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

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Abstract

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 involving the STING adaptor, TBK1 kinase and IRF3 transcription factor. Here we identified the helicase DEAD box polypeptide 41 (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 shRNA in murine or human cells inhibited the induction of innate immune genes and resulted in defective STING, TBK1 and IRF3 activation in response to c-di-GMP or c-di-AMP. These results suggest a mechanism whereby c-di-GMP and c-di-AMP are detected by the DDX41 PRR, which complexes with STING to signal to TBK1-IRF3 and activate the interferon response.

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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. performed and analyzed the biochemical experiments. R.M.T. carried out the confocal imaging and analysis. S.O., Y.J. and Z-J.L. performed the cloning, expression and purification of DDX41 and STING CTD. S.S.I. prepared peritoneal macrophages and M.S. prepared PBMCs. S.A.Z. performed and analyzed experiments on primary cells. S.Z. and W.Z. performed and analyzed the ACE binding affinity experiments and K.P. wrote the manuscript.

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The authors declare no competing financial interests.

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

The mechanism by which c-di-GMP and c-di-AMP activate the host type I IFN response remains poorly understood. Indeed, intracellular detection of c-di-GMP or c-di-AMP leads to the activation of the type I IFN response in a manner independent of the cytoplasmic PRR RIG-I (also known as DDX58) or its downstream adaptor IPS-1. Moreover, c-di-GMP or c-di-AMP require neither MyD88 nor TRIF adaptors, suggesting that the Toll-like receptor (TLR) family of PRRs also do not play a role in the detection of c-di-GMP or c-di-AMP[9]. The activation of type I IFNs by c-di-GMP and c-di-AMP was however, shown to require STING (aka MITA, MPYS, ERIS or TMEM173), suggesting that these cyclic dinucleotides are detected via a PRR that signals via STING[10, 11]. In response to certain viral nucleic acids and B-DNA, STING functions as an adaptor protein and has been demonstrated to facilitate downstream signal transmission to IRF3 and NF-κB[12-14]. Here, we provide evidence that the recently identified PRR DDX41 is the primary sensor that directly binds to c-di-GMP or c-di-AMP and can trigger the type I IFN host immune response via STING.

RESULTS

DDX41 mediates c-dinucleotide sensing in murine DCs and human monocytes

We stably knocked down DDX41 via short hairpin RNA (shRNA) in the murine splenic dendritic cell line D2SC (**Fig. 1a**) and examined IFN-β induction in response to c-di-GMP and c-di-AMP. Control cells infected with *L. monocytogenes* displayed a robust IFN-β response, whereas DDX41-shRNA cells showed a marked reduction in the IFN-β response (**Fig. 1b**). Consistent with published data[10, 11], STING-shRNA cells also showed impaired IFN-β induction in response to *L. monocytogenes* (**Fig. 1b**). Cytoplasmic delivery of either c-di-GMP or c-di-AMP via lipofection also yielded strong IFN-β activation which was largely diminished in DDX41-shRNA cells, which paralleled the impairment displayed in STING-shRNA cells (**Fig. 1c,d**). Induction of IFN-β by the synthetic DNA poly (dA:dT) but not by the RNA ligand poly (I:C) was impaired in DDX41-shRNA cells (**Supplementary Fig. 1a,b**), demonstrating the specificity of DDX41 for c-di-GMP, c-di-AMP and B-DNA. Type I IFNs mediate the innate immune response via the IFN-α/β receptor, where receptor ligation leads toward the activation of hundreds of interferon stimulated genes (ISGs)[15]. Both c-di-GMP and c-di-AMP activated the ISG Mx1 in control cells, however, induction of Mx1 was significantly reduced in DDX41-shRNA or STING-shRNA cells (**Fig. 1e,f**). Similarly, c-di-GMP-mediated activation of other ISGs was also impaired in DDX41-

