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The helicase DDX41 recognizes the bacterial secondary messengers cyclic di-GMP and cyclic di-AMP to activate a type I interferon immune response

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The induction of type I interferons by the bacterial secondary messengers cyclic di-GMP (c-di-GMP) or cyclic di-AMP (c-di-AMP) is dependent on a signaling axis that involves the adaptor STING, the kinase TBK1 and the transcription factor IRF3. Here we identified the heliase DDX41 as a pattern-recognition receptor (PRR) that sensed both c-di-GMP and c-di-AMP. DDX41 specifically and directly interacted with c-di-GMP. Knockdown of DDX41 via short hairpin RNA in mouse or human cells inhibited the induction of genes encoding molecules involved in the innate immune response and resulted in defective activation of STING, TBK1 and IRF3 in response to c-di-GMP or c-di-AMP. Our results suggest a mechanism whereby c-di-GMP and c-di-AMP are detected by DDX41, which forms a complex with STING to signal to TBK1-IRF3 and activate the interferon response.

The host innate immune system provides a critical first line of defense against invading microorganisms, including pathogenic bacteria. Through the use of germline-encoded pattern-recognition receptors (PRRs), mammalian cells detect a wide variety of highly invariant molecular structures known as 'pathogen-associated molecular patterns' that are often required by microbes for their survival or pathogenicity[1–3]. Indeed, many bacterial pathogens use cyclic diguanosine monophosphate (c-di-GMP) or cyclic diadenosine monophosphate (c-di-AMP), two key secondary messengers with essential roles in regulating metabolism, motility and virulence[4–6]. During infection with certain bacterial species, these bacteria-derived secondary messengers can also act as pathogen-associated molecular patterns, triggering a host type I interferon innate immune response characterized by activation of the transcription factors NF-κB and IRF3 (refs. 7,8).

The mechanism by which c-di-GMP and c-di-AMP activate the host type I interferon response remains poorly understood. Indeed, intracellular detection of c-di-GMP or c-di-AMP leads to activation of the type I interferon response in a manner independent of the cytoplasmic PRR RIG-I (DDX58) or its downstream adaptor IPS-1 (MAVS, Cardif or VISA). Moreover, c-di-GMP and c-di-AMP require neither the adaptor MyD88 nor the adaptor TRIF, which suggests that the Toll-like receptor family of PRRs is also not involved in the detection of c-di-GMP or c-di-AMP[9]. However, the activation of type I interferons by c-di-GMP and c-di-AMP has been shown to require the adaptor STING (MITA, MPYS, ERIS or TMEM173), which suggests that these cyclic dinucleotides are detected by a PRR that signals via STING[10,11]. In response to certain viral nucleic acids and B-form DNA (B-DNA or poly(dA:dT)), STING functions as an adaptor and has been demonstrated to facilitate downstream signal transmission to IRF3 and NF-κB[12–14]. Here we provide evidence that the PRR DDX41 (DEAD (aspartate-glutamate-alanine-aspartate)-box polypeptide 41) is the main sensor that directly binds to c-di-GMP or c-di-AMP and can trigger the type I interferon host immune response via STING.

RESULTS

DDX41 mediates cyclic-dinucleotide sensing in immunocytes

We stably knocked down DDX41 through the use of short hairpin RNA (shRNA) in the mouse splenic dendritic cell (DC) line D2SC (**Fig. 1a**) and examined the induction of interferon-β (IFN-β) in response to c-di-GMP and c-di-AMP. Control cells infected with *Listeria monocytogenes* had a robust IFN-β response, whereas cells treated with DDX41-specific shRNA had a much lower IFN-β response (**Fig. 1b**). Consistent with published data[10,11], cells treated with STING-specific shRNA also had impaired induction of IFN-β in response to *L. monocytogenes* (**Fig. 1b**). Cytoplasmic delivery of either c-di-GMP or c-di-AMP by lipofection also yielded strong activation of IFN-β that was much lower in cells treated with DDX41-specific shRNA, which paralleled the impairment of cells treated with STING-specific shRNA (**Fig. 1c,d**). Induction of IFN-β by

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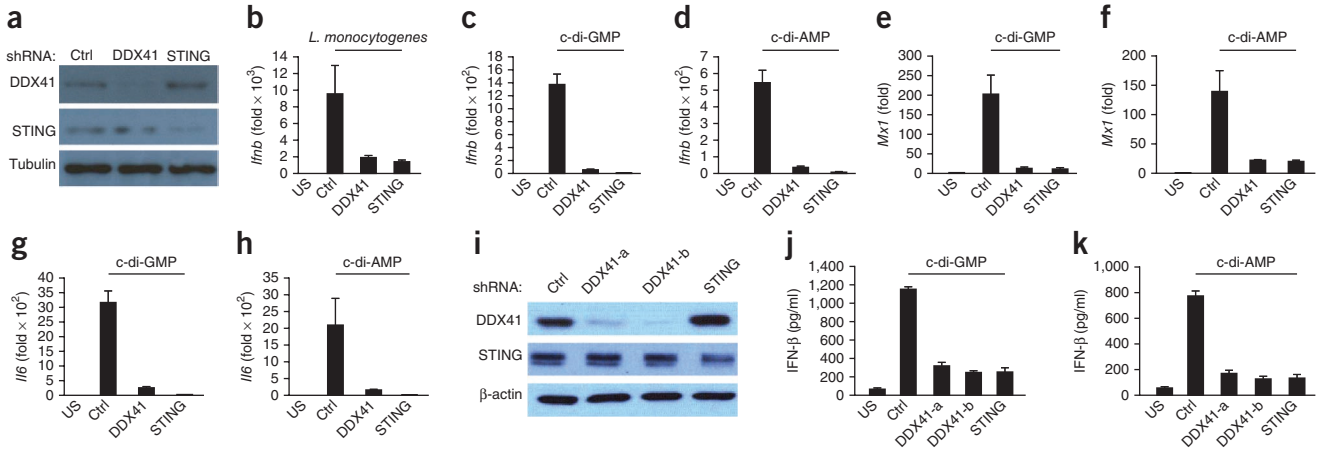

