Innate sensing of bacterial cyclic dinucleotides: more than just STING

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Bacterial cyclic dinucleotides are recognised by the innate immune system, leading to induction of type I interferons. The mammalian helicase DDX41 directly binds cyclic dinucleotides and mediates the signaling pathway to type I interferon induction.

The detection of viral and bacterial nucleic acids by the innate immune system, leading to the induction of type I interferons (IFN- α and IFN- β) is a current area of intense research. The interest is not only in determining mechanisms whereby pathogens are sensed, but also in understanding how inappropriate activation of such mechanisms by self nucleic acids can lead to autoimmunity. Host pattern recognition receptors (PRRs) that detect pathogen RNA and DNA on the cell surface, in endosomes and within the cytosol and nucleus have been proposed and identified, and in some cases, such as the Toll-like receptors (TLRs), characterised extensively. However, it has recently become apparent that small bacterial nucleic acids called cyclic dinucleotides (CDNs) are also capable of evoking an innate immune response when present in the cytosol. In a study published in this issue of *Nature Immunology*, Liu *et al.* show that the cellular DEAD-Box helicase DDX41 binds bacterial CDNs

and is responsible for activating the signaling pathway leading to type I IFN induction¹.

Bacterial CDNs, such as cyclic-di-AMP and cyclic-di-GMP, are second messengers that have a regulatory role in normal bacterial physiology. For example cyclic-di-GMP regulates motility and expression of virulence genes². Such CDNs are not thought to be produced by mammalian cells, and should only appear in the cytosol due to the presence of invading bacteria. Thus they are candidate vita-PAMPs, the recognition of which signifies the presence of live microbes to the innate immune system³. Indeed, CDNs from intracellular bacteria such as *Listeria* and *Pseudomonas* stimulate an innate immune response particularly characterised by type I IFN induction, and to do so via the STING-TBK1-IRF3 cytosolic signaling axis which is also known to be utilised for innate immune sensing of cytosolic DNA⁴⁻⁶. Here, the authors demonstrate that the helicase DDX41 operates 'upstream' of STING-TBK1-IRF3 to sense CDNs and trigger the STING-dependent pathway (**Fig. 1**)¹.

Very little was known about the function of DDX41 until recently, when this helicase was shown to sense cytosolic dsDNA, such as that from DNA viruses, leading to activation of the STING-TBK-IRF3 pathway⁷. At that time, DDX41 joined a growing list of helicase proteins implicated in innate immune sensing of RNA (e.g. RIG-I, MDA5 and DDX60) and DNA (e.g. DHX36 and DHX9)⁸. However, the current paper is the first demonstration of a role for a helicase in detecting CDNs¹. Such role was revealed when knockdown of DDX41 expression by shRNA in both murine and human cell lines blocked CDN-stimulated induction of IFN- β and other innate immune genes such as TNF¹. Further, knockdown of DDX41 also inhibited CDN- and *Listeria*-dependent gene induction in primary murine bone marrow derived dendritic cells, peritoneal macrophages and human peripheral blood mononuclear

cells¹. Given that nucleic acid sensing pathways are often cell-type specific, it is quite compelling that DDX41 was required for CDN responses in such a range of human and murine cells.

The authors also provide evidence that, as in the case of dsDNA⁷, DDX41 can also directly bind to CDNs: CDNs could pull DDX41 out of cell lysates and also colocalised with DDX41 in intact cells¹. Importantly, recombinant DDX41 protein directly bound both cyclic-di-GMP and cyclic-di-AMP, and not structurally similar molecules, *in vitro*¹. DEAD-box helicases such as DDX41 have two conserved domains, namely a DEAD domain and a helicase domain, and both domains cooperate to fulfil the key functions of helicases in binding nucleic acid (normally RNA or DNA) and hydrolysing ATP⁹. Here, binding of CDNs by DDX41 required the DEAD domain, but not the helicase domain¹.

It is still not entirely clear how DDX41 signals after engaging CDNs, but in murine cells transfected with CDNs, DDX41 and STING co-immunoprecipitated in a ligand-dependent manner¹. Further, a CDN-dependent STING-TBK1 interaction was blocked by DDX41 shRNA. Also, either STING or DDX41 shRNA inhibited CDN-stimulated TBK1 and IRF3 activation¹. These data place DDX41 ‘upstream’ of STING in the CDN sensing pathway, although whether DDX41 directly binds to STING remains to be determined.