shRNA cells (**Supplementary Fig. 1c-f**). These results indicate that DDX41 plays a critical role in c-dinucleotide-mediated activation of type I IFN and IFN-mediated signaling. Cytosolic detection of bacterial secondary messengers also leads to the activation of NF-κB, a key transcription factor important for the induction of pro-inflammatory cytokines such as interleukin 6 (IL-6) and TNF[16]. We found that IL-6 and TNF expression levels were significantly reduced in DDX41-shRNA cells compared to control-shRNA cells that were treated with c-di-GMP and/or c-di-AMP (**Fig. 1g,h** and **Supplementary Fig. 1g**). To determine whether DDX41 mediates c-di-GMP/c-di-AMP sensing and type I IFN activation in human cells, we stably knocked down DDX41 via shRNA in the human monocyte cell line THP-1 (**Fig. 1i**). Consistent with our findings in murine cells, both c-di-GMP and c-di-AMP induced the production of IFN-β in control–shRNA cells, whereas c-di-GMP or c-di-AMP-mediated production of IFN-β was significantly defective in DDX41- or STING-shRNA THP-1 cells (**Fig. 1j,k**). Induction of ISGs in response to c-di-GMP was also impaired in DDX41-shRNA THP-1 cells (**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 suggest that DDX41 functions in regulating type I IFN and pro-inflammatory gene inductions in response to c-dinucleotides in both murine and human cells.

DDX41-mediates c-dinucleotide sensing in primary cells

The role of DDX41 in facilitating c-di-GMP or c-di-AMP induced activation of type I IFN in primary cells was also examined. BMDCs (mDCs derived from bone marrow with granulocyte-macrophage colony-stimulating factor (GM-CSF)) were prepared and transfected with shRNA targeting DDX41 (**Fig. 2a**). Control-shRNA BMDCs infected with *L. monocytogenes* displayed robust production of IFN-β, while IFN-β induction in DDX41-shRNA BMDCs was markedly reduced (**Fig. 2b**). c-di-GMP and c-di-AMP stimulation also induced the production of IFN-β in control-shRNA BMDCs, while IFN-β production was highly impaired in DDX41-shRNA BMDCs (**Fig. 2c,d**). Similarly, primary thioglycollate-elicited mouse peritoneal macrophages transfected with siRNA targeting DDX41 (**Fig. 2e**) displayed reduced activation of IFN-β in response to *L. monocytogenes* infection or c-di-GMP stimulation compared to control-siRNA transfected cells (**Fig. 2f,g**). Consistently, induction of Mx1 and IL-6 were also impaired in DDX41-siRNA peritoneal macrophages infected with *L. monocytogenes* or treated with c-di-GMP (**Supplementary Fig. 2a-d**).

To determine whether DDX41 plays a role in sensing c-di-GMP in primary human cells, we transfected peripheral blood mononuclear cells (PBMCs) with an siRNA specific for DDX41 (**Fig. 2h**). While control-siRNA PBMCs infected with *L. monocytogenes* or treated with c-di-GMP elicited the activation of IFN-β, DDX41-siRNA PBMCs showed defective IFN-β activation in response to *L. monocytogenes* or c-di-GMP (**Fig. 2i,j**). Similar reductions in IFN-β activation were displayed in DDX41-siRNA PBMCs obtained from two additional human donors (**Supplementary Fig. 2e,f**). These results further indicate a critical role for DDX41 in facilitating type I IFN responses induced by c-di-GMP or c-di-AMP in primary immune cells.

DDX41 is a direct sensor of c-di-GMP

DDX41 is known not only to signal via the STING adaptor to activate type I IFN, but to also function as a PRR that directly binds viral DNA and B-DNA[17]. We therefore investigated whether DDX41 functioned as a direct sensor (PRR) for c-di-GMP or c-di-AMP. Biotin-labeled c-di-GMP or c-di-AMP pulled down ectopically expressed DDX41 from 293T cell lysates (**Fig. 3a** and **Supplementary Fig. 3a**). Immunofluorescence microscopy further revealed that DDX41 and c-di-GMP co-localized upon their co-transfection into D2SC cells (**Fig. 3b**). In addition, we found that GST-purified DDX41 or His-purified DDX41 from *E.coli* directly bound c-di-GMP (**Fig. 3c** and **Supplementary Fig. 3b**). The c-di-GMP interaction with DDX41 was specific as only unlabeled c-di-GMP, c-di-AMP and poly (dA:dT) (B-DNA), but not poly (I:C), could competitively disrupt the c-di-GMP-DDX41 interaction (**Fig. 3d**). Furthermore, the structurally similar molecules GMP and AMP, but not the c-di-GMP and c-di-AMP precursors, GTP or ATP, also disrupted the DDX41-c-di-GMP complex (**Supplementary Fig. 3c**).