Figure 1 The c-di-GMP- and c-di-AMP-mediated induction of the innate immune response in mouse DCs and human monocytes requires DDX41. (**a**) Immunoblot analysis of DDX41 and STING in D2SC mouse DCs treated with nontargeting scrambled shRNA (control (Ctrl)) or shRNA targeting DDX41 or STING (above lanes); tubulin serves as a loading control. (**b**–**d**) Quantitative PCR analysis of *Ifnb* mRNA in D2SC cells treated with control shRNA and left unstimulated (US) or treated with control shRNA or DDX41- or STING-specific shRNA and stimulated for 6 h with *L. monocytogenes* (**b**), c-di-GMP (**c**) or c-di-AMP (**d**); results are presented relative to those of *Rpl32* mRNA (which encodes the ribosomal protein L32). (**e**–**h**) Quantitative PCR analysis of *Mx1* mRNA (**e**,**f**) and *Il6* mRNA (**g**,**h**) in D2SC cells treated with shRNA as in **b**–**d** and left unstimulated or stimulated with c-di-GMP (**e**,**g**) or c-di-AMP (**f**,**h**); results are presented relative to those of *Rpl32* mRNA. (**i**) Immunoblot analysis of DDX41 and STING in THP-1 monocytes treated with control shRNA or shRNA targeting DDX41 (two shRNAs: DDX41-a and DDX41-b) or STING; β-actin serves as a loading control. (**j**,**k**) Enzyme-linked immunosorbent assay (ELISA) of IFN-β in THP-1 monocytes treated with shRNA as in **i** and left unstimulated or stimulated for 16 h with c-di-GMP (**j**) or c-di-AMP (**k**). Data are representative of at least three independent experiments (error bars (**b**–**h**,**j**,**k**), s.e.m.).

the synthetic DNA poly(dA:dT) was impaired in cells treated with DDX41-specific shRNA, but induction of IFN-β by the RNA ligand poly(I:C) was not (**Supplementary Fig. 1a**,**b**), which demonstrated the specificity of DDX41 for c-di-GMP, c-di-AMP and B-DNA. Type I interferons mediate the innate immune response via the receptor for IFN-α/β, whereby ligation of the receptor leads toward the activation of hundreds of interferon-stimulated genes[15]. Both c-di-GMP and c-di-AMP activated the interferon-stimulated gene *Mx1* in control cells; however, the induction of *Mx1* was much lower in cells treated with DDX41- or STING-specific shRNA (**Fig. 1e**,**f**). Similarly, the c-di-GMP-mediated activation of other interferon-stimulated genes was also impaired in cells treated with DDX41-specific shRNA (**Supplementary Fig. 1c**–**f**). These results indicated a critical role for DDX41 in cyclic dinucleotide–mediated activation of type I interferon and interferon-mediated signaling. The cytosolic detection of bacterial secondary messengers also leads to the activation of NF-κB, a key transcription factor important for the induction of proinflammatory cytokines such as interleukin 6 (IL-6) and tumor-necrosis factor[16]. We found that expression of the genes encoding IL-6 and tumor-necrosis factor was much lower in cells treated with DDX41-specific shRNA than in cells treated with control shRNA, when the cells were also treated with c-di-GMP and/or c-di-AMP (**Fig. 1g**,**h** and **Supplementary Fig. 1g**).

To determine whether DDX41 mediates the sensing of c-di-GMP and c-di-AMP and activation of type I interferon in human cells, we stably knocked down DDX41 through the use of shRNA in the human monocyte cell line THP-1 (**Fig. 1i**). Consistent with our findings obtained with mouse cells, both c-di-GMP and c-di-AMP induced

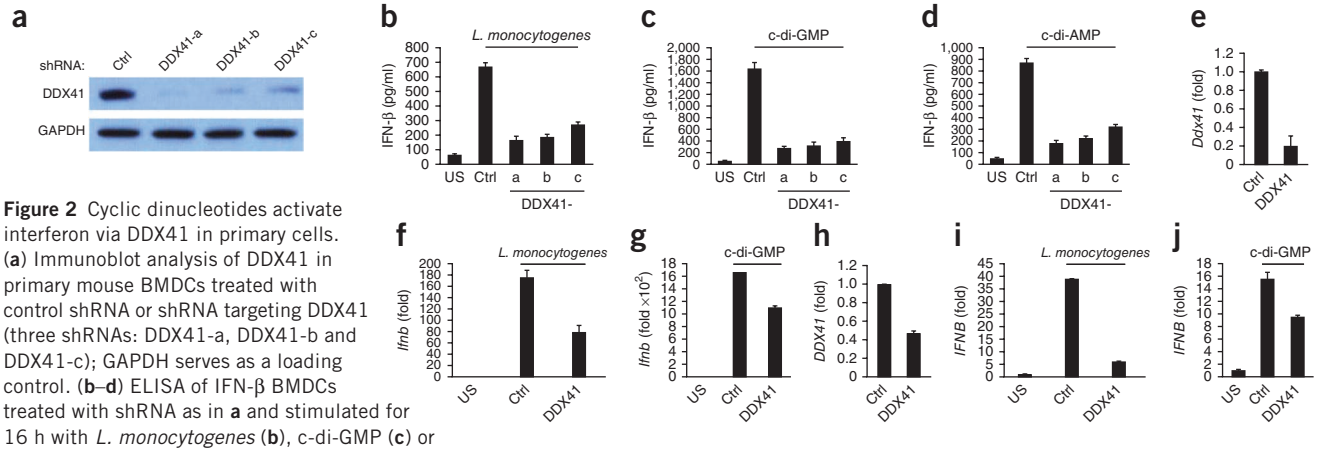

Figure 2 Cyclic dinucleotides activate interferon via DDX41 in primary cells. (**a**) Immunoblot analysis of DDX41 in primary mouse BMDCs treated with control shRNA or shRNA targeting DDX41 (three shRNAs: DDX41-a, DDX41-b and DDX41-c); GAPDH serves as a loading control. (**b**–**d**) ELISA of IFN-β BMDCs treated with shRNA as in **a** and stimulated for 16 h with *L. monocytogenes* (**b**), c-di-GMP (**c**) or c-di-AMP (**d**). (**e**–**g**) Quantitative PCR analysis of *Ddx41* mRNA (**e**) or *Ifnb* mRNA (**f**,**g**) in primary mouse peritoneal macrophages transfected with control siRNA or siRNA targeting DDX41, then given no further treatment (**e**) or stimulated for 6 h with *L. monocytogenes* (**f**) or c-di-GMP (**g**); results are presented relative to those of *Rpl32* mRNA. (**h**–**j**) Quantitative PCR analysis of *DDX41* mRNA (**h**) and *IFNB* mRNA (**i**,**j**) in primary human PBMCs transfected and stimulated as in **e** (**h**), **f** (**i**) or **g** (**j**); results are presented relative to those of mRNA encoding ribosomal protein 36B4). Data are representative of at least two independent experiments (error bars (**b**–**j**), s.e.m.).

