Molecular mechanisms and cellular functions of cGAS–STING signalling

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Contributions

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Abstract

The cGAS–STING signalling axis, comprising the synthase for the second messenger cyclic GMP–AMP (cGAS) and the cyclic GMP–AMP receptor stimulator of interferon genes (STING), detects pathogenic DNA to trigger an innate immune reaction involving a strong type I interferon response against microbial infections. Notably however, besides sensing microbial DNA, the DNA sensor cGAS can also be activated by endogenous DNA, including extranuclear chromatin resulting from genotoxic stress and DNA released from mitochondria, placing cGAS–STING as an important axis in autoimmunity, sterile inflammatory responses and cellular senescence. Initial models assumed that co-localization of cGAS and DNA in the cytosol defines the specificity of the pathway for non-self, but recent work revealed that cGAS is also present in the nucleus and at the plasma membrane, and such subcellular compartmentalization was linked to signalling specificity of cGAS. Further confounding the simple view of cGAS–STING signalling as a response mechanism to infectious agents, both cGAS and STING were shown to have additional functions, independent of interferon response. These involve non-catalytic roles of cGAS in regulating DNA repair and signalling via STING to NF-κB and MAPK as well as STING-mediated induction of autophagy and lysosome-dependent cell death. We have also learnt that cGAS dimers can multimerize and undergo liquid–liquid phase separation to form biomolecular condensates that could importantly regulate cGAS activation. Here, we review the molecular mechanisms and cellular functions underlying cGAS–STING activation and signalling, particularly highlighting the newly emerging diversity of this signalling pathway and discussing how the specificity towards normal, damage-induced and infection-associated DNA could be achieved.

Introduction

Cell intrinsic recognition and defence systems against foreign genetic material encompass an ancient and fundamental feature of living systems. A first line of defence in mammals is orchestrated by the innate immune system. Germline-encoded pattern recognition receptors (PRRs) detect various pathogen and damage-associated molecular patterns(PAMPs and DAMPs). Their activation elicits signalling cascades that lead to the initiation of cell autonomous defence mechanisms, as well as the production of soluble mediators, such as type I interferons and pro-inflammatory cytokines (Box 1). Type I interferons play a central role in impeding viral propagation, hence their production is typically governed by PRRs that have evolved to sense viral infection. By inducing the expression of interferon-stimulated genes, type I interferons boost cell autonomous defence mechanisms in an autocrine manner, and furthermore can spread antiviral immunity and activate the adaptive immune system.

Cytosolic DNA is a potent activator of a type I interferon response[1,2] (Fig. 1). Under normal conditions, DNA is confined to the nucleus and mitochondria, and is rapidly degraded by nucleases in the cytosol and endolysosomal compartments. Following infections, for example, increased amounts of intracellular DNA are detected in a pathway that involves cyclic GMP–AMP synthase (cGAS; also known as MB21D1)[3,4], a member of the nucleotidyl transferase (NTase) enzyme family[5] that functions upstream of stimulator of interferon genes (STING). cGAS normally resides as inactive protein in the cell. Upon binding to DNA, cGAS undergoes a conformational change to an active state and produces the second messenger cyclic GMP–AMP (cGAMP) from ATP and GTP[6,7,8,9], which is subsequently detected by the cyclic-dinucleotide sensor STING[3,4,10], an ~40-kDa dimeric transmembrane protein at the endoplasmic reticulum (ER)[11]. Binding of cGAMP activates STING, which then translocates to the Golgi and activates TANK-binding kinase 1 (TBK1). TBK1 then phosphorylates itself, STING and, subsequently, the interferon regulatory factor 3 (IRF3) transcription factor. IRF3 dimerizes, enters the nucleus and triggers the production of type I interferons and, subsequently, the expression of interferon-stimulated genes that together orchestrate antiviral defence mechanisms.

Over the past years it became evident that the cGAS–STING axis is activated not only by non-self DNA, such as DNA from DNA viruses or retroviruses, intracellular bacteria and protozoa, but also by extracellular, mitochondrial and nuclear DNA that gain access to the cytosol. Increased cytosolic DNA levels due to, for example, mitotic stress in cancers, radiation therapy and cellular senescence or in autoimmune disorders, such as Aicardi–Goutières syndrome and systemic lupus erythematosus, can lead to constitutive and systemic activation of cGAS–STING, resulting in chronic inflammation and pathology[12,13,14,15]. Understanding molecular and cellular details of cGAS–STING signalling is therefore of considerable biomedical importance. Intense efforts are underway to develop both inhibitors and activators of cGAS and STING, in order to, respectively, treat autoimmune and inflammatory diseases or to activate the innate immune response in immune silent ('cold') tumours to induce antitumour immunity[16] (Box 2).

Although the original models suggested that cGAS is simply activated when it is exposed to DNA in the cytosol, recent studies showed that the situation is more complex. cGAS not only is found in the cytosol but has a multifaceted cellular distribution that involves localization at the cell membrane and in the nucleus. In the nucleus, cGAS is exposed to chromosomal DNA, raising important questions of to what extent it is activated there, how it is regulated and how it distinguishes chromatin from infection-associated DNA. Both cGAS activation and STING

activation were observed to lead to formation of oligomeric structures and, in the case of cGAS, even liquid–liquid phase separation-driven condensates were described. Phase-separated condensates emerge as a new organizing principle of cells and are supramolecular assemblies or membraneless organelles with liquid-like dynamic properties, whereby the phase-separated protein and nucleic acid are enriched in the condensate and depleted otherwise[17]. In the case of cGAS and STING, oligomers and condensates could play important roles in defining substrate selectivity and ensuring a switch-like response to infections, because they bias the system towards long and accessible (that is, not covered by, for example, nucleosomes) DNA that promotes formation of large assemblies. Furthermore, type I interferons are an evolutionarily recent development in vertebrates, whereas cGAS–STING and related systems are much more ancient and are found in primitive eukaryotes and even prokaryotes, raising questions regarding the evolutionary roots and ancient functions of the cGAS–STING system.

