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Hunting down the elusive cytosolic-DNA sensor

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The 2024 Albert Lasker Basic Medical Research Award was attributed to Zhijian (James) Chen for "the discovery of the cGAS enzyme that senses foreign and self DNA, solving the mystery of how DNA stimulates immune and inflammatory responses." Bringing to bear an ingenious in vitro complementation system, an astute insight, and superlative biochemistry, Chen and colleagues identified cGAS (cGAMP synthase) as both the molecule that perceives cytosolic DNA in infected, stressed, or dying cells and the enzyme that catalyzes the synthesis of cGAMP (cyclic GMP-AMP), a critical second messenger along the route to inflammatory cytokine production. These findings cleared up the reigning confusion surrounding a major mechanism of inciting the innate immune system, with therapeutic implications for fighting infections, containing tumors, and extinguishing autoimmune and inflammatory diseases.

The immune system has two arms. The innate arm is the body's first line of defense against invaders, mobilizing cells primarily of the myeloid lineage to effect a rapid, generic response. The adaptive arm, the purview of B and T cells, mounts a delayed response against specific molecular targets known as antigens. In both cases, it is critical for the invader to distinguish self from nonself in order to avoid potentially disastrous self-reactivity.

As an "early responder," the innate immune system must be able to rapidly sense intruders from without and stressed or dying cells from within. Several families of germline-encoded receptors have evolved to meet these needs, each with a distinct cell-type distribution, intracellular locations, recognition specificities for repeated molecular patterns, and downstream consequences upon engagement. For example, the Toll-like receptor (TLR) family collectively recognizes a diversity of molecular patterns characteristic of viruses, bacteria, fungi, and parasites including, for example, those contained within lipopolysaccharide, flagellin, or zymosan. In addition, the retinoic-acid-inducible gene I (RIG-I)-like family of helicases detects viral RNAs in various forms located in the cytoplasm of infected cells.

Especially advantageous would be sensors that can detect anomalous DNA because most infectious agents carry DNA and all cells contain DNA, within the nucleus and mitochondria of normal cells but in the cytoplasm of infected, stressed, or dying cells.

The Hunt for the Cytosolic-DNA Sensor

DNA outside the nucleus or mitochondria is a sign of peril that needs to be dealt with posthaste, thus triggering the cell to mount an inflammatory response by secreting cytokines such as interleukin (IL)-1, tumor necrosis factor (TNF)α, and type-I interferons (IFNs). Sensing of foreign DNA launches an innate immune response against invading pathogens while perception of "misplaced" self DNA can instigate an autoimmune or autoinflammatory response. TLR9 is expressed on internalized endosomes of dendritic cells and B cells, where it binds DNA preferentially containing unmethylated CpGs, hence of viral or bacterial origin. However, as early as 2006, Stetson and Medzhitov recognized that there is a more broadly expressed cytosolic-DNA sensor that acts independently of TLR9 but shares with it some elements of the downstream signaling pathways culminating in inflammatory cytokine production (1). This recognition spurred a fast and furious hunt for the cytosolic-DNA sensor.

During the ensuing seven years, an array of candidates was proposed: the Z-DNA binding protein, DAI; the Aim-2-like protein, IFI16; the DDX41 helicase; DNA-dependent protein kinase; RNA polymerase III; and the DNA-damage sensor, Mre11, to name some of them. Ultimately, none of these leads proved fertile, causing great confusion in the field.

Equally confusing was the role of a multimoniker endoplasmic reticulum (ER) protein eventually determined to be a key signaling element of the cytosolic-DNA-sensing pathway (reviewed in ref. 2). This membrane tetraspanner was initially named MPYS and was thought to be involved in transduction of cell-death signals via engagement of major histocompatibility class II molecules rather than in DNA-triggered induction of inflammatory cytokines. Ishikawa and Barber termed the same molecular entity "stimulator of interferon genes" (STING), establishing that DNA-induced IFN responses were compromised in its absence, but mistakenly concluding that it was linked to the RIG-I pathway dedicated to sensing microbial RNAs. A similar error was made by Shu and colleagues, naming the molecule MITA, and Jiang et al, calling it ERIS. Barber's group eventually reported strong evidence that STING is an important component of the cytosolic-DNA-sensing pathway, activated in a diversity of contexts including DNA transfection, DNA-virus infection, bacterial infection, and plasmid