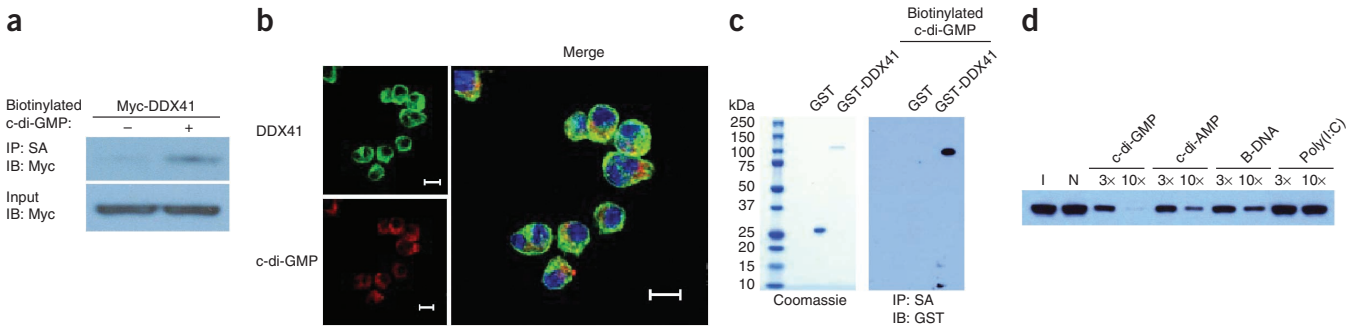

Figure 3 DDX41 is a direct sensor of c-di-GMP. (**a**) Immunoprecipitation (IP) and immunoblot analysis (IB) of the interaction of biotinylated c-di-GMP with Myc-tagged DDX41 in 293T cells. SA, streptavidin. (**b**) Confocal microscopy of the colocalization of c-di-GMP and DDX41 in D2SC cells transfected with expression vector for Myc-tagged DDX41 and with biotinylated c-di-GMP. Scale bars, 10 μm. (**c**) Precipitation and immunoblot analysis (right) of the interaction of biotinylated c-di-GMP with glutathione *S*-transferase (GST) alone or GST-tagged DDX41. Left, SDS-PAGE of GST and GST-DDX41 (without biotinylated c-di-GMP), followed by Coomassie staining (far left, molecular size, in kilodaltons (kDa)). (**d**) Precipitation and immunoblot analysis of input GST-tagged DDX41 (I) and of the interaction of biotinylated c-di-GMP (2.0 μM) with GST-tagged DDX41 with no competitor (N) or with increasing amounts of unlabeled ligand (above blot: 3×, 6.0 μM; 10×, 20 μM). Data are representative of at least two independent experiments.

the production of IFN-β in THP-1 cells treated with control shRNA, whereas c-di-GMP- or c-di-AMP-mediated production of IFN-β was very defective in THP-1 cells treated with DDX41- or STING-specific shRNA (**Fig. 1j**,**k**). The induction of interferon-stimulated genes in response to c-di-GMP was also impaired in THP-1 cells treated with DDX41-specific shRNA (**Supplementary Fig. 1h–l**). In addition, the DDX41-STING signaling axis was required for the induction of IFN-β in THP-1 cells stimulated with B-DNA (**Supplementary Fig. 1m**). These results suggested that DDX41 functioned in regulating the induction of genes encoding type I interferons and proinflammatory molecules in response to cyclic dinucleotides in both mouse and human cells.

DDX41 mediates cyclic-dinucleotide sensing in primary cells

We also examined the role of DDX41 in facilitating the activation of type I interferon induced by c-di-GMP or c-di-AMP in primary cells. We prepared BMDCs (mouse DCs derived from bone marrow through the use of granulocyte-macrophage colony-stimulating factor) and transfected the cells with shRNA targeting DDX41 (**Fig. 2a**). BMDCs treated with control shRNA and infected with *L. monocytogenes* had robust production of IFN-β, much greater than that of BMDCs treated with DDX41-specific shRNA and infected similarly (**Fig. 2b**). Stimulation with c-di-GMP or c-di-AMP also induced the production of IFN-β in BMDCs treated with control shRNA, whereas IFN-β production was considerably impaired in BMDCs treated with DDX41-specific shRNA and stimulated similarly (**Fig. 2c**,**d**). Similarly, thioglycollate-elicited primary mouse peritoneal macrophages transfected with small interfering RNA (siRNA) targeting DDX41 (**Fig. 2e**) had less activation of IFN-β in response to infection with *L. monocytogenes* or stimulation with c-di-GMP than did cells transfected with control siRNA and infected or stimulated similarly (**Fig. 2f**,**g**). Consistent with that, the induction of *Mx1* and *Il6* was also impaired in peritoneal macrophages transfected with DDX41-specific siRNA and infected with *L. monocytogenes* or treated with c-di-GMP (**Supplementary Fig. 2a–d**).

To determine whether DDX41 has a role in sensing c-di-GMP in primary human cells, we transfected peripheral blood mononuclear cells (PBMCs) with siRNA specific for DDX41 (**Fig. 2h**). Whereas transfection of PBMCs with control siRNA followed by infection with *L. monocytogenes* or treatment with c-di-GMP elicited activation of IFN-β, PBMCs transfected with DDX41-specific siRNA showed defective IFN-β activation in response to *L. monocytogenes* or c-di-GMP (**Fig. 2i**,**j**). PBMCs obtained from two additional human donors and transfected with DDX41-specific siRNA had similarly lower IFN-β activation (**Supplementary Fig. 2e**,**f**). These results further indicated a critical role for DDX41 in facilitating type I interferon responses induced by c-di-GMP or c-di-AMP in primary cells of the immune system.