Here, we review the emerging multifaceted nature of both upstream and downstream parts of cGAS–STING signalling, discuss models of activation that explain cytosolic and nuclear functions, and point to current limitations and open questions in understanding the regulation of this pathway.

Discovery of DNA sensing by cGAS–STING

STING (also named MITA, MPYS and ERIS) is an evolutionary conserved transmembrane protein that is found beyond vertebrates, down to primitive metazoa and protozoa[18]. It was discovered by two independent cDNA overexpression screens aimed at identifying open reading frames that would trigger antiviral gene expression[11,19]. Around the same time, it was proposed that STING (there referred to as MPYS) would be associated with major histocompatibility complex class II (MHC II) and be involved in pro-apoptotic signalling[20]. Although all of the initial studies agreed on the fact that STING is a transmembrane protein, there was no consensus on its subcellular location and its exact membrane topology. Subsequent studies, however, confirmed the initial concept that STING is an ER-resident[11]membrane passing molecule with its carboxy-terminal (C-terminal) part facing the cytosol[21]. Functional studies agreed on the fact that STING exerted TBK1–IRF3-dependent antiviral activity, yet the respective upstream signal leading to its signalling function remained unclear. Although STING was postulated to be generally involved in DNA and RNA virus recognition[11,19], a more pronounced phenotype was indeed observed for DNA viruses and synthetic double-stranded DNA (dsDNA) ligands. In fact, stimulation with double-stranded RNA or AT-rich dsDNA proved to exert STING-independent antiviral activity via the PRRs MDA5 and RIG-I, respectively[22]. Overall, STING emerged as the long-sought link between cytosolic DNA recognition and antiviral immunity, and initially it was postulated that STING directly detects DNA itself[23]. However, subsequent studies revealed that STING acts as a receptor for bacterial cyclic dinucleotides[10], which appeared to be at odds with its function in DNA recognition. The solution to this conundrum came from the pioneering work of Chen and co-workers with the discovery of cGAS, which acts as a direct DNA receptor and produces the endogenous cyclic dinucleotide second messenger molecule cGAMP to, in turn, activate STING[3,4]. In metazoans, there are enzymes that are highly related to cGAS — on the level of sequence, structure and function — including other MAB21 family proteins (such as MAB21L1, MAB21L2 and MAB21L3) as well as RNA-recognizing 2′–5′-oligoadenylate synthases (OASs).

Interestingly, cGAMP is structurally related to bacterial cyclic dinucleotides (for example, c-di-GMP and c-di-AMP), highlighting the role of these molecules in signalling across phylogenetic kingdoms. However, cGAMP contains an unusual 2′–5′ phosphodiester bond between the 2′O of guanosine and the 5′O of adenosine that appears to be, up until now, specific to eukaryotes (a feature also represented by OASs)[6,7,8] (Fig. 1). cGAS is also related to, for example, bacterial dinucleotide cyclase DncV, comprising a pathogenicity factor from *Vibrio cholerae*. Together, these different enzymes form a 'CD-NTase' (cGAS-DncV) subclade of NTases[24]. Recent results uncover an increasing number of nucleotide-based small molecules produced from these enzymes[25] and reveal widespread bacterial cGAS–STING (or cGAS-like enzymes coupled to other effectors) as components of antiviral defence systems[26].

In hindsight, the discovery of the activity of cGAS as a DNA receptor explained earlier observations that overexpression of cGAS (then known as C6Orf150) showed broad antiviral effects[27]. In the following sections, we discuss in more detail how cGAS and STING function.

cGAS structure and localization

cGAS, an ~520-amino-acid protein containing an unstructured, highly basic ~160-amino-acid amino-terminal (N-terminal) domain along with a globular ~360-amino-acid domain, was first purified from cytosolic extracts of a murine fibrosarcoma cell line using a functional assay for DNA-induced cGAMP synthase activity and shown to function as a cytosolic DNA sensor with direct DNA-binding activity[3]. Subsequent X-ray crystallography allowed visualization of the cGAS structure and how it interacts with DNA. Concomitantly, subcellular distribution of cGAS was investigated, revealing that in addition to the initially described cytoplasmic localization, cGAS is also bound to the inner leaflet of the plasma membrane and present in the nucleus.

Basic overview of cGAS structural features

The catalytic domain of cGAS consists of two structural lobes with the active site at their interface. Lobe 1 includes the evolutionary conserved core β-sheet of NTases, also found in, for example, DNA polymerases, that harbours the conserved acidic residues involved in the Mg^{2+}-dependent catalytic transfer of the nucleoside phosphate onto the hydroxyl acceptor. Lobe II completes the active site and adds interactions for nucleoside–triphosphate donor binding.

Initial structural studies showed that free cGAS (not bound to DNA) does not have a suitably structured active site[8,28,29], whereas DNA binding induces a global conformational change and structures a catalytic activation loop in the interface between the two lobes, such that substrate ATP and GTP and metal ions can bind in a catalytically proficient manner[8,29]. Subsequent work showed that in order to adopt a stable active conformation, cGAS needs to assemble into a dimer, with two DNA strands sandwiched between the two cGAS protomers[30,31] (Fig. 1). This cGAS dimer formation is an allosteric process that is coupled to switching cGAS from the catalytic inactive state to the catalytic active state and is promoted by further clustering[32,33] (see below). Each protomer of the dimer harbours two DNA binding sites denoted 'A' and 'B'[30,31], and each of the two DNA molecules engaged by the dimer is bound to site A of one cGAS protomer and to site B of the respective other protomer. In total, cGAS covers around 16–18 bp of DNA and this binding occurs in a sequence-independent fashion.

cGAS subcellular localization

Although initial studies detected a minor amount of cGAS in nuclear and perinuclear regions, it was concluded that cGAS is a cytoplasmic protein and that this cytoplasmic compartmentalization dictates specificity of cGAS towards non-self DNA, in that it prevents cGAS from interacting with nuclear or mitochondrial DNA (which are also recognized by cGAS; see next section). Recent reports challenge this simplistic view by showing that cGAS can be found within the nucleus (Fig. 2). Nuclear cGAS foci have been observed initially in human fibroblasts and keratinocytes transfected with plasmid DNA[34], in the context of nuclear membrane breakdown[35,36], but also in non-mitotic cells under certain conditions, where cGAS was observed to enter the nucleus upon DNA damage[37]. More recent work indicates that cGAS constitutively resides in the nucleus[38,39,40], and even that cGAS is predominantly localized within the nucleus and tightly sequestered to chromatin[40]. The N-terminal, unstructured domain was shown to constitute a dominant cytoplasmic retention signal, which — when functionally perturbed — resulted in increased nuclear localization[39,41]. The nuclear localization of cGAS was ascribed to its DNA binding activity[38,39] and to a highly basic patch on the catalytic domain involved in chromatin retention[40].