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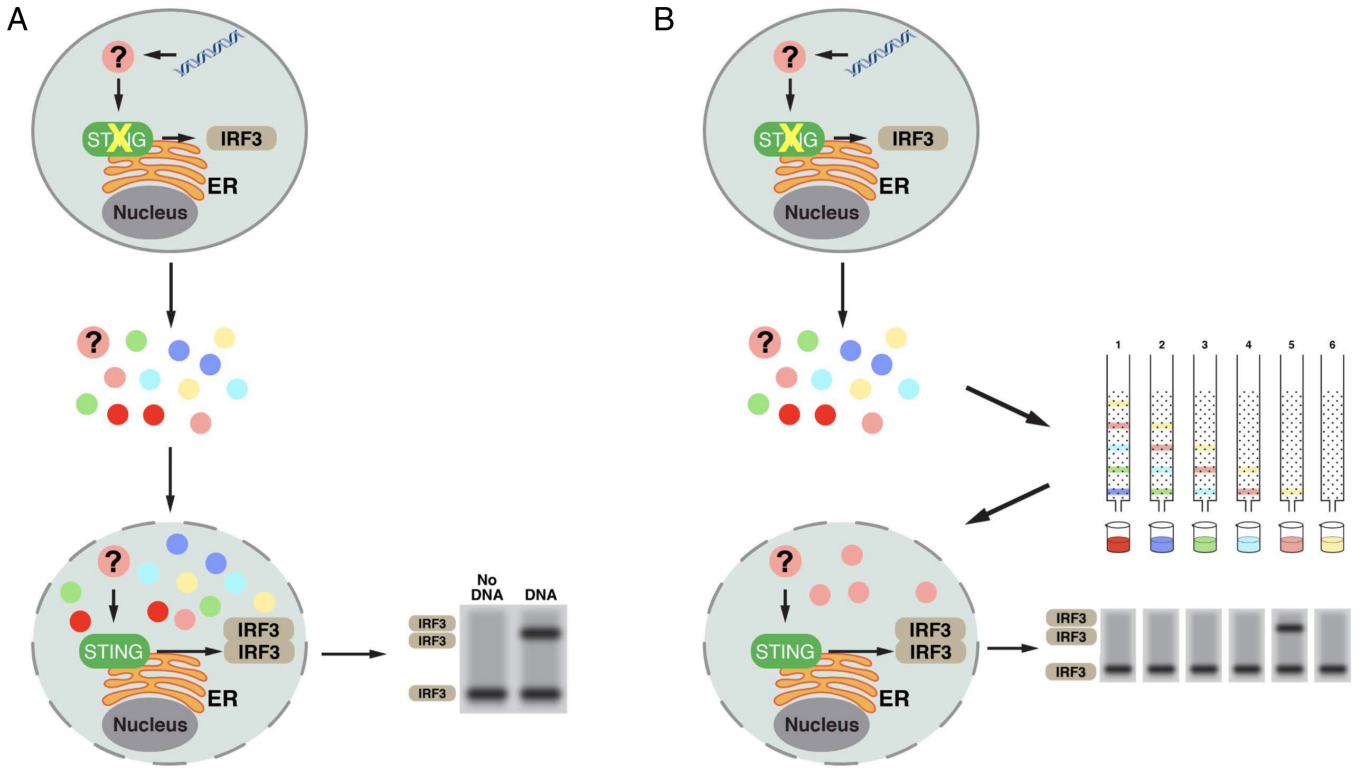

Fig. 1. A complementation assay designed to evidence and identify a cytosolic-DNA-induced signal to activate STING. (*A*) Evidencing the signal. DNA is introduced into the cytoplasm of a donor cell lacking STING activity by transfection or DNA-virus infection, from which a cytoplasmic extract is made. The extract is added to a permeabilized recipient cell to test for STING-dependent activation of IRF3. The question mark (?) denotes a putative DNA sensor. (*B*) Identifying the signal. As per panel A except column fractions of the DNA-supplemented donor-cell extract are added to the permeabilized recipient cell. Image credit: Zhijian (James) Chen (UT Southwestern, Dallas, TX).

vaccination (3); but, mistakenly, they subsequently proposed that STING itself is the cytosolic DNA sensor. Finally, Vance's group made the intriguing observation that STING directly binds cyclic dinucleotides (4), intriguing because it had recently been shown that such molecules, which are conserved signaling elements in bacteria, could induce a STING-dependent type-I IFN response in mice (4, 5). Because they could identify STING mutations that impacted the response to cyclic dinucleotides but not to DNA, the group concluded that STING acts as a direct sensor of cyclic dinucleotides. However, it was not evident how STING's sensing of cyclic dinucleotides related to its role in the response to cytosolic DNA.