Previous studies have suggested that STING itself is the cytosolic receptor for CDNs. Thus the idea was that in contrast to dsDNA, no further ‘upstream’ sensor is required for CDNs to activate the STING-TBK1-IRF3 axis⁵. Several recent papers described the structure of one molecule of c-di-GMP bound to a dimer of the carboxyl terminal domain of human STING¹⁰. In order to compare the relative importance of DDX41 and STING for cyclic dinucleotide sensing the authors compared the

affinities of cyclic-di-GMP and cyclic-di-AMP for both proteins. Data obtained from cell lysates, intact cells and *in vitro* binding assays with recombinant proteins all suggested that DDX41 has a higher affinity for CDNs than STING¹. Furthermore, DDX41 shRNA, strongly inhibited the ability of STING in cell lysates to associate with cyclic-di-GMP, and STING-cyclic-di-GMP colocalisation in intact cells¹. In contrast, reducing STING expression did not affect DDX41-cyclic-di-GMP colocalisation¹. Thus the authors propose DDX41 as the primary sensor of CDNs, and also that DDX41 is required for STING to bind CDNs in intact cells. This would suggest that STING might be a secondary receptor or co-factor for CDN sensing. It is also possible that the DDX41 pathway may require CDNs to bind to STING for full activation of the DDX41-dependent response to CDNs.

The work raises other interesting questions relating to the mechanism whereby CDNs activate the STING-TBK1-IRF3 axis, as it is currently unclear how CDNs, either bound to DDX41 or directly to STING, stimulate STING to activate TBK1. One hallmark of STING activation after dsDNA or CDN stimulation is its movement from the endoplasmic reticulum and possibly the mitochondria to perinuclear punctate structures that remain ill defined. This movement of STING is required for recruitment of TBK1 to STING and downstream signalling (**Fig. 1**). Previous work using *in vitro* reconstitution of the STING-TBK1-IRF3 axis has shown that the C-terminal tail of STING acts as a scaffold that binds both TBK1 and IRF3 in order to direct TBK1 to phosphorylate and activate IRF3¹¹. Therefore engagement of CDNs with DDX41 and STING would be expected to trigger STING movement to perinuclear structures, and also cause a conformational change in STING, which would expose the C-terminal tail to allow TBK1 recruitment. However such a

mechanism has not yet been clarified, although the authors do show here that CDNs stimulate recruitment of TBK1 to a STING-containing complex in cells.

In the wider context, the exact mechanism whereby DEAD-box proteins function as cytosolic PRRs for nucleic acids remains a fascinating enigma. Unlike other PRRs such as TLRs and RIG-I-like receptors, DDX41 does not yet have a clearly defined signaling domain that is distinct from its nucleic acid binding domain making it difficult currently to explain how dsDNA⁷ or CDN¹ binding to the DEAD domain would trigger recruitment of signaling adaptors (or STING directly) and hence stimulate downstream signaling. Previously, the DEAD domain itself was shown to be required for DDX41 to bind to STING after dsDNA stimulation, so this may also be the case for the CDN pathway⁷. This raises the possibility that other DEAD-Box proteins might also sense CDNs and act via STING, possibly in different cell types, since the DEAD domain is quite conserved in the DDX protein family.

Further structural studies will be required in order to understand how DDX41 engages with DNA and CDNs, and whether the interaction with STING is direct or whether other signaling proteins are involved. It will be of particular interest in the future to test DDX41-deficient mice in order to determine whether DDX41 is essential or redundant with other nucleic acid sensors in mice *in vivo*, notwithstanding the fact that nucleic acid sensing pathways in mice and humans are likely to be somewhat distinct. Given its role in DNA sensing, it might be expected that similar to other nucleic acid PRRs, DDX41 may have a role in mediating autoimmunity in certain contexts. Finally, CDNs, acting via DDX41 and STING, may prove to be useful vaccine adjuvants. Thus we can expect continued intense interest in elucidating the relative roles of DDX41 and STING, and their exact mechanism of action, in nucleic acid sensing.

Figure 1. Mechanism whereby DDX41 senses bacterial cyclic dinucleotides and dsDNA to mediate type I IFN induction. The presence of dsDNA and the cyclic dinucleotides (CDNs) cyclic-di-GMP and cyclic-di-AMP indicate the presence of invading bacteria to the cellular innate immune response. These are sensed by DDX41, via binding of the dsDNA or CDNs to the DEADc domain. Ligand binding to DDX41 then causes association of DDX41 with a STING dimer at the endoplasmic reticulum, and subsequent movement of STING to perinuclear punctate structures, via a currently ill-defined mechanism. This STING movement is necessary to recruit and activate TBK1 to phosphorylate IRF3, causing transcription factor dimerization and induction of type I interferons.

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