Another study also found that cytosolic cGAS does not appear to reside in a free pool but is largely localized to the inner leaflet of the plasma membrane[41] (Fig. 2). Here, the N-terminal domain appears to be responsible for membrane sequestration and shown to interact preferentially, but not exclusively, with phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P^2) on the inner plasma membrane leaflet in the steady state. cGAS lacking the membrane-localizing N-terminal domain showed increased spontaneous activity and higher nuclear retention, but decreased activation by modified vaccinia Ankara (a dsDNA virus). Based on these findings, it was proposed that not only could membrane sequestration help keep cGAS away from endogenous DNA but membrane localization could help cGAS detect more rapidly or efficiently DNA from viruses that enter the cell through endocytosis[41].

Clearly, more quantitative studies of cellular localization are still needed to obtain a better understanding of cGAS cellular distribution. Nevertheless, a picture emerges where cGAS is not only found in the cytosol but appears to be constitutively, and perhaps even preferentially, located in the nucleus, suggesting that detection of self-DNA is not prevented through confinement to compartments or organelles, but rather by sequestering it to structures such as chromatin. The mode in which cGAS that does reside in the nucleus is kept inactive despite interacting with DNA has not yet been revealed. However, sequestration in an inactive state could be an important mechanism for an optimal dynamic range and a rapid response without the need for de novo protein synthesis: it could serve to limit the ‘noise’ of the system by keeping soluble cGAS concentrations low, and allowing rapid replenishment of the soluble fraction when agonistic DNA is recognized, thereby ensuring a high dynamic range of subsequent signalling.

cGAS recognized DNA sources

The foundational studies of cGAS identified its requirement for responding to DNA virus infection[3] and microbial DNA remains the primary trigger for cGAS activation. However, besides infection-associated DNA entering the cytosol from viral or bacterial sources, cGAS has now been found to interact or at least co-localize with various types of endogenous self-

DNA. These include cytosolic DNA of nuclear and mitochondrial origin, DNA in cytosolic micronuclei and chromatin in the nucleus (Fig. 2). On a structural level, DNAs that activate cGAS range from long dsDNA molecules[32,42,43] to single-stranded DNA (ssDNA) with local secondary structure and short, synthetic DNA with G-rich single-stranded overhangs[43] as well as RNA–DNA hybrids (but RNA–DNA hybrids constitute suboptimal agonists)[44]. Here, we describe which forms of DNA cGAS encounters in different compartments and what is known about their role in cGAS activation.

Microbial DNA

Known viral non-self DNAs detected by cGAS include, among others, DNA from herpes simplex virus and vaccinia virus[45], HIV[46] and cytomelagovirus[47,48] (reviewed previously[49]). The cGAS–STING axis has also been shown to play an important role in RNA virus control, but this likely seems to be an indirect effect though mitochondrial DNA (mtDNA) release (see below). cGAS also senses DNA from intracellular bacteria including *Chlamydia trachomatis*[50], *Mycobacterium tuberculosis*[51,52,53], *Francisella novicida*[54], *Listeria monocytogenes*[55] and *Neisseria gonorrhoeae*[56]. However, some intracellular bacteria also release cyclic dinucleotides that can directly activate STING[57,58], so which PAMP is predominantly detected by the cGAS–STING axis for intracellular bacteria detection is still a matter of debate. Finally, cGAS contributes to parasite control[59] by detecting the genomic DNA of protozoans such as *Plasmodium*.

Microbial DNAs are often shielded from accessing the cytosol and are packaged into, for example, nucleoids or tight protein–DNA structures that, like chromatin, are presumably poor activators of cGAS. It is generally assumed that occasional ruptures of the bacteria cells or viral structures could lead to exposed cytosolic DNA that allows binding of cGAS and switching it to an active state. Similar considerations can be drawn for engulfed tumour-derived DNA (see below), which probably no longer has a fully assembled, proper nucleosomal packaging once it enters the cytosol.

Although it is often assumed that recognition of microbial DNA occurs in the cytosol, the substantial nuclear presence of cGAS opens the possibility for nuclear sensing of viruses. Indeed, in the case of HIV-2, DNA sensing by cGAS occurs in the nucleus, assisted by a host factor denoted NONO (non-POU domain-containing octamer binding protein)[60]. NONO, a multifunctional protein, binds nucleic acids and HIV capsid proteins in the nucleus and thus might direct cGAS to HIV DNA. Nuclear localization of cGAS suggests now that detection of viral DNA could be a more general feature as many DNA and retroviruses replicate in the nucleus and might have more accessible DNA during the integration and replication phases.

Besides NONO, other host proteins could contribute to sensing of viruses and the diverse nature of their non-self ssDNA and dsDNA (Table 1). Polyglutamine binding protein 1 (PQBP1), a host protein involved in RNA splicing, has been found in screens in dendritic cellsto interact with reverse-transcribed HIV-1 ssDNA and cGAS for efficient IRF3 activation[61]. CCHC-type zinc-finger protein 3 (ZCCHC3) and GTPase-activating protein SH3 domain-binding protein 1 (G3BP1) were identified as cofactors that enhance cGAS binding to dsDNA[62,63]. The recognition of viral DNA by cGAS is likely more complex, as cGAS, NONO and PQBP1 are physically linked through a nuclear ribonucleoprotein protein complex containing NEAT1 long non-coding RNA that regulates cGAS–STING activation[64]. The mechanisms underlying these RNA and protein cofactors and the regulatory complexes of cGAS-mediated pathogenic DNA recognition need further investigation and could range from their more direct roles in stabilizing

macromolecular interactions of nucleic acids with cGAS to indirect functions in regulating cellular localization, DNA access or formation of cGAS biomolecular condensates (see section Mechanism of cGAS activation).