Thus, it was clear by 2011 that STING was a key element of the cytosolic-DNA-sensing pathway culminating in inflammatory cytokine production. However, the sensing events upstream of STING engagement were tenebrous—too many players, too many names! In contrast, the signaling events downstream of STING were quite well understood by that time: STING recruits and activates a pair of cytosolic kinases, IKK and TBK1, which in turn activate a pair of transcription factors, respectively, NFκB and IRF3. The two transcription factors then migrate to the nucleus, where they cooperate to induce the expression of inflammatory mediators.

Success for an Intrepid Hunter

With back-to-back papers published in 2013, James Chen and colleagues cleared away the confusion surrounding the DNA sensor upstream of STING (6, 7). They hypothesized that cytosolic DNA binds to and activates a dedicated cytosolic sensor, which activates STING either by directly binding to it or via a second messenger, which then engages the downstream signaling pathway culminating in inflammatory cytokine induction. To test this hypothesis, they set up an ingenious (and courageous!) in vitro complementation system (Fig. 1*A*): DNA was introduced into donor cells lacking STING activity, and then cytoplasmic extracts of these cells were mixed with permeabilized recipient cells, permitting cytoplasmic constituents to diffuse into and out of the cells, while retaining ER-displayed STING. Should the donor cells sense cytosolic DNA and produce a molecule capable of activating STING in the permeabilized recipient cells, IRF3 activation should ensue. Introduction of DNAs in various forms into donor cells of various types was indeed able to induce IRF3 activation of diverse types of permeabilized recipient cells in a STING-dependent manner. The process was independent of DNA sequence and relied, quite unexpectedly, on a factor that was not a protein, RNA, or DNA.

The next challenge was to purify and identify the cytosolic-DNA-dependent, STING-activating factor. Chen and colleagues met this challenge by performing nanoliquid chromatography mass spectrometry (nano-LC-MS) on the cytosolic-DNA-containing cell extracts (Fig. 1*B*). Noting the Vance group's findings that bacterial c-di-GMP and c-di-AMP were bound by STING (4, 5), they searched for evidence of these two circular dinucleotides in the MS data—alas unsuccessfully. However, they made the extraordinarily insightful observation that certain of the MS signals approximated the average of the signals characteristic of the two dinucleotides. c-GMP-AMP (or cGAMP) had previously been found in *Vibrio cholera* but never before in eukaryotic cells.

Nonetheless, several biochemical approaches—chemical synthesis above all—confirmed the identity of cGAMP; and gain-of-function, loss-of-function, and direct binding studies all showed that cGAMP-driven activation of IRF3 operated through STING engagement of cGAMP. Importantly, Chen and colleagues went on to demonstrate that infection of cells with a DNA virus such as Herpes Simplex Virus (HSV)-1 elevated cGAMP levels while infection with an RNA virus, vesicular stomatitis virus (VSV)-1, did not even though it could strongly induce type-I IFN production through the RIG-I pathway. This dichotomy was true of both murine and human cells.

But how is cGAMP made? Chen's group used a combination of conventional column chromatography and MS to identify an enzyme capable of catalyzing cGAMP synthesis. Extracts from a cell type known to contain cGAMP-synthesizing activity were applied to a series of columns and the fractions incubated with ATP, GTP, and DNA (to generate cGAMP) before introduction into the standard complementation assay. The protein that emerged from their efforts, which they named cGAMP synthase (or cGAS), had sequence features in common with members of the nucleotidyl-transferase family—in particular OAS-1, involved in sensing RNAs downstream of the RIG-I pathway. cGAS expression levels in various mouse and human cell lines correlated with the cells' ability to produce cGAMP and to induce IFN production in response to cytosolic DNA. A combination of gain- and loss-of-function experiments firmly established that, in response to DNA transfection or DNA-virus infection, cGAS drove IRF3 activation and type-I IFN production in a cGAMP- and STING-dependent manner, while cGAS with putative active-site mutations did not. Previously proposed DNA sensors such as IFI16, DDX41, and DAI also did not manifest these activities. Moreover, when DNA was added to purified recombinant cGAS protein in vitro, cGAMP activity capable of activating IRF3 was generated. Multiple DNA forms, but not RNA, could stimulate cGAMP synthesis in this assay, reflecting the fact that DNA, but not RNA, was directly bound by cGAS.

