Recognition of Cytosolic DNA by cGAS and other STING-dependent sensors

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Abstract

The presence of DNA in the cytoplasm of mammalian cells is perceived as a danger signal, alerting the host to the presence of microbial infection. In response to the detection of cytoplasmic DNA, the immune system mounts a programmed response that involves the transcription of anti-viral genes such as type I interferons and production of inflammatory cytokines such as interleukin (IL)-1β. The recent discovery of the cGAS-cGAMP second messenger pathway as well as IFI16 and additional sensors collectively provide critical insights into the molecular basis behind the sensing of cytoplasmic DNA. The insights obtained from these important discoveries could unveil new avenues to understand host-immunity, improve vaccine adjuvancy and allow development of new treatments for inflammatory diseases associated with abberant sensing of DNA.

Introduction

Germline-encoded pattern recognition receptors are required for the generation of an efficacious host response to microbial infection [1–3]. These receptors monitor extracellular, endosomal and intracellular compartments for signs of infection. Molecular signatures characteristic of microbial infection (e.g. LPS) or those released from abnormal, damaged, or dying cells (e.g. ATP) engage distinct and overlapping sensors in these compartments [4–6]. Once pathogen-derived signals are detected, a rapid, relatively generic, innate immune response ensues, leading to the production of pro-inflammatory cytokines, type I interferons (IFNs) and chemokines. These events allow the host to curb growth and spread of infectious agents and clear them by activating adaptive immunity [1, 2].

Nucleic acids have been shown to be particularly potent molecular triggers of the innate immune response [7–9]. Microbe-derived nucleic acids commonly find their way into sub-cellular compartments of immune cells during infection [9, 10]. Immune cells are equipped with a plethora of nucleic acid receptors, each specific for a particular polynucleotide species and a specific expression pattern within cellular compartments. Examples of these receptors include RIG-I-like receptors (RLRs) such as RIG-I and MDA-5, which detect 5′

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triphosphate RNA and dsRNA, respectively, in the cytosol; and Toll-like receptors (TLR) 3 (dsRNA sensitive); TLRs 7 and 8 (ssRNA sensitive); and TLR9 (CpG DNA sensitive) located in the endosomal compartment [7]. Signaling pathways of RNA sensing by TLRs and cytosolic RLRs have been studied extensively and reviewed recently in great detail [7, 11]. An area that has received particular focus in recent years is DNA sensing. Sensors of DNA include TLR9, which recognize unmethylated CpG in endosomes, as well as a number of more recently defined sensors including AIM2, IFI16, DDX41 and cGAS [10, 12]. Detection of cytosolic DNA results in two major types of pro-inflammatory responses. In one of these pathways, Absent in Melanoma-2 (AIM2) binds microbial DNA and recruits the adaptor protein ASC, facilitating the formation of a complex called the inflammasome [13–16]. This, in turn leads to activation of caspase-1, that subsequently mediates maturation of the pro-inflammatory cytokines IL-1β and IL-18. AIM2 is one of four proteins which constitute the PYHIN (PYD and HIN domain) containing proteins [17]. While the inflammasome is important in host-defense, the crucial response in nucleic acid sensing and antiviral immunity involves the transcriptional activation of type I IFN and other pro-inflammatory cytokine genes [12]. This activates phagocytic cells, such as macrophages and dendritic cells, and NK cells which destroy infected cells and reduce viral loads, thus bringing about the initial control of infection. In addition, type I IFNs induce the transcription of scores of interferon-stimulated genes (ISGs), whose products establish a general antiviral state by amplifying IFN responses and inhibiting viral replication [18, 19].

Understanding how DNA elicits the type I IFN response is important, since a range of pathogenic organisms appear to be detected by this pathway [12]. In addition, the DNA-sensing pathway is also important in DNA vaccination. Evidence from mouse studies in particular indicate that the adjuvancy of DNA vaccines rely on engagement of these mechanisms [20, 21]. Finally, a better understanding of these pathways has direct relevance for inflammatory disease. It has become clear over the past few years that host DNA present in the cytosol can also trigger an immune response, leading to debilitating inflammatory diseases, such as Aicardi-Goutieres syndrome (AGS), systemic lupus erythematosis (SLE) and other lupus-like diseases [3, 22]. In this review, we discuss recent progress in uncovering the mechanisms of DNA sensing in the cytosol with special emphasis on the role of cytosolic DNA receptors and associated signaling pathways resulting in type I IFN responses. We attempt to explore the importance of newly identified receptors all of which converge on a common adapter molecule called STING.