In addition to triggering cGAS activation in the infecting cell, microbial infection can also promote the sorting of their DNA into extracellular vesicles, which are taken up by bystander, neighbouring cells to induce paracrine 'spreading' of cGAS activation between cells. Such paracrine cGAS–STING signalling was shown to occur between cells infected by intracellular bacteria and activated T cells, and resulted in compromised antibacterial defence owing to increased apoptosis of T cells in response to cGAS–STING signalling[65].

Extracellular self-DNA

Extracellular self-DNA can enter the cytosol through increased extracellular DNA supply, for example in the context of increased cell death, or perturbed phagocytic digestion. Here, well-studied paradigms that imply involvement of DNA as a substrate are deficiencies for extracellular or lysosomal DNases, which result in the translocation of self-DNA into the cytoplasm and subsequent cGAS–STING activation[12]. At the same time, self-DNA can also be delivered into the cytosol via extracellular vesicles (exosomes and microvesicles) (Fig. 2). For instance, exosomes can carry tumour-derived DNA to dendritic cells[66]. Once delivered to the dendritic cell — either via exosomes or more directly from the extracellular space via endocytosis — such tumour-derived DNA can trigger dendritic cell activation through the cGAS–STING pathway in the tumour microenvironment and increase antitumour immunity[66,67,68]. Yet, in this context, it has to be noted that tumour cell-derived cGAMP has also been shown to trigger STING activation in the tumour microenvironment, leading to an antitumour response of natural killer cells[69].

Mitochondrial DNA

A potential source of cytosolic, cGAS-agonistic self-DNA is mtDNA[70] (Fig. 2). Leakage of mtDNA into the cytosol occurs in the context of intrinsic apoptosis, where mitochondrial membrane integrity is compromised by the formation of BAK/BAX macropores and mitochondrial outer membrane permeabilization that mainly serves to release mitochondria-sequestered cytochrome *c*, which initiates the apoptotic caspase cascade. However, in the case of apoptotic stimuli, mitochondrial outer membrane permeabilization has been shown not only to lead to cytochrome *c* release but also to allow extrusion of the inner mitochondrial membrane to the cytosol, which is associated with mitochondrial inner membrane permeabilization and mtDNA efflux to the cytosol, where it could bind to cGAS[71](Fig. 2).Yet, under normal conditions, this apoptotic mtDNA efflux does not result in cGAS activation, because concomitant activation of the intrinsic apoptosis cascade specifically antagonizes cGAS–STING signalling by proteolytic cleavage of cGAS by effector caspases[72,73]. Interestingly, the ability of cGAS to be activated by mtDNA can be functionally disconnected from the apoptotic cascade. Apparently, mtDNA leakage leading to cGAS activation and cytochrome *c* release resulting in intrinsic apoptosis seem to obey to different thresholds. In that regard, it has been observed that cGAS activation by leaked mtDNA can already occur before the concomitant cytochrome *c* release results in apoptotic cell death. Numerous scenarios have been described in which such mechanisms seem to be at play. For example, depletion of the mtDNA packaging factor TFAM and subsequent mtDNA leakage activates the cGAS–STING axis[70], revealing a cellular response to mitochondrial stress through detection of mtDNA. Also, microbial pathogens can indirectly trigger cGAS–STING activation through the induction of mitochondrial stress that results in mtDNA leakage. In this regard, herpes simplex virus[70]

infection triggers the release of mtDNA, activating cGAS. A similar scenario is true for dengue virus, a positive single-stranded RNA virus that does not produce DNA in the course of its life cycle and, thus, has no capacity for autonomous cGAS induction. Yet, by triggering the release of mtDNA, dengue virus mounts an antiviral response in a cGAS–STING-dependent fashion[74,75]. These observations likely explain how the DNA sensor cGAS counteracts the replication of numerous RNA viruses[76] and why the cGAS–STING axis is specifically targeted by certain RNA viruses[77]. Finally, mtDNA release can also be triggered by cytokine signalling cascades[78], but the mechanisms by which mtDNA is released from the mitochondria in these circumstances remains unclear, warranting further studies in this area. Overall, these studies suggest that mitochondrial stress can be a broadly triggered cue in eliciting a cellular response through cGAS–STING, effectively widening the surveillance role of this cascade.

Nuclear chromatin

As mentioned above, recent work implies that cGAS is constitutively nuclear and chromatin sequestration is in fact a critical process to prevent its spurious activation[38,39,40] (Fig. 2). A first report using longer reconstituted nucleosomal arrays implied cGAS activation by chromatin in vitro[79], suggesting that cGAS is activated by nucleosomes. However, two recent studies reported that nucleosome core particles (mononucleosomes without linker DNA) do not lead to cGAS activation although cGAS binds them with high affinity[36,38]. The discrepancy in these studies is as yet unclear, but it is conceivable that cGAS activation by nucleosomal arrays was not caused by nucleosomes, but by linker DNA or residual free DNA.

On the one hand, cGAS sequestration to chromatin is highly salt resistant, suggesting that cGAS interaction with chromatin is not, or not only, mediated by DNA but might involve protein–protein interactions as well[40]. On the other hand, cGAS residues critically involved in nuclear retention are part of a prominent DNA-binding site of cGAS (site B, see above and Fig. 1), raising the possibility that nucleosomal DNA binds cGAS involving this cluster, but does not trigger an active conformation. In any case, the mode of how cGAS is sequestered to chromatin is still unknown and requires future studies.