The pathway linking a cell's perception of cytosolic DNA to its production of inflammatory cytokines and thereby its mobilization of the innate immune system was now clear, as illustrated in Fig. 2, and was rapidly confirmed by multiple investigators. The sensor, cGAS, and the secondary messenger, cGAMP, are the indisputable instigators in this scenario. This watershed achievement by James Chen and his group was propelled by several features of their 2013 papers: an ingenious complementation assay, an astute insight, and superlative biochemistry.

Postscript

A flurry of work, from both the Chen and other laboratories soon followed. One key set of findings was structural (reviewed in ref. 9). Within a year of the identification of cGAS, six groups (including Chen's) reported the crystal structure of murine or human DNA:cGAS complexes with or without bound cGAMP, revealing the mechanism by which binding of DNA activates cGAS to synthesize cGAMP and, in concert with biochemical studies, identifying the precise structure of the cGAMP product as cyclic [G (2′, 5′ pA (3′, 5′)p] or 2′3′ cGAMP. This cyclic hetero-dinucleotide second messenger is distinct from the known bacterial 3′, 5′ cyclic dinucleotide messengers. The crystal structure of the cGAMP:STING complex was also

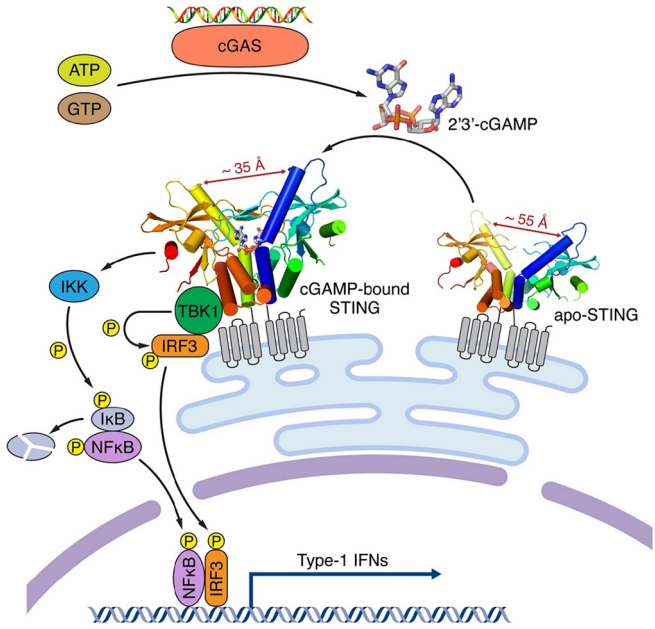

Fig. 2. The cGAS-cGAMP-STING pathway of the innate-immune response. Cytosolic DNA binds to and activates the enzyme cGAS (cGAMP synthase), which then catalyzes the synthesis of a unique isoform of cGAMP (cyclic GMP-AMP). cGAMP functions as a second messenger that binds to and activates the ER membrane protein STING, which in turn activates the protein kinases IKK and TBK1. IKK and TBK1 activate NF-kB and IRF3, respectively, which enter the nucleus and cooperate to turn on the production of type-I interferons and other inflammatory mediators. Image credit: Modified from ref. 8 with permission from Elsevier.

quickly solved by the Chen and Patel groups, revealing the basis of the high-affinity interaction between the two components as well as demonstrating a ligand-induced conformational change in STING likely underlying its activation.

Another important set of follow-on observations was functional (reviewed in ref. 10). Chen's and Rice's groups generated cGAS-deficient mice and used them to demonstrate that cGAS is the predominant innate-immune sensor for DNA viruses in vivo. cGAS also proved critical for the innate immune response to retroviruses such as HIV: cytosolic retroviral RNA is converted by reverse transcriptase to double-stranded DNA, which activates cGAS to synthesize cGAMP. Indeed, it soon became clear that the cGAS–cGAMP–STING pathway is the major route by which most metazoans launch an immune response to DNA viruses, retroviruses, and bacteria.

Therapeutic Applications

As the known influence of the cGAS–cGAMP–STING pathway has burgeoned, so has interest in developing therapies to impact it (reviewed in ref. 11), an especially attractive proposition given the obvious potential of developing small-molecule modulators of STING activity. As illustrated in Fig. 3, these efforts currently span the areas of host defense, antitumor immunity, cellular senescence, autoimmune and inflammatory responses, and neurodegeneration.