To determine whether c-di-GMP binding was specific to the DDX41 helicase, we compared c-di-GMP binding with another DEAD box helicase PRR. c-di-GMP bound to DDX41, but showed no interaction with DDX58 (**Supplementary Fig. 3d**). To define which domain of DDX41 was important for binding c-di-GMP or c-di-AMP, we used a series of deletion mutants of DDX41 (**Fig. 4a,b**) and evaluated their interactions with biotinylated c-di-GMP or c-di-AMP in 293T cells. c-di-GMP and c-di-AMP failed to interact with DDX41 lacking the central DEAD box domain (**Fig. 4c,d**). To determine the physiological relevance of this domain in terms of type I IFN induction, we reconstituted DDX41-shRNA THP-1 cells with either full length DDX41 or the DDX41 deletion mutant lacking the DEAD box domain (**Fig. 4e**). As expected, DDX41-shRNA cells displayed defective IFN-β production in response to c-di-GMP or c-di-AMP compared to control-shRNA cells (**Fig. 4f,g**). However, DDX41-shRNA cells reconstituted with full length DDX41, but not the DDX41 deletion mutant lacking the DEAD box domain, “rescued” the defective IFN-β production in DDX41-shRNA cells in response to c-di-GMP or c-di-AMP stimulation (**Fig. 4f,g**). Thus c-dinucleotide mediated induction of IFN-β requires the central DEAD box domain of DDX41.

DDX41-dependent signaling downstream of c-di-GMP and c-di-AMP

DDX41 interacts and co-localizes with the STING adaptor (**Supplementary Fig. 4a,b**) to facilitate DNA ligand dependent signal transduction[17]. Introduction of either c-di-GMP or c-di-AMP into D2SC cells led to enhanced DDX41-STING complex formation (**Fig. 5a**). In DNA dependent signaling pathways, STING further binds to the downstream kinase TBK1 to activate the type I IFN response[18, 19]. Both c-di-GMP and c-di-AMP when transfected into control D2SC cells activated the formation of a STING-TBK1 complex, however, c-di-GMP and c-di-AMP mediated activation of the STING-TBK1 complex was almost completely abrogated in DDX41-shRNA cells (**Fig. 5b**). Consequently, c-di-GMP and c-di-AMP mediated activation of TBK1, IRF3 and the downstream type I IFN effector, STAT1 was impaired in DDX41-shRNA cells (**Fig. 5c and Supplementary Fig. 5**). Activation of NF-κB was also impaired in DDX41-shRNA cells in response to either c-di-GMP or c-di-AMP (**Fig. 5c**). Together, our findings suggest that DDX41 is a critical PRR for c-di-GMP

and c-di-AMP mediated IFN induction, and that its absence generates a defect in downstream STING-dependent signaling.

c-di-GMP signals to the STING adaptor via DDX41

A recent study found that c-di-GMP could bind to the C-terminal domain of STING (STING CTD) and suggested that the STING adaptor could function as an immune sensor of c-di-GMP[20]. We therefore performed binding assays to determine the affinities of c-di-GMP or c-di-AMP for DDX41 and for STING in parallel. Biotin-labeled c-di-GMP pulled down ectopically expressed DDX41 with greater affinity over ectopically expressed STING from 293T cell lysates (**Fig. 6a**). Physiologically, binding of c-di-GMP with endogenous DDX41 was also greater than the association between c-di-GMP and endogenous STING (**Fig. 6b**). Immunofluorescence imaging further revealed greater co-localization between c-di-GMP and DDX41 in comparison to STING (34.13% vs 6.25%) (**Fig. 6c**). Affinity capillary electrophoresis (ACE) experiments were also performed to examine the binding affinities between c-di-GMP and recombinant DDX41 or recombinant STING CTD. c-di-GMP bound DDX41 with a K_d of ~5.65 μM, whereas c-di-GMP associated with STING CTD with a K_d of ~14.54 μM (**Fig. 6d**). Consistent with these findings, c-di-GMP bound to purified recombinant DDX41 with stronger affinity than purified recombinant STING CTD in pulldown binding assays (**Fig. 6e**). We therefore hypothesized that DDX41 is the major primary sensor of c-di-GMP and c-di-AMP, operating upstream of STING, and upon binding these PAMPs, yields enhanced complex formation with STING to facilitate downstream signaling and type I IFN activation (**Supplementary Fig. 6**). Accordingly, c-di-GMP was significantly impaired in its capacity to associate with ectopically expressed STING in 293T cells transfected with siRNA targeting DDX41 (**Fig. 7a**). Consistently, co-localization between c-di-GMP and STING was largely reduced in DDX41-shRNA cells, whereas c-di-GMP-DDX41 interactions remained intact in STING-shRNA cells (**Fig. 7b,c**). Taken together, our findings indicate that DDX41 is the primary PRR for c-di-GMP and c-di-AMP, which signals via STING for type I IFN induction.