Cytosolic chromatin and micronuclei

Important sources of agonistic, cell-intrinsic self-DNA detected by cGAS are cytosolic micronuclei as well as isolated cytosolic chromatin or DNA structures arising from defective DNA replication, repair and mitosis[79,80,81,82,83] (Fig. 2). Micronuclei contain chromatin fragments surrounded by a nuclear envelope-like structure and formed as a result of, for example, DNA double-strand breaks, mitotic errors or problems in DNA replication, leading to chromatin fragments, or they may even contain entire non-segregated chromosomes that recruit their own nuclear envelopes during mitosis. Micronuclei often undergo envelope collapse due to structural defects in the underlying lamina[84], enabling cGAS to access the DNA. However, it was recently found that cGAS in micronuclei can originate from nuclear cGAS, implying that membrane collapse may not even be necessary for cGAS activation by micronuclei[38].

In ageing and in senescent cells, extranuclear chromatin fragments might enter the cytosol through nuclear envelope blebbing, loss of nuclear envelope integrity[85,86] or even some nuclear export processes[87]. Among other characteristics, such as chromatin restructuring and altered metabolic activity, a hallmark of senescence is the senescence-associated secretory phenotype (SASP), characterized by secretion of various cytokines and chemokines that trigger a pro-inflammatory programme to clear senescent cells by the immune system[88].

Several studies have linked cytosolic chromatin and cGAS–STING to the regulation of SASP[35,80,82].

cGAS also detects cytosolic DNA arising from dysfunctional telomeres[89,90,91]. Precancerous cells escaping senescence-associated cell cycle arrest enter replicative crisis due to shortening, deprotection and fusion of telomeres. The resulting mitotic delay and problems with dicentric chromosomes lead to increased amounts of cytosolic chromatin and micronuclei that are detected by cGAS[90]. Extrachromosomal telomere repeat DNA — a hallmark of cancer cells, which is caused by activation of the alternative lengthening of telomeres (ALT) pathway — is also sensed by cGAS[91]. Consequently, the cGAS–STING pathway is found inactivated in ALT-proficient tumour cells[89]. Importantly, as activation of the cGAS–STING axis can lead to autophagy (see below), further transformation of precancerous cells can be prevented by cGAS activation via induction of autophagy-dependent cell death[90,91]. Thus, activation of cGAS–STING signalling in precancerous and senescent cells, or in cells with mitotic problems, could be a mechanism to remove these cells, broadening the functional role of cGAS as an important sensor not only of pathogen-associated intracellular DNA but also of certain forms of endogenous aberrant and potentially pathological chromosomal states and genotoxic stress.

It is so far puzzling why cGAS is suppressed when bound to the abundant nuclear chromatin, but activated by the interaction with chromatin fragments in micronuclei. Micronuclei contain marks for heterochromatin (trimethylated histone H3: H3K9me3 and H3K27me3) and DNA damage (γ-H2AX), suggesting that they are derived from transcriptionally silent regions of the genome and presumably feature DNA damage[81,82], which could be associated with potent cGAS induction by micronuclear chromatin. Indeed, inactivation of DNA replication, repair and recombination genes, including RecQ-like helicase BLM (Bloom syndrome)[83], RNase-H2 (ref.[92]) and the tumour suppressor BRCA2 (refs[93,94]), or increase of chromosomal damage by radiation, topoisomerase II[95,96] or inhibitors of poly-ADP ribose polymerase 1 (PARP1; factor involved in DNA repair)[97,98,99] leads to activation of the cGAS–STING pathway. Although cGAS–STING activation associated with genome instability is currently attributed to formation of cytosolic micronuclei, mere co-compartmentalization of cGAS and micronuclear chromatin does not allow one to unequivocally infer that cGAS is activated by this aberrant chromatin. In fact, cGAS may not even require dsDNA to be activated. For instance, it was recently proposed that nuclear RNA–cDNA hybrid intermediates of LINE1 (long interspersed nuclear element 1) arising from RNase-H2 deficiency could account for cGAS–STING activation[100]. Thus, an important future direction is to investigate a possible activation of cGAS by damaged chromosomal DNA, and to address which specific ligands present in micronuclei, but absent from the nucleus, are responsible for the activation of cGAS specifically by micronuclear chromatin.

Mechanism of cGAS activation

Not all DNAs activate cGAS although they bind to cGAS. This is particularly true for most short DNA elements. Recent work suggests that DNA-induced dimer formation of cGAS by short DNAs is weak. Full activation and stabilization of active cGAS–DNA complexes proceeds by forming oligomeric structures or condensates and are strongly promoted by DNA beyond a certain length threshold that allows two or more cGAS molecules to bind to the same DNA. In

the following, we discuss structural and mechanistic aspects of cGAS–DNA binding and catalysis, along with current models of cGAS clustering that help understand how cGAS could gain specificity towards infection-associated DNA.

Oligomers, clustering and condensates

All structures of active, DNA-bound cGAS obtained so far showed a dimer conformation. Here, binding of the two DNA molecules to the catalytic domain via sites A and B repositions lobe I relative to lobe II, enabling proper formation of the cGAS dimer with two sandwiched DNA molecules. Binding of DNA ligand also induces ordering in the activation loop, which is required for proper formation of ATP and GTP pockets and placement of catalytic residues (Fig. 3a). Although it is well established that DNA-mediated cGAS dimer formation is necessary to adopt a stable active state, the functional role of the peculiar cGAS dimer arrangement with two nearly parallel DNA ligands has remained unclear.

The dimer structure suggests that ~16–18 bp of dsDNA should, in principle, be sufficient to fully activate cGAS. However, generic DNA <20 bp bound poorly to the cGAS catalytic domain and was capable of inducing cGAS catalytic activity in vitro only if cGAS was present at very high concentrations[30,33]. Short dsDNA substrates are likely not efficient in activating cGAS as they do not promote dimer formation at low cGAS concentrations. A notable exception are short dsDNA oligonucleotides contain G-rich ssDNA overhangs[43]. There are also species-specific variations, with murine cGAS being more active towards shorter DNA than human cGAS, attributable to a species-specific variation in site A[101]. DNA <40 bp also fails to activate cGAS in human cells even at high concentrations[32,42]. In fact, the potency of DNA as a cGAS agonist increases with its length[32,42,102]. It is possible that a certain amount of DNA protruding from the core binding sites on the cGAS dimer is required for binding of the N-terminal domain, which helps stabilize cGAS dimerization[33]. However, the catalytic domain alone also displays this length dependency in vitro[32], suggesting that activation of cGAS by DNA is strongly enhanced if the DNA is long enough to bind at least two cGAS catalytic domains.