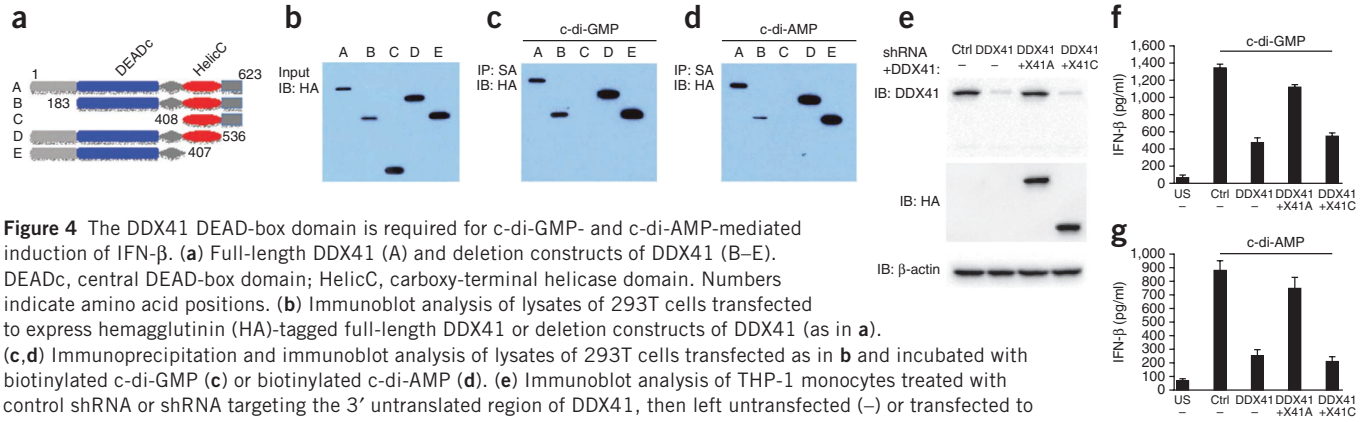

Figure 4 The DDX41 DEAD-box domain is required for c-di-GMP- and c-di-AMP-mediated induction of IFN-β. (**a**) Full-length DDX41 (A) and deletion constructs of DDX41 (B–E). DEADc, central DEAD-box domain; HelicC, carboxy-terminal helicase domain. Numbers indicate amino acid positions. (**b**) Immunoblot analysis of lysates of 293T cells transfected to express hemagglutinin (HA)-tagged full-length DDX41 or deletion constructs of DDX41 (as in **a**). (**c**,**d**) Immunoprecipitation and immunoblot analysis of lysates of 293T cells transfected as in **b** and incubated with biotinylated c-di-GMP (**c**) or biotinylated c-di-AMP (**d**). (**e**) Immunoblot analysis of THP-1 monocytes treated with control shRNA or shRNA targeting the 3′ untranslated region of DDX41, then left untransfected (–) or transfected to express hemagglutinin-tagged full-length DDX41 (+X41A) or DDX41 lacking the DEAD-box domain (+X41C). (**f**,**g**) ELISA of IFN-β in THP-1 cells treated with control shRNA or shRNA targeting DDX41, then left unstimulated or treated for 16 h with c-di-GMP (**f**) or c-di-AMP (**g**) and then reconstituted as in **e** (below graphs). Data are representative of at least two independent experiments (error bars (**f**,**g**), s.e.m.).

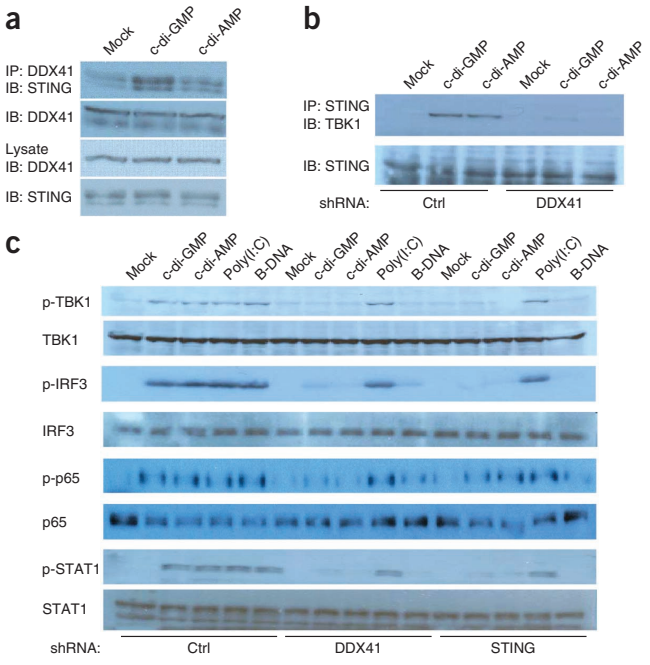

Figure 5 Both c-di-GMP and c-di-AMP require DDX41 for STING-dependent signaling. (**a**) Immunoprecipitation and immunoblot analysis (top two blots) of the DDX41-STING interaction in D2SC cells mock-transfected (Mock) or transfected for 4 h with c-di-GMP or c-di-AMP. Below (Lysate), immunoblot analysis of DDX41 and STING in lysates without immunoprecipitation. (**b**) Immunoprecipitation and immunoblot analysis of the STING-TBK1 interaction in D2SC cells treated with control shRNA or DDX41-specific shRNA (below lanes) and transfected as in **a** (above lanes). (**c**) Immunoblot analysis of phosphorylated (p-) and total TBK1, IRF3, NF-κB subunit p65 and STAT1 in D2SC cells treated with control shRNA or DDX41- or STING-specific shRNA (below lanes) and transfected for 4 h with c-di-GMP, c-di-AMP, poly(I:C) or B-DNA (above lanes). Data are representative of at least two independent experiments.

DDX41 is a direct sensor of c-di-GMP

DDX41 is known not only to signal via STING to activate type I interferon but also to function as a PRR that directly binds viral DNA and B-DNA[17]. We therefore investigated whether DDX41 functions as a direct sensor (PRR) for c-di-GMP or c-di-AMP. Biotin-labeled c-di-GMP or c-di-AMP precipitated ectopically expressed DDX41 from lysates of human embryonic kidney 293T cells (**Fig. 3a** and **Supplementary Fig. 3a**). Immunofluorescence microscopy further showed that DDX41 and c-di-GMP localized together after transfection of D2SC cells with expression vector for DDX41 and with biotinylated c-di-GMP (**Fig. 3b**). In addition, we found that DDX41 purified from *Escherichia coli* through the use of glutathione *S*-transferase or histidine directly bound c-di-GMP (**Fig. 3c** and **Supplementary Fig. 3b**). The interaction of c-di-GMP with DDX41 was specific, as only unlabeled c-di-GMP, c-di-AMP and poly(dA:dT) (B-DNA) competitively disrupted the DDX41–c-di-GMP interaction, but poly(I:C) did not (**Fig. 3d**). Furthermore, the structurally similar molecules GMP and AMP also disrupted the DDX41–c-di-GMP complex, but the c-di-GMP and c-di-AMP precursors GTP and ATP, respectively, did not (**Supplementary Fig. 3c**).