As mentioned above, the cGAS–cGAMP–STING pathway is now recognized to be the major innate-immune pathway mobilized in response to DNA viruses, retroviruses, and bacteria. Hence, the adjuvant activity of STING agonists has the potential to augment the effectiveness of vaccines against a variety of pathogens.

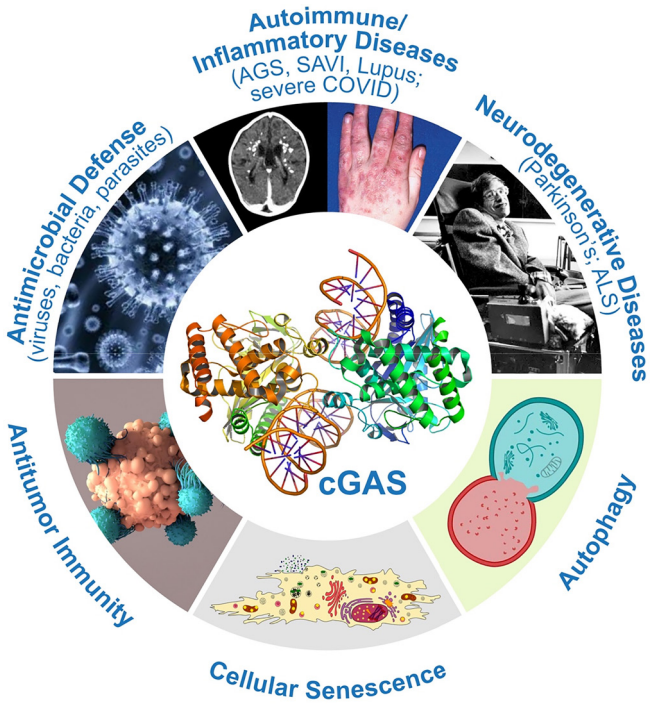

Fig. 3. cGAS plays a pivotal role in numerous physiological and pathological processes. Shown in the center is the crystal structure of a cGAS dimer bound to two molecules of DNA. This DNA binding activates the cGAS enzyme to produce the second messenger cyclic GMP-AMP (cGAMP), which then induces protective immunity against microbial infections and cancer. Under some pathological conditions, aberrant activation of cGAS by self DNA leads to maladaptive immune responses that underlie a variety of autoimmune, inflammatory, and neurodegenerative diseases. AGS: Aicardi–Goutieres Syndrome; SAVI: STING-Associated Vasculopathy with onset in Infancy; ALS: Amyotrophic Lateral Sclerosis. Image credit: Zhijian (James) Chen (UT Southwestern, Dallas, TX).

Agonists of the cGAS–cGAMP–STING pathway might also enhance tumor elimination. Preclinical studies have demonstrated that this pathway is required for the antitumor effects of immune-checkpoint inhibitors, X-irradiation, chemotherapy, and several other antitumor modalities, likely reflecting the ability of the inflammatory mediators it induces to "wake-up" the adaptive immune system. Such a boost might prove particularly valuable in the heretofore elusive elimination of solid tumors.

On the other hand, antagonists of the cGAS–cGAMP–STING pathway are likely to have important applications in the treatment of autoimmune or inflammatory diseases. Hyperactivity of STING underlies two rare genetic autoinflammatory disorders: Aicardi–Goutierès Syndrome (or AGS) and STING-Associated Vasculopathy with onset in Infancy (or SAVI). Mice with a defective *Trex* gene, which encodes an exonuclease and is mutated in patients with AGS and in some patients with Systemic Lupus Erythematosus, develop a lethal autoimmune disease. These mice show a cGAS-dependent accumulation of cGAMP in multiple tissues. Mutation of *Cgas* on top of the defective *Trex* mutation rescues the overabundant cGAMP and autoimmune phenotypes. Given the growing implication of inflammation in neurodegenerative disorders, it is not surprising that cGAS activation has been associated with age-related macular degeneration, Parkinson's Disease, Alzheimer's Disease, and Amyotrophic Lateral Sclerosis. More generally, there is increasing evidence that a frequent trigger of autoimmune and inflammatory diseases is genetically determined or stress-induced mitochondrial dissolution (12), releasing DNA into the cytosol, which would be sensed by cGAS and thereby induce an inflammatory response.

This vast range of potential therapeutic applications has, of course, been recognized by the biopharma enterprise. Many companies are developing or have already developed agonists or antagonists of the cGAS–cGAMP–STING pathway and numerous clinical trials have already been launched. We await their results with great hope.

Data, Materials, and Software Availability. There are no data underlying this work.

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