The DNA length-dependent activation of cGAS could be explained by the recent observation that cGAS dimers further assemble into oligomers, with unique biophysical properties of liquid-like droplets[102], that could have important roles in regulating cGAS activation (Fig. 3). First insights into the formation of oligomeric assemblies of cGAS came from structural studies of cGAS with 39-bp DNA, showing that two cGAS dimers can assemble in a ‘ladder-like’ configuration, where they resemble ‘steps’ along the two DNA arms[32](Fig. 3a,c). In this configuration, the nearby cGAS dimers can reinforce each other along the DNA by multivalent interactions, which was proposed to stabilize cGAS through cooperative effects as it will lead to high local cGAS concentrations. Subsequently, it was shown in cells that multivalent interactions involving the cGAS catalytic domain and the N-terminal domain lead to formation of liquid-like phase-separated condensates[102] (Fig. 3c). In vivo, formation of such condensates was augmented by zinc[102]. Accordingly, negative-stain electron microscopy showed that the formation of the phase-separated structures requires a proper zinc-thumb dimerization element in cGAS, corresponding to a loop region that is stabilized through coordination of zinc and that forms a critical cGAS:cGAS dimer interface[33]. Interestingly, a third DNA binding site, termed site ‘C’, was recently identified and shown to promote mesh-like interactions or even formation of lattice-like structures in such condensates[103] (Fig. 3a–c). The microstructures underlying the large condensates that can be visualized in cells need to be studied in more detail, but might include local cooperative, ‘nucleation’ elements, such as short ladders and lattice-like elements, in a macroscopically more amorphic and gel-like assembly as suggested

by the electron microscopy data. Zinc ions, released through, for example, the ER and mitochondria, might help activate formation of these condensates through the zinc-thumb dimerization loop. Manganese was also found to increase cGAS activity, and accelerates the overall catalytic activity by an as yet unknown mechanism[104,105], altogether suggesting that multiple divalent metals are necessary for optimal cGAS activation.

Taking all data together, a unifying principle for cGAS catalytic activation emerges that could explain both cytosolic and nuclear functions of cGAS (Fig. 3c). In the absence of DNA, cGAS catalytic domains form a weak or transient dimer only. In a first step towards activation, cGAS binds DNA, but DNA binding per se does not yet trigger a catalytic active state. For instance, as discussed above, cGAS binds readily to short DNA, but short DNAs are generally poor activators of cGAS at low cGAS concentrations as they do not strongly promote dimer formation; nucleosome interactions might simply prevent dimer formation for other reasons, but detailed mechanisms await further structural analysis. Elements that support the formation of ladders[32], DNA restructuring via DNA-bending proteins[32], DNA interactions via site C[103] and additional DNA-binding interactions through the N-terminal domain[33] all comprise modes to potentially stabilize dimeric cGAS structures on DNA, leading to activation of cGAS that resembles an allosteric or cooperative switch, whereby formation of one cGAS dimer will enhance formation of additional cGAS dimers in its vicinity by condensating and prearranging DNA[32,33]. Multivalent interactions between assembled cGAS dimers and other DNA elements through, for example, site C and the N-terminal domain will promote formation of large, phase-separated condensates where cGAS and DNA are highly concentrated, strongly favouring the dimeric, active state of cGAS[102]. In the future, more mechanistic analyses of the structural nature and composition of condensates are necessary. Recent work suggests, for instance, that formation of cGAS–DNA condensates requires G3BP1 (a cofactor that enhances cGAS binding to dsDNA) and is promoted by RNA-dependent kinase PKR[106]. The latter is particularly interesting in light of the key role RNA plays in the formation of many other condensate structures. Finally, it needs to be investigated in more detail whether condensates are necessary for cGAS activation, whether condensate formation is required for cGAS activation and whether condensates have additional functions for cGAS activation apart from concentration of cGAS molecules and DNA.

Two-stage catalysis

Once switched into the active state, cGAS catalyses the generation of 2′,3′-cGAMP, which involves two distinct chemical reactions at the same catalytic site. In the first reaction, cGAS takes ATP and GTP as substrates and transfers ATP onto the 2′OH of GTP, generating the linear heterodinucleoside phosphate pppG(2′–5′)pA (ref.[7]). GTP and ATP are preferred substrates for this reaction over other ribonucleotides and they reinforce each other in a cooperative binding event[88]. However, cGAS products have been found to contain 10–20% linear homodinucleotides (pppG(2′–5′)pG and pppA(3′–5′)pA) in addition to the canonical intermediate pppG(2′–5′)pA, so side reactions occur at non-negligible rates[7,107]. Although linear homodinucleotides might, in principle, also occur in the cell, their conversion to cyclic homodinucleotides is negligible and these products presumably do not represent physiologically relevant species[108].

In the second step, the linear pppG(2′–5′)pA is taken as a substrate, and cGAS transfers the GTP moiety intramolecularly onto the 3′OH of the adenosine nucleoside phosphate. Hereby, adenine and guanine bind in the reversed order to the purine binding pockets; that is, the guanine of pppG(2′–5′)pA occupies the prior adenine binding site, and vice versa. The steric

constraints imposed by binding of the linear intermediate and active site features are responsible for using the 2′OH in the first catalytic step and the 3′OH in the second. cGAS was engineered to produce 3′,3′-cGAMP using the bacterial homologue DncV as a structural guide for mutations around the active site, as DncV naturally produces 3′,3′-cGAMP (by using the 3′OH rather than the 2′OH position of the acceptor nucleotide in the first reaction step that generates pppG(3′–5′)pA instead of pppG(2′–5′)pA)[108]. This engineering approach necessitated only few targeted mutations. In light of this finding and the fact the cGAS-related RNA sensors OASs also produce a 2′–5′ linkage, it could be concluded that the formation of this specific linkage is an evolutionary adaptation in metazoans, allowing generation of metazoan-specific signalling molecules for the activation of immune responses.