DISCUSSION

Many bacterial pathogens including Staphylococcus, Streptococcus, Pseudomonas, Yersiniae, Listeria and *Mycobacterial* species employ key secondary messengers including c-di-GMP or c-di-AMP that play essential modulatory roles in bacteria[4, 7]. Although several substrates and effectors have been identified for these cyclic dinucleotide monophosphate species within the bacterial cell, our understanding of these bacteria-specific secondary messengers on the innate immune response within the mammalian host cell is just beginning to emerge. c-di-GMP and c-di-AMP activate the host type I IFN response in a manner dependent on the STING adaptor[10, 11] and our findings indicate that the DNA sensor helicase DDX41 functions as a direct PRR for these cyclic dinucleotides in both murine and human cells. Our results showed that unlabeled c-di-GMP or c-di-AMP could disrupt the DDX41-c-di-GMP interaction. We found that GMP and AMP, but not the bulkier GTP or ATP molecules, could also competitively disrupt the DDX41-c-di-GMP complex. Although two molecules of GMP or AMP are structurally similar to c-di-GMP or c-di-AMP, respectively, they are not known to function as PAMPs in mammalian cells[9]. It will

therefore be of further interest to determine how these species play a modulatory role in the type I IFN response. Our competition experiments additionally revealed that B-DNA could disrupt 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 revealed by co-crystallization studies and point mutation analysis will be the subject of future investigation.

Our results additionally indicated that c-di-GMP and c-di-AMP mediated activation of innate signaling and type I IFN induction were similarly defective between cells in which DDX41 or STING had been knocked down , suggesting DDX41 and STING share a common signaling pathway. STING-deficient cells displayed a very modest defect in NF-κB activation 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 (also known as p204) was also shown to facilitate some viral DNA triggered signaling via the STING adaptor[21, 22]. Although a role for IFI16 cannot be ruled out in the c-di-GMP and c-di-AMP signaling pathway, it is however unlikely that IFI16 functions as a primary sensor for these molecules since its' basal expression is low and is rather induced in a type I IFN-dependent manner. DDX41 expression on the other hand, is greater at the basal state and is not modulated by type I IFNs[17].

Our data suggests that DDX41 serves as the PRR for c-di-GMP and c-di-AMP, which upon receptor binding signals to TBK1-IRF3 via the STING adaptor. Lending further credibility as a scaffolding molecule, STING was recently 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, with lower affinity than DDX41. Although the physiological relevance of this interaction requires further investigation, our data shows that c-di-GMP interaction with STING is significantly enhanced in the presence of DDX41 in cells. The solved structure of the C-terminal domain of STING in complex with c-di-GMP revealed that one molecule of c-di-GMP binds one dimer of STING[24-28]. We propose that c-di-GMP detection and binding to DDX41 promotes enhanced DDX41-STING interactions leading to an increase in binding affinity of STING toward c-di-GMP, ultimately driving downstream signaling events. Thus, STING may function as a secondary receptor or co-factor in the cyclic dinucleotide signaling pathway.

