS in PBS, 1 mM NaF, 1 mM Na_3VO_4, and protease inhibitors) and incubated for 1 hr at 4°C. After centrifugation, cell lysates were precipitated with 10 μl biotin-conjugated dsDNA90 agarose beads at 4°C for 3 hr. The agarose beads were washed five times by wash buffer (10 mM CHAPS in PBS, 300 mM NaCl, 1 mM NaF, and 1 mM Na_3VO_4) and proteins were eluted with SDS sample buffer by boiling for 10 min. Western blot was developed by Supersignal West Pico Chemiluminescent Substrate (Thermo Scientific).

In Vitro DNA Binding Analysis

Proteins were produced by TNT Coupled Wheat Germ Extract System (Promega). Proteins were diluted with TNE buffer (50 mM Tris [pH 7.5], 150 mM NaCl, 1 mM EDTA, 1% NP-40, and protease inhibitors). Lysates were precipitated with 10 μl biotin-conjugated dsDNA90 or ssDNA agarose beads at 4°C overnight. The agarose beads were washed five times with wash buffer (50 mM Tris [pH 7.5], 300 mM NaCl, 1 mM EDTA, 1% NP-40), and proteins were eluted with SDS sample buffer by boiling for 10 min. Western blot analysis of pull-down materials were detected with Supersignal West Femto Maximum Sensitivity Substrate (Thermo Scientific).

ChIP Assay

ChIP assay was performed with the Chromatin Immunoprecipitation Assay Kit (Millipore) according to the manufacturer's instructions. DNA was purified by phenol/chloroform extraction and PCR was performed with Vent DNA polymerase (NEB). The following primers were used for dsDNA90 PCR: forward, 5′-CTAAGGGTGTGGCCCTTCCGCATAGAACTGTACAGATCTACTAGTGATCT-3′; reverse, 5′-CCCTGGAAGATGGAAGCGTTTTGCAACCGCATGTAGATCATGTACAGATC-3′.

Competition Assay

In vitro translation products were incubated with dsDNA90 (Sigma Aldrich), ssDNA90 (Sigma Aldrich) or Poly(dA:dT) (Sigma Aldrich) (0.01 mg/ml, 0.1 mg/ml, or 1.0 mg/ml, respectively) in TNE buffer (50 mM Tris [pH 7.5], 150 mM NaCl, 1 mM EDTA, 1% NP-40, and protease inhibitors) and biotin-conjugated dsDNA90 agarose beads at 4°C. After 2 hr, the agarose beads were washed five times with wash buffer (50 mM Tris [pH 7.5], 300 mM NaCl, 1 mM EDTA, and 1% NP-40) and precipitated with SDS sample buffer by boiling for 10 min.

Protein Purification and DNA Binding Assay

293T cell were transiently transfected with STING-HA or GFP-HA by calcium phosphate method. After 24 hr, cells were washed with PBS and lysed in TNE lysis buffer (50 mM Tris [pH 7.5], 150 mM NaCl, 1 mM EDTA, 1% NP-40, and protease inhibitors). Supernatants were precipitated by anti-HA resin (Sigma Aldrich) for overnight at 4°C and washed with TNE buffer. Resin was suspended by elution buffer (TNE lysis buffer, 100 μg/ml HA peptide [Sigma Aldrich], and protease inhibitors). For STING c-term (6xHis) (aa 152–379) purification from *E. coli*, a single colony was inoculated in Luria-Bertani (LB) medium with kanamycin and grown at 37°C until an optical density of 0.5. isopropylthio-β-galactoside (IPTG; a final concentration 0.2 mM) was added and culture was incubated at 16°C overnight. Cultures were pelleted and resuspended in lysis buffer (20 mM Tris [pH 7.5], 300 mM NaCl, 10% glycerol, 5 mM DTT, and protease inhibitors). The suspension was lysed with French Press (AVESTIN). After centrifugation, supernatants were added with 50mM imidazole and transferred to HisTrap HP (GE Healthcare), washed with washing buffer (lysis buffer with 50 mM imidazole) and eluted in elution buffer (lysis buffer with 300 mM imidazole). For STING c-term (6xHis) (aa 181–379) purification from *E. coli*, a single colony was inoculated in LB medium with kanamycin and grown at 37°C until an optical density of 0.7, and then IPTG (a final concentration 1 mM) was added. After 3 hr, culture were pelleted and resuspended in phosphate buffer (20 mM sodium phosphate buffer [pH 8.0], 50 mM imidazole, and protease inhibitors). The suspension was sonicated. After centrifugation, supernatants were transferred to HisTrap HP (17-5247-01; GE Healthcare, Piscataway, NJ), washed with phosphate buffer, and eluted in elution buffer (20 mM sodium phosphate buffer [pH 8.0] and 250 mM imidazole). For purified protein pull-down, 50 ng purified proteins was diluted with 10 μl TNE buffer and incubated with 0.1 nmol biotin-conjugated dsDNA90 at 4°C for 2 hr. Protein and DNA complex were then added to 10 μl streptavidin beads prewashed in TNE buffer and incubated at 4°C for 2 hr. The beads were washed five times with TNE buffer, and proteins were eluted with SDS sample buffer by boiling for 10 min. Images were obtained with Supersignal West Pico Chemiluminescent Substrate (Thermo Scientific).

Surface Plasmon Resonance Assay

SPR assay was performed by Affina Biotechnologies (Stamford, CT) with the BIAcore 3000 system (GE Healthcare). The certification-grade streptavidin chip (SA5; GE Healthcare) was equilibrated with HBS-EP buffer (10 mM HEPES, 150 mM NaCl, 3 mM EDTA, 1 mg/ml BSA, and 0.05% surfactant P-20). Biotinylated DNA90 was immobilized at the flow rate of 30 μl/min to the level of 530 RU dsDNA90. Solutions of C-term region of STING in the HBS-EP were injected across the surface at the flow rate of 30 μl/min at 25°C. The sample injection was followed by a 5–60 min dissociation phase at the same flow rate. The sensor chips were regenerated under 6 M urea and 1 M NaCl in the HBS-EP buffer. BIAnalysis evaluation software (BIAEval 3.2) was used for calculation of the rate and equilibrium constants. The equilibrium binding constant, K_D, was determined as the ratio of the dissociation rate constant, k_{diss}, and k_{ass}, the association rate constant.

Fluorescence Anisotropy Assay

C-term region of STING (aa 152–379) protein stock was diluted in 20 mM sodium phosphate buffer (pH 8.0) to a consistent volume of 90 μl. To each protein concentration, 10 μl 3 nM FAM-DNA (30-mer, FAM-TACAGATCTACTAGTGATCTATGACTGATC; 18-mer, FAM-TACAGATCTACTAGTGAT) was added, yielding a final FAM-DNA concentration of 300 pM. The fluorescence polarization was measured with a Beacon 2000 equipped with a 490 nm band-pass filter for excitation and a 535 nm filter for emission collection. The mean and standard deviation of the anistropy were calculated in Excel for each protein concentration and exported to Origin for plotting. The Kd is estimated from a nonlinear least-squares fit to the function y = Amin + (Amax – Amin) × [Protein] / (Kd + [Protein]).

Statistics

A Student's t test was used to analyze data. p values of 0.05 or less were considered to donate significance.

ACCESSION NUMBERS

The GEO accession number for the microarray data reported in this paper is GSE43482 (including subseries GSE43479, GSE43480, and GSE43481).

SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures and can be found with this article online at http://dx.doi.org/10.1016/j.molcel.2013.01.039.

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