Kinetic dissection of the different reaction steps revealed that, during the two-step catalysis, a minority of the linear dinucleotides is directly converted to cGAMP without leaving the active site[107] and a majority of the linear intermediates leaves the active site before the second reactions occurs. These linear products, once dissociated from the enzyme, probably cannot efficiently compete with the much higher concentrated ATP and GTP for the active site of cGAS. This would eventually lead to large accumulation of linear intermediates that could exceed cGAMP production, for which there is currently no evidence. It is possible that an unknown nuclease degrades these linear intermediates. However, an alternative or additional mechanism could be provided by the formation of cGAS oligomers and condensates. In such concentrated structures, linear intermediates will reach much higher local concentrations and thereby could much more efficiently compete with ATP and GTP; this would be further reinforced by reduced diffusion rates of the nucleotides into the condensates. At the same time, the condensates are associated with high concentrations of cGAS active sites, meaning that even if they dissociate, linear intermediates would easily encounter a cGAS active site nearby, which would foster efficient binding to cGAS and progression of the reaction. Finally, DNA at site C partially blocks the entry to the active site[103] and could lower the dissociation rate of linear intermediates to favour cGAMP formation even further. Thus, cGAS–DNA condensates might act as a reaction chamber for more efficient cGAMP formation, whereas cGAS activated outside condensates could preferentially produce only linear intermediates that rapidly diffuse away from cGAS. Such a mechanism could effectively lower the ‘noise’ of the system, caused by possible low-level production of cGAMP by cGAS at chromatin or cytosolic DNA fragments under normal steady-state cell states.

Regulation and modifications

Although cGAS was initially identified as an interferon-stimulated gene[27], its level of expression is largely constitutive in most cell types. As such, unlike for other PRR systems, the level of cGAS, and hence its activatability, is not regulated at the transcriptional level. Instead, numerous post-translational mechanisms have been shown to play an important role in regulating the activity of this receptor towards its ligand, its enzymatic activity or its half-life. cGAS is subject to different types of post-translational modifications, involving proteolysis, acetylation, glutamylation, ubiquitylation, sumoylation and phosphorylation (Table 2). Some modifications inhibit cGAS by directly modifying or cleaving at active site residues (Ser305 (ref.[109]), Asp319 (ref.[110]) and Lys414 (refs[111,112])), although it is somewhat unclear how the modifying/cleaving enzymes reach some of these residues in a folded cGAS. Several modifications are important in the context of nucleosomal interactions, dimer formation and condensation. Although site B and the loop involved in chromatin retention appear not to be regulated by the currently known set of modifications, site A and the dimer interface are subject to modifications by acetylation (Lys384 and Lys394 (ref.[111])), sumoylation (Lys231

(ref.[113]), Lys347, Lys384 and Lys394 (ref.[114])) and ubiquitylation (Lys173 and Lys384 (ref.[115])). These modifications might specifically prevent formation of active dimers and condensates (for example, via introducing electrostatic repulsion or inducing steric obstruction of dimer formation), but could still enable chromatin sequestration through site B. Similar effects are expected to result from cleavage of the N-terminal domain by caspase 1 in the context of apoptosis[116]. However, other regulatory means might exist as sumoylation target Lys479 (ref.[113]) is not part of the known DNA binding or oligomerization interfaces.

Functions of cGAS

cGAS has a well-described catalytic function in producing the second messenger and STING activator cGAMP as detailed above. Recent work, however, showed that cGAS also possesses non-catalytic functions, in particular a non-catalytic function in inhibiting homologous recombination of DNA breaks in the nucleus.

Catalytic functions

The main catalytic function of cGAS is to produce cGAMP for the activation of STING[3,4]. Activation of STING then activates IRF3 and NF-κB to drive antiviral and pro-inflammatory immune responses; however, at the same time it also triggers effector functions that are independent of de novo gene expression (see below). cGAMP can trigger antiviral responses in bystander cells as well. It can diffuse through gap junctions to neighbour cells[117] or enter distant cells through incorporation into viral capsids[118] (Fig. 2). Some cGAMP can be released into the microenvironment (for example, upon cell damage or necrotic cell death) and this extracellular cGAMP can enter other cells, most likely via channel-dependent mechanisms. For example, a transmembrane transporter, SLC19A1, has been found to serve as a direct importer for cGAMP[119,120]. Of note, extracellular cGAMP is degraded by a specific mammalian phosphodiesterase, ENPP1 (ref.[121]), thereby controlling cGAMP direct uptake by cells. A host-encoded, intracellular phosphodiesterase degrading cGAMP has not yet been identified and the metabolic fate of cGAMP inside cells is still unclear. In any case, taking into account the intercellular transmission of both cGAMP and cGAS DNA substrates discussed above, catalytic function of cGAS has local, microenvironmental and perhaps even more systemic roles to elicit defence mechanisms by activating STING (see next section) and its downstream pathways leading to interferon expression or cell death.

Non-catalytic functions

In addition to its primary catalytic function, cGAS also has been found to possess various non-catalytic functions. For instance, cGAS was observed to promote sensing of extracellular cyclic dinucleotides, which enter the cell through endocytosis or importers. Interestingly, these extracellular cyclic dinucleotides enhance the formation of the catalytic active, dimeric form of cGAS[122], suggesting that they might be able to shift the equilibrium towards cGAS dimers, thereby facilitating cGAS activation.