To determine whether the binding of c-di-GMP was specific to DDX41, we assessed the binding of c-di-GMP to DDX58, another helicase PRR. We found that c-di-GMP bound to DDX41 but showed no interaction with DDX58 (**Supplementary Fig. 3d**). To define which domain of DDX41 was important for the binding of c-di-GMP or c-di-AMP, we used a series of deletion mutants of DDX41 (**Fig. 4a**,**b**) and evaluated their interaction with biotinylated c-di-GMP or c-di-AMP in 293T cells. We found that c-di-GMP and c-di-AMP did not interact with DDX41 lacking the central DEAD-box domain (**Fig. 4c**,**d**). To determine the physiological relevance of this domain in terms of the induction of type I interferon, we treated THP-1 cells with DDX41-specific shRNA and reconstituted the cells with either full-length DDX41 or the DDX41 deletion

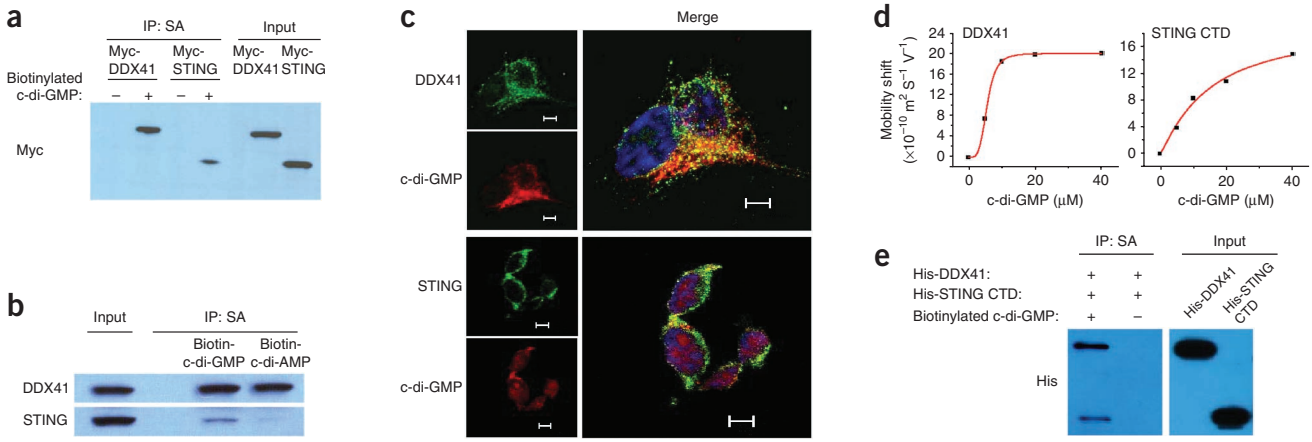

Figure 6 The binding affinity of c-di-GMP for DDX41 is greater than that of c-di-GMP for STING. (**a**) Precipitation and immunoblot analysis of the interaction of biotinylated c-di-GMP with Myc-tagged DDX41 or STING in 293T cells. Right (Input), immunoblot analysis of Myc-tagged DDX41 or STING in cells without c-di-GMP. (**b**) Immunoprecipitation and immunoblot analysis of the interaction of biotinylated c-di-GMP or c-di-AMP with endogenous DDX41 and STING in D2SC cells. Far left (Input), immunoblot analysis of DDX41 or STING in cells without c-di-GMP. (**c**) Confocal microscopy of the interaction of c-di-GMP with DDX41 or STING in 293T cells transfected with biotinylated c-di-GMP and with expression vector for Myc-tagged DDX41 (top) or Myc-tagged STING (bottom). Scale bars, 5 μm. (**d**) Affinity capillary electrophoresis analysis of the binding affinity of recombinant DDX41 and c-di-GMP (left; $r^2 = 0.99992$) or recombinant carboxy-terminal domain of STING (STING CTD) and c-di-GMP (right; $r^2 = 0.98342$). (**e**) Immunoprecipitation and immunoblot analysis of the interaction of biotinylated c-di-GMP with bacterially purified histidine (His)-tagged DDX41 and carboxy-terminal domain of STING. Right (Input), immunoblot analysis of without immunoprecipitation. Data are representative of at least two independent experiments.