DNA sensing in the cytosol

The molecular basis of DNA sensing has been the focus of intense investigation for several years. Early studies showed that cells recognize DNA in the cytosol by TLR9-independent means, which ignited rapid and exciting progress in the field [23, 24]. DNA of microbial or host origin can gain access to the cytosol through different modes. During viral infection, genetic material from viral genomes or viral replication intermediates can access cytosolic compartments. Some bacterial pathogens such as *Francisella tularensis* replicate within the cytosol while others such as *Mycobacterium tuberculosis* introduce immunostimulatory DNA into the host cytosol through its type VII secretion system [25, 26]. These responses are not unique to viruses and bacteria, since protozoan DNA, too, has been shown to gain

entry into the cytosol, as demonstrated in *Plasmodium falciparum* infections [27]. Failure to generate the requisite type I IFN can result in pervasive DNA virus infection, for example, the immune system fails to curb HSV-1 infection if these pathways are not engaged effectively [28–30].

The principal pathway of type I IFN induction is through activation of the stimulator of interferon gene (STING). STING, also known as TMEM173, MPYS, MITA and ERIS, is localized to the endoplasmic reticulum [31–33]. STING contains four transmembrane helices and a globular carboxy-terminal domain (CTD), which facilitates interaction of STING with the Inhibitor of kappa B kinase (IKK)-related kinase, called TANK binding kinase 1 (TBK1). TBK1 in turn phosphorylates and activates IRF3, an important transducer of IFN gene transcription [34]. Studies in mice lacking STING have revealed the importance of this protein both in recognition of cytosolic dsDNA and in controlling susceptibility to herpesvirus infections [28, 33]. In addition to its role as an adaptor in the DNA-sensing pathway, STING has also been described as a direct sensor of cyclic di-guanylate monophosphate (c-di-GMP) and cyclic-di-adenylate monophosphate (c-di-AMP) [35]. These cyclic dinucleotides regulate bacterial motility and biofilm formation. STING binds these small molecules through its CTD, leading to activation of TBK1 and IRF-3, which in turn results in transcription of IFN-β and associated genes [34]. STING is also subject to negative regulation by the E3 ligase, RING-finger protein 5 (RNF5). RNF5 ubiquitinates and degrades STING upon activation of antiviral response through STING, thus preventing excessive STING-mediated cytokine response [36]. STING has also been shown to coordinate activation of STAT6, which drives transcription of certain ISGs independently of IFN-β, which are important for antiviral immunity [37].

Although STING has been suggested to bind DNA, the prevailing paradigm of how the IFN response is initiated indicates that one or more upstream DNA-binding proteins couple DNA recognition to STING signaling [38]. Several cytosolic DNA-binding proteins have been identified and in a number of cases have been shown to function upstream of STING. These molecules are depicted in Figure 1 and include DNA-dependent activator of IRFs (DAI), DDX41, IFI16 and cGAS. DAI (also known as ZBP1), binds Z-form DNA and induces STING-mediated IFN-β response [39]. However, DAI was found to be dispensable for IFN-β induction, as the IFN-β response to cytosolic DNA was normal in in vitro studies in hematopoietic cell lines with DAI expression knocked down, and in in vivo studies using DAI-deficient mice, although the response was DAI-dependent in fibroblasts of human and murine origin [21, 39–41]. These data either imply DAI does not play a role in vivo and in cells of hematopoietic origin, or that redundant mechanisms come into play.

The AIM2-like receptor, IFI16 and its mouse ortholog p204

Following the discovery of AIM2 and elucidation of its role as a DNA-binding receptor for inflammasome signaling, the related PYHIN proteins IFI16 (human) and p204 (murine) [17], which share a common domain organization, were both linked to DNA-induced IFN responses [14, 15, 42]. IFI16 is primarily a nuclear localized protein, previously linked to p53-mediated apoptosis and DNA damage signaling [43, 44]. IFI16 was identified in an affinity purification screen for cytosolic DNA binding proteins in THP-1 cells [42]. As

depicted in Figure 1, IFI16 binds dsDNA, and studies in monocytic cells indicated that a pool of IFI16 localized in the cytosol co-localizes with dsDNA during HSV-1 infection. Structural studies confirmed that both IFI16 and AIM2 HIN domains bind the sugar-phosphate backbone of dsDNA through electrostatic interactions [45]. Whether STING binds IFI16 directly is currently unknown. Unlike AIM2, which engages ASC via PYD-PYD domain interactions, IFI16 was shown to form a complex with STING in monocytes and coordinate type I IFN responses in various cell types [29, 42, 46].