The significance of type I IFN induction in the context of anti-bacterial innate immunity is currently unclear and somewhat controversial, particularly due to conflicting reports on whether type I IFN either functions to support or inhibit bacterial growth[29-32]. It will therefore be of interest to further study how different bacteria and host cells use secondary messengers and DDX41 as virulence factors and innate immune receptors, respectively, in their battle of infection and immunity[33-35]. As such, cyclic dinucleotide species and DDX41 represent new targets such that modulation of their interaction during certain bacterial infections can alter the host immune response in a manner to suppress bacterial replication and spread.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/natureimmunology/.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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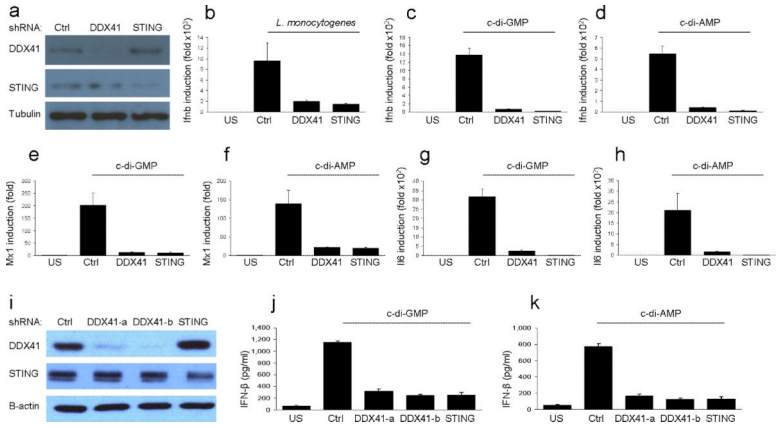

Figure 1.

c-di-GMP and c-di-AMP mediated induction of the innate immune response in murine DCs and human monocytes requires DDX41. (**a**) Immunoblot analysis of DDX41 and STING in D2SC mDCs transfected with non-targeting scrambled shRNA, or shRNAs targeting DDX41 or STING. (**b-d**) Expression of IFN-β mRNA measured via qPCR in control-shRNA D2SC mDCs left unstimulated (US) or in control-shRNA, DDX41-shRNA or STING-shRNA mDCs stimulated with *L. monocytogenes* (b), c-di-GMP (c) and c-di-AMP (d) for 6 h. (**e-h**) Expression of Mx1 mRNA (**e,f**) or IL-6 mRNA (**g,h**) measured by qPCR in D2SC cells treated as in **c-d** and stimulated with c-di-GMP (**e,g**) and c-di-AMP (**f,h**). (**i**) Immunoblot analysis of DDX41and STING in THP-1 monocytes treated with non-targeting scrambled shRNA control, or shRNAs targeting either DDX41 (two shRNAs: DDX41-a and DDX41-b) or STING. (**j,k**) ELISA of IFN-β cytokine production in control-shRNA, DDX41-shRNA or STING-shRNA THP-1 monocytes 16 h after stimulation with c-di-GMP (j) or c-di-AMP (k). Error bars indicate standard error. Data are representative of at least three independent experiments.

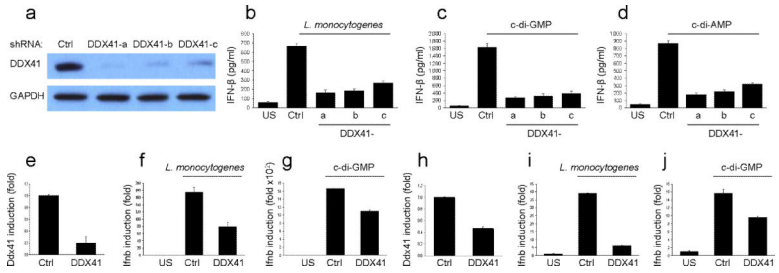

Figure 2.

Cyclic dinucleotides activate IFN via DDX41 in primary cells. (**a**) Immunoblot analysis of DDX41 in primary mouse BMDCs treated with non-targeting scrambled shRNA, or shRNAs targeting DDX41 (three shRNAs: DDX41-a, DDX41-b and DDX41-c). (**b-d**) ELISA of IFN-β cytokine production in control-shRNA or DDX41-shRNA BMDCs treated with *L. monocytogenes* (b), c-di-GMP (c) or c-di-AMP (d) for 16 h. (**e-g**) Quantification of DDX41 mRNA (**e**) or IFN-β mRNA induction (**f,g**) by qPCR in primary mouse peritoneal macrophages transfected with either control siRNA or siRNA targeting DDX41 (e), then stimulated with *L. monocytogenes* (f) or c-di-GMP (g) for 6 h. (**h-j**) Quantification of DDX41 mRNA (**h**) and IFN-β mRNA induction (**i,j**) via qPCR performed as in **e-g** using primary human peripheral blood monocytes electroporated with either control siRNA or siRNA targeting DDX41 (h), then stimulated with *L. monocytogenes* (i) or c-di-GMP (j) for 6 h.. Error bars indicate standard error. Data are representative of at least two independent experiments.