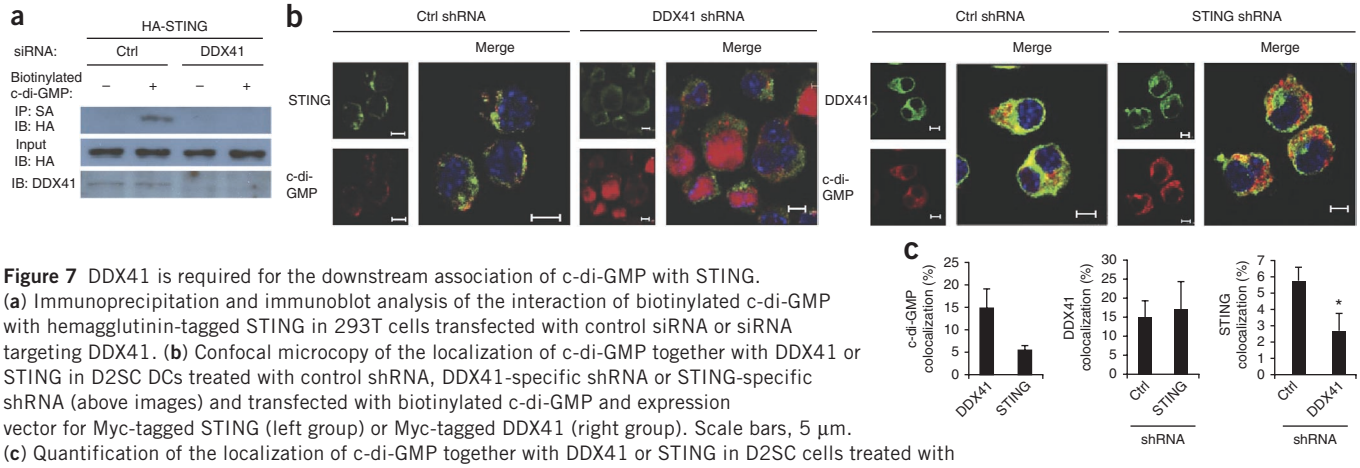

Figure 7 DDX41 is required for the downstream association of c-di-GMP with STING. (**a**) Immunoprecipitation and immunoblot analysis of the interaction of biotinylated c-di-GMP with hemagglutinin-tagged STING in 293T cells transfected with control siRNA or siRNA targeting DDX41. (**b**) Confocal microcopy of the localization of c-di-GMP together with DDX41 or STING in D2SC DCs treated with control shRNA, DDX41-specific shRNA or STING-specific shRNA (above images) and transfected with biotinylated c-di-GMP and expression vector for Myc-tagged STING (left group) or Myc-tagged DDX41 (right group). Scale bars, 5 μm. (**c**) Quantification of the localization of c-di-GMP together with DDX41 or STING in D2SC cells treated with control shRNA (left), with DDX41 in D2SC cells treated with control or STING-specific shRNA (middle) or with STING in D2SC cells treated with control or DDX41-specific shRNA (right). Data are representative of at least two independent experiments (error bars (**c**), s.e.m.). *$P = 0.04$.

mutant lacking the DEAD-box domain (**Fig. 4e**). As expected, cells treated with DDX41-specific shRNA had less production of IFN-β in response to c-di-GMP or c-di-AMP than did cells treated with control shRNA (**Fig. 4f**,**g**). However, in cells treated with DDX41-specific shRNA, reconstitution with full-length DDX41 restored IFN-β production in response to stimulation with c-di-GMP or c-di-AMP, but reconstitution with the DDX41 deletion mutant lacking the DEAD-box domain did not (**Fig. 4f**,**g**). Thus, the cyclic dinucleotide–mediated induction of IFN-β required the central DEAD-box domain of DDX41.

Requirement for DDX41 in cyclic dinucleotide signaling

DDX41 interacted with and localized together with STING (**Supplementary Fig. 4a**,**b**) to facilitate DNA ligand–dependent signal transduction[17]. The introduction of either c-di-GMP or c-di-AMP into D2SC cells led to enhanced formation of the DDX41-STING complex (**Fig. 5a**). In DNA-dependent signaling pathways, STING further binds to the downstream kinase TBK1 to activate the type I interferon response[18,19]. When transfected into control D2SC cells, both c-di-GMP and c-di-AMP activated formation of the STING-TBK1 complex; however, c-di-GMP- or c-di-AMP-mediated activation of the STING-TBK1 complex was almost completely abrogated in cells treated with DDX41-specific shRNA (**Fig. 5b**). Consequently, c-di-GMP- or c-di-AMP-mediated activation of TBK1, IRF3 and the downstream type I interferon effector STAT1 was impaired in cells treated with DDX41-specific shRNA (**Fig. 5c** and **Supplementary Fig. 5**). Activation of NF-κB was also impaired in cells treated with DDX41-specific shRNA in response to either c-di-GMP or c-di-AMP (**Fig. 5c**). Together our findings suggested that DDX41 was a critical PRR for c-di-GMP- and c-di-AMP-mediated induction of interferon and that its absence generated a defect in downstream STING-dependent signaling.

Signaling by c-di-GMP to STING via DDX41

A published study has shown that c-di-GMP binds to the carboxy-terminal domain of STING (amino acids 139–379) and suggested that STING might function as an immunological sensor of c-di-GMP[20]. We therefore used binding assays to determine the affinity of c-di-GMP or c-di-AMP for DDX41 and for STING in parallel. Biotin-labeled c-di-GMP precipitated more ectopically expressed DDX41 than ectopically expressed STING from lysates of 293T cells (**Fig. 6a**), indicative of the greater affinity of c-di-GMP for DDX41 than for STING. Physiologically, the binding of c-di-GMP to endogenous DDX41 was also greater than the association of c-di-GMP with endogenous STING (**Fig. 6b**). Immunofluorescence imaging further showed more colocalization of c-di-GMP and DDX41 (34.13%) than of c-di-GMP and STING (6.25%; **Fig. 6c**). We also used affinity capillary electrophoresis to examine the binding affinity of c-di-GMP for recombinant DDX41 or a recombinant carboxy-terminal domain of STING. We found that c-di-GMP bound DDX41 with a dissociation constant of ~5.65 μM, whereas c-di-GMP associated with the carboxy-terminal domain of STING with a dissociation constant of ~14.54 μM (**Fig. 6d**). Consistent with those findings, in precipitation binding assays, c-di-GMP bound with stronger affinity to purified recombinant DDX41 than to the purified recombinant carboxy-terminal domain of STING (**Fig. 6e**). We therefore hypothesized that DDX41 was the main sensor of c-di-GMP and c-di-AMP and operated upstream of STING, and after binding those pathogen-associated molecular patterns, yielded enhanced complex formation with STING to facilitate downstream signaling and activation of type I interferon (**Supplementary Fig. 6**).

In agreement with those findings, c-di-GMP was considerably impaired in its ability to associate with ectopically expressed STING in 293T cells transfected with siRNA targeting DDX41 (**Fig. 7a**). Consistent with that, the localization of c-di-GMP together with STING was much lower in cells treated with DDX41-specific shRNA, whereas DDX41–c-di-GMP interactions remained intact in cells treated with STING-specific shRNA (**Fig. 7b**,**c**). Together, our findings indicated that DDX41 was the main PRR for c-di-GMP and c-di-AMP and signaled via STING for the induction of type I interferon.

DISCUSSION

Many bacterial pathogens, including *Staphylococcus, Streptococcus, Pseudomonas, Yersiniae, Listeria* and *Mycobacteria* species, use key secondary messengers such as c-di-GMP or c-di-AMP, which have essential modulatory roles in bacteria[4,7]. Although several substrates and effectors have been identified for these cyclic dinucleotide monophosphate species in the bacterial cell, understanding of how these bacteria-specific secondary messengers modulate the innate immune response in the mammalian host cell is just beginning to emerge. Both c-di-GMP and c-di-AMP activate the host type I interferon response in a manner dependent on STING[10,11], and our findings have indicated that the DNA sensor and helicase DDX41 functioned as a direct PRR for these cyclic dinucleotides in both mouse and human cells.