The murine PYHIN family comprises 13 family members [17]. One of these, p204 has also been linked to DNA-induced type I IFN responses [42]. The importance of IFI16 in mediating anti-viral responses to dsDNA is underscored by recent evidence indicating that viruses such as HSV1 encode proteins that target IFI16 for proteasomal degradation [47]. Nuclear IFI16 has been linked to recognition of HSV1 DNA during productive infection of human foreskin fibroblasts, leading to a type I IFN response. In this model, nuclear localized IFI16 detects nuclear HSV dsDNA and subsequently triggers a cytosolic STING pathway. Studies using p204 siRNA in mice have also linked p204 to control of HSV1 infection in an ocular model of HSV keratitis [29]. A broader analysis of the murine and human PYHIN proteins revealed that multiple members of this protein family can engage STING or ASC dependent pathways [48]. These findings further support a role for this family of PYHIN proteins as activators/regulators of the antiviral response.

DExD/H helicase DDX41

In addition to DAI and IFI16, DDX41, a DExD/H box helicase, was shown to induce IFN-β response upon stimulation with poly(dA:dT), HSV-1, *Listeria monocytogenes*, and adenovirus in macrophages as well as myeloid dendritic cell lines [49, 50]. Zhang et al. identified DDX41 by screening all DEAD box helicases for their role in this pathway [49]. Knockdown of DDX41 resulted in a profound decrease in IFN-β induction in mDCs upon stimulation by dsDNA ligands. This IFN-β induction by DDX41 was shown to occur via STING-mediated phosphorylation of TBK1 and IRF3. In another study, DDX41 was also found to bind to and control the IFN response to cyclic-dinucleotides (CDNs) such as cyclic-di-AMP and cyclic-di-GMP [51]. Biochemical studies indicate that DDX41 associates with STING through its DEADc domain [49]. Further evidence supporting a role for DDX41 in DNA sensing came from studies of TRIM21. TRIM21 is an E3 ligase that is induced in cells by IFN-β. TRIM21 ubiquitinates and subsequently degrades DDX41, providing the STING-mediated cytosolic surveillance pathway with a negative feedback mechanism, as shown in Figure 1 [52, 53].

OAS1-like nucleotidyl transferase, cGAS

Seminal recent studies identified a cyclic di-nucleotide called cyclic GMP-AMP in cells exposed to dsDNA and DNA viruses [54]. Using elegant biochemical purification and reconstitution strategies, the Chen lab identified this second messenger in the cytoplasm of cells exposed to DNA [54]. cGAMP is structurally similar to c-di-GMP and c-di-AMP. As with these c-di nucleotides, cGAMP binds STING and turns on TBK1-IRF3 signaling leading to type I IFN gene transcription. In addition to defining this second messenger, in a biochemical tour de force, the authors also used complementary protein purification

strategies combined with quantitative mass spectrometry to identify the enzyme responsible for cGAMP generation. Their studies identified an uncharacterized mouse gene with significant structural homology to the catalytic domain of human oligoadenylate synthase (OAS1), a nucleotidyl transferase that is part of a larger family of NTases that include adenylate cyclase [55]. The authors named this enzyme cGMP-AMP synthase "cGAS". cGAS binds DNA and catalyzes the synthesis of cGAMP from ATP and GTP via the mechanism outlined in Figure 2. The identification of cGAMP has reconciled prior observations that indicated STING could function both as an adapter for DNA sensing and a receptor for cyclic dinucleotides [35, 56].

cGAS contains a nucleotidyl transferase (NTase) domain that partially overlaps with a C-terminal male abnormal 21 (Mab21) domain, as depicted in Figure 2. Recent structural insights into the enzyme have expanded our understanding of this unique second messenger system. Structures of the cGAS NTase and Mab21 domains reveal a bilobal scaffold, with an N-terminal NTase catalytic core that adopts a mixed α/β fold, and a C-terminal α-helical lobe. This bears significant structural similarity to 2′-5′-oligoadenylate synthase (OAS), with the exception of a unique "zinc thumb," located between the two lobes of cGAS, that is essential for DNA binding. DNA binding to cGAS brings about a conformational change in the NTase domain of cGAS making it accessible to ATP and GTP in the cytosol, which are then utilized to generate cGAMP [54].