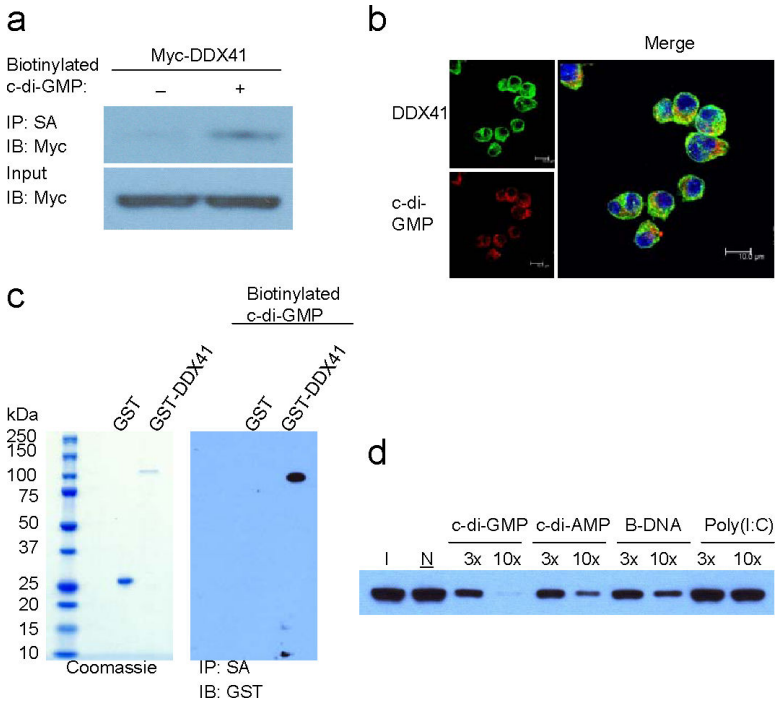

Figure 3.

DDX41 is a direct sensor of c-di-GMP. (**a**) Pulldown and immunoblot analysis of biotinylated c-di-GMP interactions with Myc-DDX41 from 293T cell lyasates. (**b**) Confocal imaging of c-di-GMP-DDX41 colocalization in D2SC cells co-transfected with Myc-DDX41 and biotinylated c-di-GMP. (**c**) Pulldown and immunoblot analysis between biotinylated c-di-GMP and GST-DDX41. (**d**) Pulldown and immunoblot analysis of biotinylated c-di-GMP (2.0 μM) interactions with GST-DDX41 alone or with increasing amounts of unlabeled ligands as indicated, (3× = 6.0 μM, 10× = 20 μM). Immunoanalysis was performed as in **c**. I, input GST-DDX41; N, no competitor. Data are representative of at least two independent experiments.

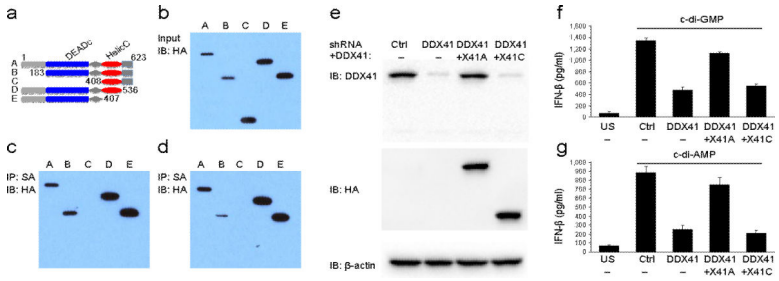

Figure 4.

DDX41 DEAD box domain is required for c-di-GMP and c-di-AMP mediated induction of IFN-β. (**a**) Schematic of deletion constructs of DDX41. (**b**) Input immunoblot of HA-tagged DDX41 deletion mutants used in co-immunoprecipitation experiments (for **c** and **d**). (**c,d**) Co-immunoprecipitation and immunoblot analysis of biotinylated c-di-GMP (**c**) or biotinylated c-di-AMP (**d**) incubated with lysates from 293T cells transfected with HA-DDX41-full length or deletion constructs labeled A-E as shown. (**e**) Immunoblot of THP-1 monocytes treated with control shRNA or shRNA targeting the 3'UTR of DDX41, then transfected with HA-DDX41 full length (X41A) or HA-DDX41 lacking the DEAD box domain (X41C). (**f,g**) ELISA for IFN-β cytokine production from THP-1 cells treated with control shRNA or shRNA targeting DDX41 that were reconstituted as in **e** following treatment with c-di-GMP (f) or c-di-AMP (g) for 16h. Error bars indicate standard error. Data are representative of at least two independent experiments.