Our results have shown that unlabeled c-di-GMP or c-di-AMP disrupted the DDX41–c-di-GMP interaction. We found that GMP and AMP also competitively disrupted the DDX41–c-di-GMP complex, but the bulkier GTP or ATP did not. Although two molecules of GMP or of AMP are structurally similar to c-di-GMP or c-di-AMP, respectively, they are not known to function as pathogen-associated molecular patterns in mammalian cells[9]. It will therefore be of further interest to determine how these species serve a modulatory role in the type I interferon response. Our competition experiments additionally showed that B-DNA disrupted the DDX41–c-di-GMP complex. Indeed, DDX41 has been shown to function as a sensor for B-DNA as well[17]. The mechanism by which DDX41 binds B-DNA, as well as cyclic dinucleotides, as demonstrated by co-crystallization studies and point-mutation analysis, requires further investigation.

Our results additionally indicated that the activation of innate signaling and induction of type I interferons mediated by c-di-GMP and c-di-AMP were similarly defective in cells in which DDX41 or STING had been knocked down, which suggested that DDX41 and STING share a common signaling pathway. STING-deficient cells had a very modest defect in the activation of NF-κB in response to c-di-GMP or c-di-AMP. The reason for this phenomenon is not entirely clear[10]; however, it may be possible that there is redundancy or compensation in signaling to NF-κB. Another DNA sensor, IFI16 (p204), has also been shown to facilitate some viral DNA–triggered signaling via the STING adaptor[21,22]. Although the possibility of a role for IFI16 in the c-di-GMP and c-di-AMP signaling pathway cannot be ruled out, it is unlikely that IFI16 functions as a chief sensor for these molecules, as its basal expression is low and it is induced in a type I interferon–dependent manner. The expression of DDX41, in contrast, is greater in the basal state and is not modulated by type I interferons[17].

Our data suggested that DDX41 served as the PRR for c-di-GMP and c-di-AMP, which, after binding to the receptor, signaled to TBK1-IRF3 via STING. Lending further credibility to the proposal that it serves as a scaffolding molecule, STING has been shown to bridge TBK1-IRF3 interactions for optimal signaling[23]. Nevertheless, consistent with a published report[20], we also found that c-di-GMP associated with STING; however, the interaction of c-di-GMP with DDX41 was much greater. Although the physiological relevance of this interaction requires further investigation, our data have shown that the interaction of c-di-GMP with STING was substantially enhanced in the presence of DDX41 in cells. The solved structure of the carboxy-terminal domain of STING in complex with c-di-GMP has shown that one molecule of c-di-GMP binds one dimer of STING[24–28]. We propose that the detection of c-di-GMP and its binding to DDX41 promote enhanced DDX41-STING interactions, leading to an increase in the binding affinity of STING for c-di-GMP, which ultimately drives downstream signaling events. Thus, STING may function as a secondary receptor or coactivator in the cyclic-dinucleotide signaling pathway.

The importance of the induction of type I interferon in the context of antibacterial innate immunity is unclear and somewhat controversial at present, particularly because of conflicting reports about whether type I interferon functions to support or to inhibit bacterial growth[29–32]. It will therefore be of interest to further study how various bacteria and host cells use secondary messengers and DDX41 as virulence factors and receptors of the innate immune system, respectively, in their battle of infection and immunity[33–35]. Thus, cyclic-dinucleotide species and DDX41 represent new targets for modulation of their interaction during certain bacterial infections to alter the host immune response to suppress bacterial replication and spread.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Supplementary information is available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

K.P., Z.Z., R.L.M., Y.L. and G.C. designed the research; K.P. and Z.Z. did and analyzed the biochemical experiments; R.M.T. did the confocal imaging and analysis; S.O., Y.J. and Z.-J.L. did the cloning, expression and purification of DDX41 and the STING carboxy-terminal domain; S.S.I. prepared peritoneal macrophages; M.S. prepared PBMCs; S.A.Z. did and analyzed experiments with primary cells; S.Z. and W.Z. did and analyzed the affinity capillary electrophoresis binding affinity experiments; and K.P. wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Cells and biological reagents. D2SC, THP-1 and 293T cells were cultured in IMDM (Invitrogen-Gibco), RPMI-1640 medium (Invitrogen-Gibco) and DMEM (Mediatech), respectively, with 10% (vol/vol) heat-inactivated FCS (Omega Scientific) and 1% (vol/vol) penicillin-streptomycin (Invitrogen-Gibco). Plasmids encoding Myc- or hemagglutinin-tagged DDX41 (and its truncation mutants) and STING have been described[17]. The following antibodies were used: antibody to DDX41 (anti-DDX41; 2F4; Novus Biologicals); anti-STING (LS-C108557; Life Span); anti-TBK1 (3013), antibody to phosphorylated TBK1 (4583), anti-STAT1 (9172), antibody to phosphorylated STAT1 (9171), antibody to phosphorylated IRF3 (4947), antibody to phosphorylated p65 (3033) and anti-Myc (2276; all from Cell Signaling); anti-hemagglutinin (sc-805), anti–glutathione *S*-transferase (sc-138), anti-IRF3 (sc-9082) and anti-p65 (sc-372; all from Santa Cruz); anti-tubulin (B-5-1-2; Sigma); and anti-β-actin (ab8226; Abcam). The reagents c-di-GMP, c-di-AMP, biotinylated c-di-GMP and biotinylated c-di-AMP were from BIOLOG Life Science Institute. Poly(I:C) and poly(dA:dT) were from Invivogen.

Lentiviral infection and RNA-mediated interference. D2SC and THP-1 cells were infected with a pLKO.1 lentivirus vector carrying a target gene sequence (for DDX41 or STING) or scrambled shRNA (Open Biosystems). At 24 h after culture, cells were selected by the addition of puromycin (2.5 ng/ml) to the medium. The 293T cells were transfected with 60 pmol of control scrambled siRNA or siRNA targeting DDX41 (Bioneer) through the use of Lipofectamine RNAiMAX (Invitrogen). For rescue experiments, knockdown of DDX41 in THP-1 cells was achieved with shRNA targeting the 3′ untranslated region of human DDX41 (Sigma).