A series of follow-up biophysical and biochemical studies have since identified unique features of the endogenous cGAMP (generated by cGAS) that distinguish it from cGAMP and other c-di-nucleotides of microbial origin [54, 56–58]. The cGAMP produced by cGAS contains mixed phosphodiester linkages. A phosphodiester linkage between 2′-OH of GMP and 5′-phosphate of AMP and another between 3′-OH of AMP and 5′-phosphate of GMP constitutes endogenous or non-canonical cGAMP. This non-canonical molecule binds to STING with higher affinity than microbial cGAMPs or other cyclic di-nucleotides and elicits potent IFN-β responses. In further studies, Chen and colleagues generated cGAS-deficient mice [30]. Cells from cGAS-deficient (cGas$^{-/-}$) mice, including fibroblasts, macrophages, and dendritic cells are compromised in their ability to produce type I interferons and other cytokines in response to DNA transfection or DNA virus infection [30]. cGas$^{-/-}$ mice were also shown to be more susceptible to lethal infection with herpes simplex virus 1 (HSV1) than wild-type mice. Finally, a follow-up study by the same group also implicated cGAS as a sensor for retroviruses including HIV, murine leukemia virus (MLV) and SIV [59]. cGAS-synthesized cGAMP can also be transferred from producing cells to neighboring cells through gap junctions, where it promotes STING activation and antiviral immunity independently of type I IFN signaling [60].

Significance of the STING-activating cytosolic DNA sensors

The discovery of the cGAS-cGAMP pathway and the recent structural, biophysical and genetic advances made in understanding how this second messenger system operates have profoundly increased our understanding of the mechanisms of DNA sensing and host responses to DNA viruses. It is likely that these discoveries will pave the way for future insights related to DNA-driven immune responses. As with all innate immune pathways, a

network of regulatory checkpoints must exist to tightly regulate these pathways to prevent excessive inflammation arising from unchecked signaling. Unlike other microbial products, such as LPS, which is unique to bacteria, nucleic acids are not distinct from their host counterparts. Therefore, sensing of nucleic acids needs to be carefully regulated such that the delicate balance between protective responses against pathogens and destructive over-reaction to self-nucleic acids or their intermediates is not perturbed. Endogenous DNA from damaged or dying cells that are engulfed by phagocytes should normally be degraded inside cellular phagosomes. However, if the enzymes such as DNase II, which normally degrade this DNA are inefficient or impaired, this DNA is inefficiently degraded and appears to leak into cytosolic compartments to drive IFN dependent pathology[61]. DNA that accumulates from endogenous retroelements can also pose a serious problem if this is not cleared properly. AGS is with a case in point, where mutations in TREX1 prevent the clearance of DNA from endogenous retroelements which then triggers STING dependent IFN responses, resulting in lupus-like symptoms [3, 22, 62]. Small molecule inhibitors targeting the cGAS pathway could prove very useful in treating this and potentially other diseases. Given that cGAS is an enzyme, therapeutic targeting of this pathway should be feasible.

Despite the compelling evidence indicating essential roles for cGAS in DNA driven immunity, the role of the other STING associated sensors still remains to be resolved. It will be important to determine if IFI16, DDX41 and perhaps some of the other molecules function in the context of cGAS or play roles in distinct cell types or cellular locations. It is plausible that these sensors function in a cell type specific fashion, or in temporally distinct stages of the IFN-β response. Future research endeavors might uncover important cross-talk between these disparate components.