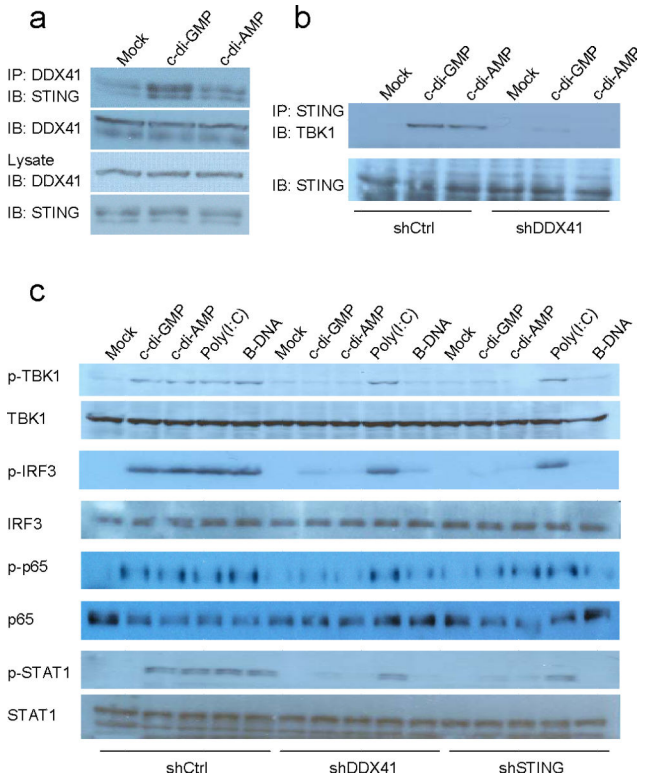

Figure 5.

c-di-GMP and c-di-AMP require DDX41 for STING dependent signaling. (**a**) Immunoprecipitation and immunoblot analysis of DDX41-STING interactions is D2SC cells transfected with c-di-GMP or c-di-AMP for 4 h. (**b)** Immunoprecipitation and immunoblot analysis of STING-TBK1 interactions in control-shRNA or DDX41-shRNA D2SC cells transfected as in **a**. (**c**) Immunoblot analysis of TBK1, IRF3, p65 and STAT1 phosphorylations in control-shRNA, DDX41-shRNA or STING-shRNA D2SC mDCs transfected with c-di-GMP, c-di-AMP, Poly (I:C) or B-DNA for 4 h. Data are representative of at least two independent experiments.

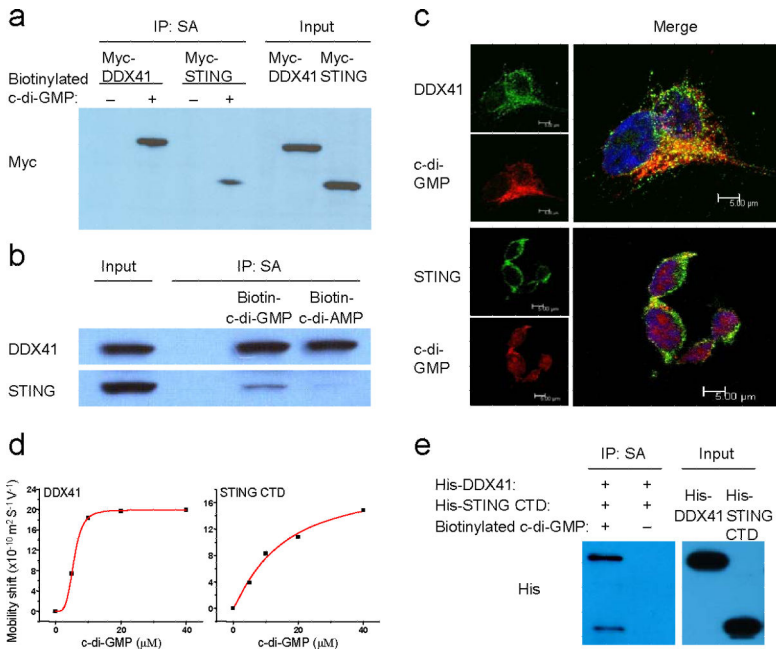

Figure 6.