***In vitro* culture of primary cells.** BMDCs were cultured as described[17]. For the isolation and culture of thioglycollate-elicted peritoneal macrophages, 5 ml of 3% (w/v) Brewer's thioglycollate media (Difco) was injected into the peritoneal cavity of 6-week-old C57Bl/6 mice. After 3 d, the peritoneum was lavaged with DMEM. Cells were lysed with ammonium chloride–potassium bicarbonate lysis buffer (Invitrogen), and mononuclear cells were obtained by centrifugation over Ficoll-Plaque (Pharmacia). Primary thioglycollate-elicited peritoneal macrophage cells were transfected with the Nucleofector approach according to the manufacturer's instructions (VPA-1009; Lonza). Cells (1×10^6) were suspended in 100 µl Nucleofector solution and were added to 200 pmol of control or DDX41-specific siRNA (Bioneer). Cells and siRNA were transferred to a cuvette and transfection was done with program Y-01 in the Nucleofector 2b device. Cells were immediately transferred to 5 ml RPMI medium supplemented with 10% FBS and 1% penicillin-streptomycin. Cells were plated in six-well tissue culture plates and treated appropriately (as noted in figures). For the isolation of primary human monocytes, whole blood was obtained from healthy donors who provided informed consent (University of California, Los Angeles, Institutional Review Board, 92-10-591-31). PBMCs were isolated with Ficoll gradient centrifugation (GE Healthcare) and samples were further enriched for monocytes with a Percoll density gradient (GE Healthcare). Monocytes obtained by Percoll-enrichment were transfected with 100 pmol siRNA oligo (SMARTpool, Thermo Scientific, Dharmacon) through the use of the Amaxa Nucleofection System and a Human Monocyte Nucleofector Kit (Lonza) according to the manufacturers' recommendations. Cells were cultured for 24 h in RPMI medium and 10% FCS (Omega Scientific) before activation and/or infection.

Real-time quantitative PCR and ELISA. RNA was isolated with TRIzol reagent as described by the manufacturer (Invitrogen). RNA (1 µg) was used to generate cDNA via the iScript cDNA Synthesis Kit (Bio-Rad). The mRNA was quantified with an iCycler thermocycler (Bio-Rad). Transcript abundance was normalized to that of mRNA encoding the ribosomal protein L32 for D2SC cells and peritoneal macrophages; mRNA encoding glyceraldehyde phosphate dehydrogenase for THP-1 monocytes; and mRNA encoding the ribosomal protein 36B4 in PBMCs. IFN-β was quantified in THP-1 cells and BMDCs with human- or mouse-specific ELISA kits (PBL InterferonSource).

Immunoassays. For immunoblot analysis, cells were collected in Triton lysis buffer (50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100 and 5% glycerol) containing complete protease inhibitors (Roche). For coimmunoprecipitation, pre-cleared lysates of 293T cells were incubated overnight at 4 °C with biotinylated c-di-GMP or biotinylated c-di-AMP, followed by the addition of streptavidin beads for an additional 4 h. Complexes were washed three times with Triton lysis buffer and analyzed by immunoblot by standard methodologies. Proteins were detected by enhanced chemiluminescence (Pierce). Endogenous proteins immunoprecipitated from lysates of D2SC cells were captured with protein A agarose beads (Roche) and analyzed by immunoblot as described above. For the detection of IRF3 dimers, lysates were separated on a 7.5% nondenaturing gel, followed by immunoblot analysis with anti-IRF3.

Transfection and bacterial infection. FuGene 6 (Roche) was used for transient transfection of plasmid DNA into 293T cells. Then, c-di-GMP (8 µg), c-di-AMP (2 µg), poly(I:C) (6 µg) or poly(dA:dT) (1 µg) was delivered into cells in a six-well format via Lipofectamine 2000 (Invitrogen). *L. monocytogenes* was grown for 16 h in brain-heart-infusion medium, followed by subculture for an additional 2–3 h to mid-log phase in fresh brain-heart-infusion medium. Cells plated in a six-well format were infected for 1 h with *L. monocytogenes* at a multiplicity of infection of 10, followed by complete replacement of the medium.

Two-color immunofluorescence and confocal microscopy. Imaging was done 24 h after transfection unless otherwise indicated. Immunofluorescence was done by serial incubation of cells with anti-Myc (immunoglobulin G1; 2276; Cell Signaling) and anti-hemagglutinin (sc-805; Santa Cruz), followed by incubation with isotype-specific, fluorochrome (Alexa Fluor 488)-labeled goat anti-mouse (A-11001; Molecular Probes) or goat anti-rabbit immunoglobulin (A-11008; Molecular Probes). Alexa Fluor 568–labeled streptavidin (S-11226; Molecular Probes) was used for the detection of biotinylated c-di-GMP (Molecular Probes). Controls included staining with isotype-matched antibodies (immunoglobulin G1; M5284; Sigma) as described[36]. Nuclei were stained with DAPI (4′,6′-diamidino-2-phenylindole). Double immunofluorescence of D2SC cells and 293T cells was examined by with a Leica-TCS-SP MP inverted single confocal laser-scanning and a two-photon laser microscope (Leica) at the Advanced Microscopy/Spectroscopy Laboratory Macro-Scale Imaging Laboratory, California NanoSystems Institute, University of California at Los Angeles. The ImageJ 1.37c program was used for quantification of colocalization, calculated as a percentage of colocalization of the green channel with the red channel.

Capillary electrophoresis. A P/ACE MDQ capillary electrophoresis system equipped with a diode array detector (Beckman Coulter) was used for all capillary electrophoresis experiments. The fused-silica capillary column (inner diameter, 75 µm; outer diameter, 365 µm; PolymicroTechnologies) used for separation was 50 cm in length with an effective length of 40 cm. Before injection, the capillary was sequentially rinsed at 30 psi with 0.1 M NaOH for 2 min, deionized water for 1 min, and the running buffer (17.5 mM phosphate, pH 7.5) for 6 min. All capillary electrophoresis separations were done at 25 kV at room temperature.

The affinity of c-di-GMP for DDX41 or STING was measured with the affinity capillary electrophoresis mode by the addition of various concentrations of c-di-GMP to the running buffer and injection of the protein. Protein concentrations injected were 0.044 mg/ml for DDX41 and 0.08 mg/ml for STING (amino acids 139–379). The interaction between recombinant proteins and c-di-GMP induced a change in protein electrophoretic mobility, which was plotted against c-di-GMP concentration. By fitting of this curve by the Hill equation dissociation equilibrium constant, the dissociation constant of the c-di-GMP–protein complex was obtained.

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