Acknowledgments

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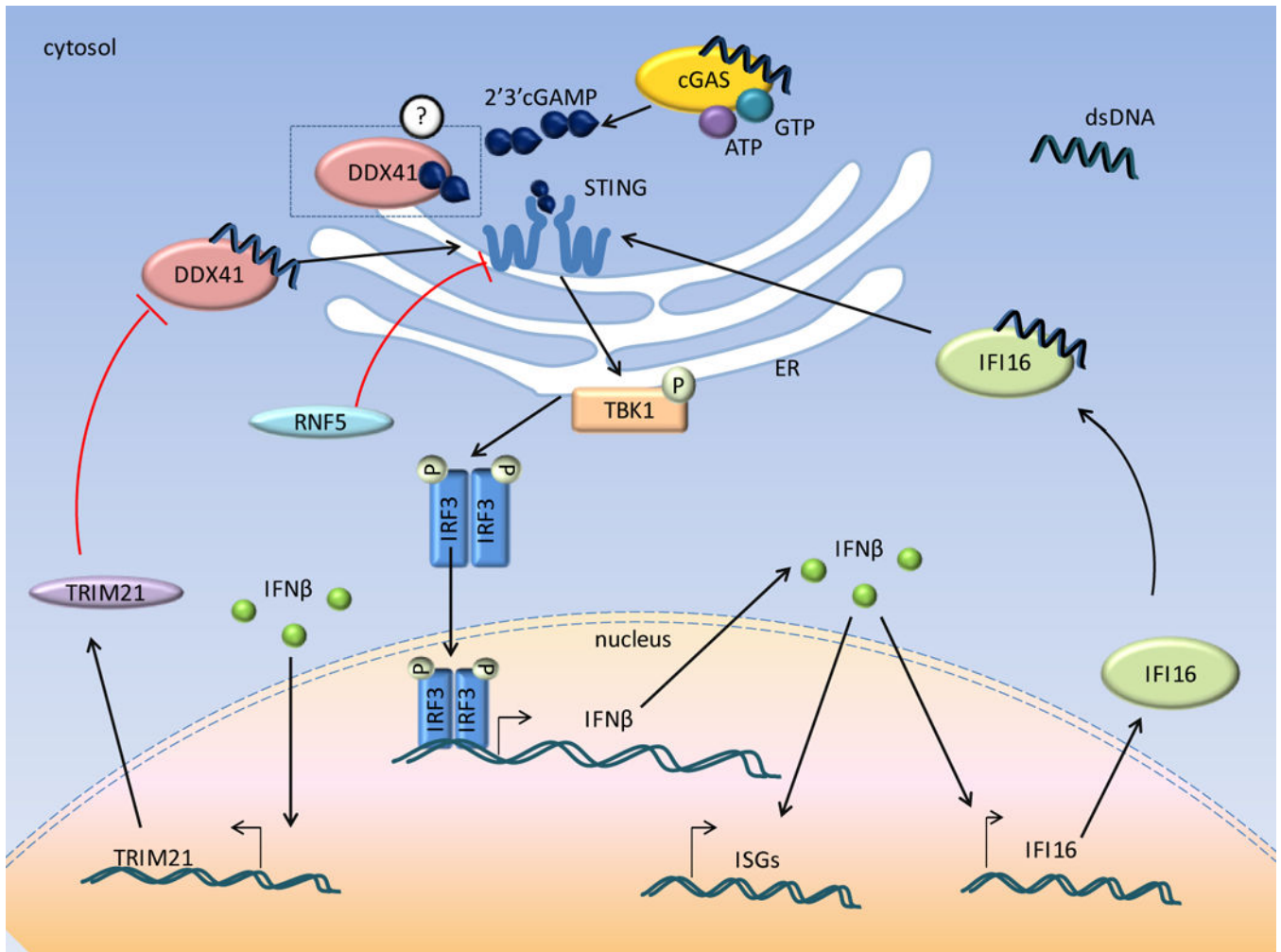
Figure 1. cGAS and other receptors of cytosolic DNA activate IFNβ transcription via activation of STING