c-di-GMP binds DDX41with a greater affinity than STING (**a)** Pulldown and immunoblot analysis of biotinylated c-di-GMP interactions with Myc-DDX41 or Myc-STING from 293T cell lysates. (**b**) Pulldown and immunoblot analysis of biotinylated c-di-GMP or biotinylated c-di-AMP interactions with endogenous DDX41 and STING from D2SC cell lysates. (**c**) Confocal microscopy indicating c-di-GMP interactions with DDX41 or STING in 293T cells co-transfected with Myc-DDX41 and biotinylated c-di-GMP (top) or Myc-STING and biotinylated c-di-GMP (bottom). (**d**) Hill plots of Affinity Capillary Electrophoresis analysis showing binding affinities between recombinant DDX41 and c-di-GMP (left panel) or recombinant STING CTD (139-379) and c-di-GMP (right panel). DDX41-c-di-GMP K_d =5.65 μM, R^2 = 0.99992. STING CTD-c-di-GMP K_d = 14.54 μM, R^2 = 0.98342. K_d, dissociation constant. (**e**) Pulldown and immunoblot analysis of biotinylated c-di-GMP interactions with bacterially purified DDX41 and STING CTD. Data are representative of at least two independent experiments.

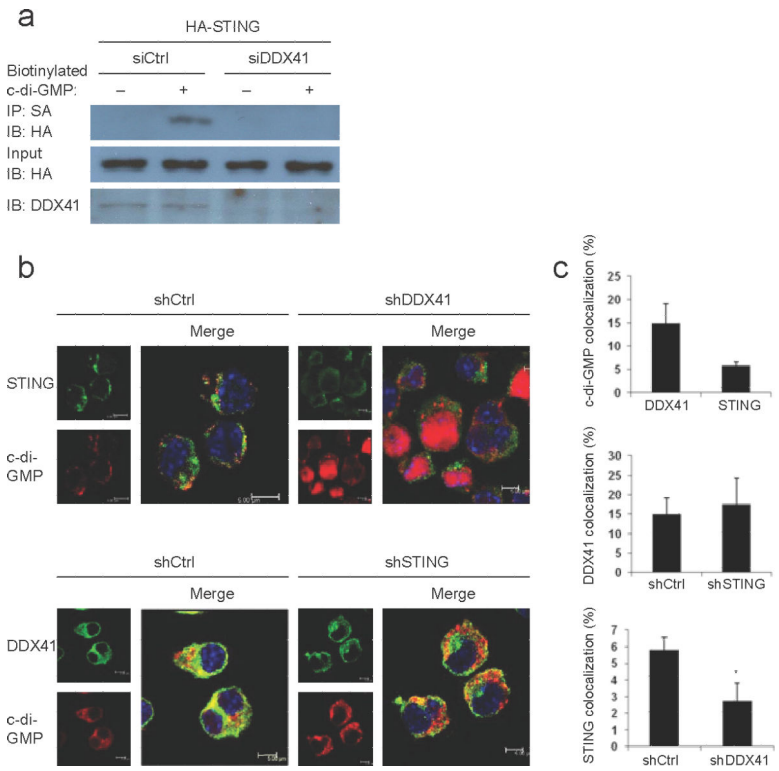

Figure 7.

DDX41 is required for c-di-GMP downstream association with STING. **(a)** Pulldown and immunoblot analysis of biotinylated c-di-GMP interactions with HA-STING from lysates of 293T cells transfected with control siRNA or siRNA targeting DDX41. (**b**) Confocal analysis showing c-di-GMP colocalizations with DDX41 or STING in control-shRNA or DDX41-shRNA (upper right panel) and STING-shRNA (lower right panel) D2SC mDCs co-transfected with biotinylated c-di-GMP and Myc-STING (upper panels) or biotinylated c-di-GMP and Myc-DDX41 (lower panels). (**c**) Quantification of c-di-GMP colocalizations with DDX41 or STING in control-shRNA (upper panel), c-di-GMP colocalization with DDX41 in STING-shRNA (middle panel) or c-di-GMP colocalization with STING in DDX41-shRNA (lower panel) D2SC cells from **b**. Error bars indicate standard error. Data are representative of at least two independent experiments.