Cytosolic dsDNA binds cGAS and generates 2′3′cGAMP from the substrates ATP and GTP. 2′3′cGAMP in turn binds to and activates the STING dimer. This leads to phosphorylation of TBK-1, IRF3 which are translocated to the nucleus and activate IFN-β transcription. In addition, other receptors, such as, DDX41 and IFI16 also bind to cytosolic dsDNA and lead to IFN-β induction via the common STING pathway. IFN-β in turn induces expression of ISGs including IFI16. Different cytosolic receptors of dsDNA might be cell-type specific or specific to the stage of IFN-β induction.
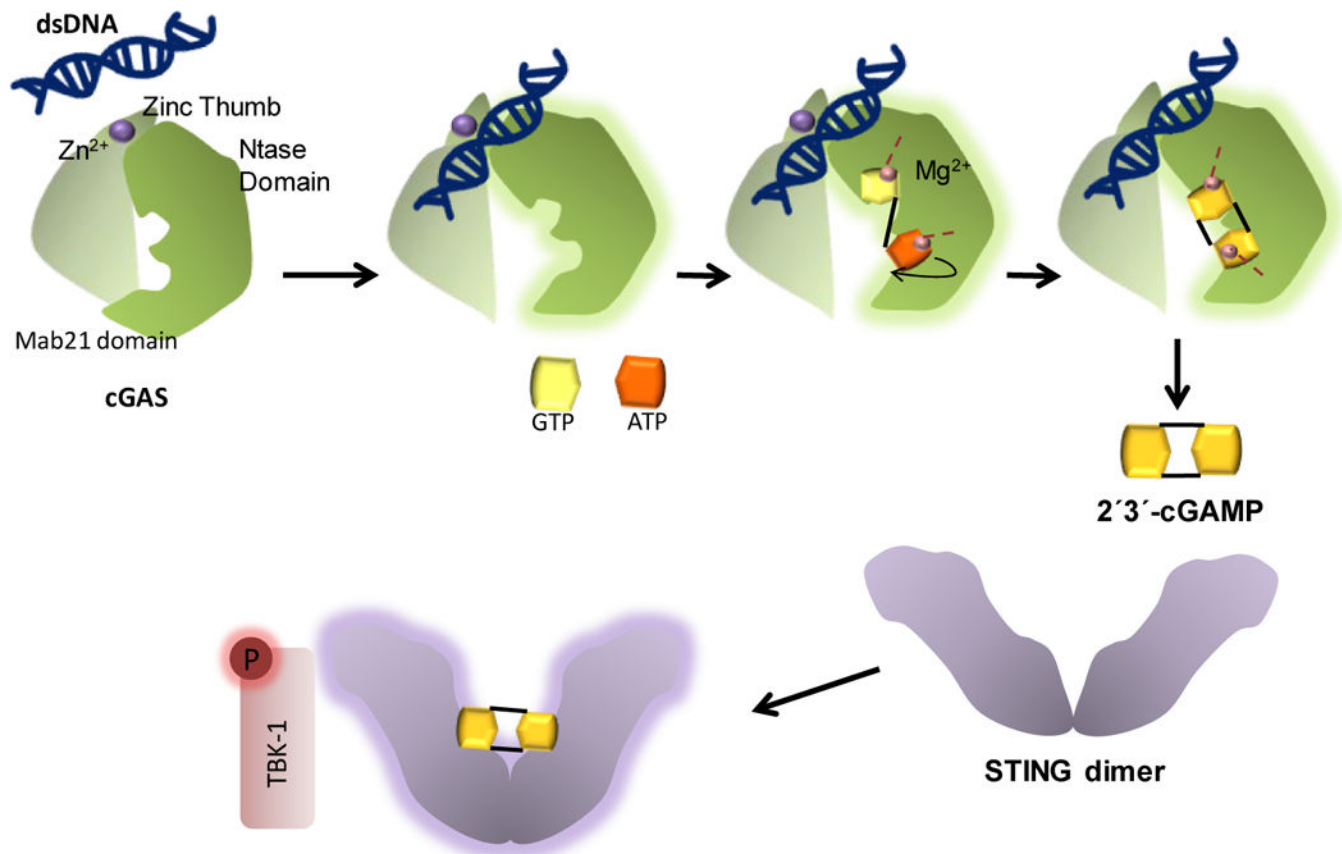
Figure 2. Mechanism of cytosolic dsDNA sensing by cGAS

Cytosolic dsDNA binds cGAS via the basic surface of the bilobal scaffold of cGAS and the zinc thumb. Binding results in a conformational change in the NTase domain, allowing entry and binding of ATP and GTP to the catalytic core. Substrate binding coordinates two Mg^{2+} ions (depicted by purple spheres) to a specific tyrosine residue in the catalytic core causing a flip-over (shown by a curved arrow) in the linear intermediate of the enzymatic reaction between ATP and GTP, resulting in 2′3′cGAMP formation. 2′3′cGAMP binds to the inactive STING dimer at the dimer interface and triggers a conformational change which activates STING and leads to subsequent phosphorylation of TBK-1 and IRF3 thus activating transcription of IFN-β.