In the nucleus, cGAS has been found to inhibit the repair of DNA double-strand breaks by homologous recombination in a mechanism that neither requires STING nor catalytic activity of cGAS[37,38] (Fig. 2). In homologous recombination, the DNA ends are resected to 3′ single-strand tails, onto which the RAD51 recombinase is loaded in a BRCA2-dependent manner (reviewed previously[123]). RAD51 catalyses strand pairing with the homologous template,

where the broken strand is extended by DNA synthesis. A first study showed that DNA damage increases nuclear localization of cGAS, which occurs by active cGAS import primed by dephosphorylation of Tyr215 (ref.[37]) (Table 2) (a site phosphorylated by B-lymphoid tyrosine kinase (BLK) to facilitate cytosolic retention). Tyr215 is located in a DNA binding site of cGAS and the phosphomimetic Tyr215Glu mutation reduces DNA binding, suggesting that dephosphorylated Tyr215 is necessary for nuclear cGAS functions by allowing its proper interactions with DNA[38]. cGAS was found at sites of chromosomal damage marked by PARP1 and γ-H2AX, where it interacted with γ-H2AX and poly-ADP ribose (PAR)[37] (but these interactions are not sufficient to recruit cGAS to strand breaks independently from DNA binding)[38]. At these sites, cGAS perturbed formation of the PARP1–TIMELESS complex that is involved in homologous recombination. However, subsequent work suggested an even more direct inhibitory function of cGAS on homologous recombination. Here, cGAS formed oligomeric clusters with the homologous dsDNA template and prevented pairing of RAD51–DNA filaments and strand invasion of the broken DNA strand into the homologous strand[38]. As cGAS was found to be chromatin-associated throughout the cell cycle[40], inhibition of homologous recombination is not simply an effect of nuclear import upon damage, as initially proposed[37], but likely involves relocalization of chromatin-bound cGAS to sites of recombination where it competes with RAD51 for DNA. Competitive interplay between proteins at recombination intermediates (RAD51, replication protein A (RPA)) and cGAS had been found before the role of cGAS in homologous recombination was revealed, and RAD51 and RPA were suggested to sequester DNA fragments resulting from DNA damage and repair in the nucleus in order to prevent cGAS activation[124]. However, ssDNA — the substrate for RPA and RAD51 — does not activate cGAS in vitro, so the nature of activating nucleic acids for cGAS in the context of DNA damage needs further study.

Interestingly, cGAS has little effect on the non-homologous end-joining (reviewed previously[123]) DNA repair pathway[37,38], which is executed through a different chromatin structure than homologous recombination, does not involve homology search and occurs with a smaller amount of free dsDNA on a DNA template. Thus, it is possible that simply the presence of sufficiently long accessible dsDNA is enough to enable high-affinity binding of cGAS oligomers to DNA and subsequent activity.

In the future, it will be interesting to investigate the biology of cGAS activity in DNA repair in more detail. This function could simply represent a tolerated by-product of nuclear sequestration of cGAS. Nevertheless, it could also have a more active role. For instance, it was shown that knockdown of cGAS suppresses DNA damage and inhibits tumour growth both in vitro and in vivo[37], suggesting cGAS as an antitumour factor. An important clue to resolving this question could be specific recruitment of cGAS to sites of damage, but this is still controversial. Another important question is whether cGAS is active while at homologous recombination sites. If this was the case, this could, for instance, trigger defensive programmes to help eliminate cells with DNA breaks. Finally, the role of cGAS in sequestering exposed dsDNA from the DNA repair machinery could also be a function that has evolved to detect and interfere with DNA virus replication in the nucleus, which often proceeds by engaging host DNA damage response and repair factors[125]. Finally, although there is currently no evidence that formation of larger, phase-separated condensates plays a role in nuclear functions of cGAS as seen in the cytoplasm, the ability to self-oligomerize is necessary for inhibition of homologous recombination[38]. Thus, perhaps a related clustering mechanism of cGAS is important for its nuclear functions as well, but on a different scale (involving smaller or less stable condensates).

STING activation and signalling

STING activation by binding of cGAMP and subsequent signal transduction is best understood for its capacity to trigger IRF3 phosphorylation, which drives the expression of a plethora of antiviral genes. Notably, we came to appreciate that, in fact, this best-studied role in IRF3 activation represents the most recent evolutionary step in STING downstream signalling, and that STING also carries out additional, evolutionarily more ancient functions, which can be mechanistically and functionally separated from IRF3 activation. In the following, we briefly discuss four independent STING functions: IRF3 activation, NF-κB activation, autophagy regulation and induction of lysosomal cell death (LCD) via STING lysosomal trafficking.

Structural mechanisms of STING and subsequent IRF^3 activation

Recent cryo-electron microscopy studies revealed the structure and domain organization of full-length chicken and human STING[126] and — at low resolution — its complex with TBK1 (ref.[127]). STING spans the ER membrane four times with its N-terminal portion. Both the C-terminal part of STING that contains the ligand binding domain (LBD) and the C-terminal tail (CTT) face the cytosol (Fig. 4a). Under steady-state conditions, ER-bound STING forms a dimer so that their LBDs generate a V-shaped ligand binding pocket for one cyclic dinucleotide ligand, as previously revealed by crystallographic studies[6,128,129,130,131]. In this dimer, the transmembrane helices of the two STING molecules are arranged so that they form an integrated, domain-swapped architecture (Fig. 4a,b). A connector helix tethers the last transmembrane helix of STING with its LBD. The two connector helices of one STING dimer form a right-handed crossover, which results in close intermolecular interactions at the junction (Fig. 4b). The CTT of STING contains a highly conserved TBK1-binding motif[127,132] that is directly adjacent to a pLxIS motif, which is important for IRF3 activation[133,134] (Fig. 4c). Binding and imaging studies suggest that a considerable amount of TBK1 is already present on these preformed, inactive STING dimers on the ER[127]. However, despite two TBK1 molecules being bound in close proximity on one STING dimer, TBK1 perhaps does not become activated when bound to STING dimers as sterical hindrance prevents phosphorylation in *trans*. Upon cGAMP binding, the STING dimer undergoes a conformational switch, which results in a 180° clockwise rotation of its LBDs in relation to the transmembrane portion (Fig. 4b). This rotation relieves the right-handed crossover of the connector helices and also allows the closure of a lid structure around cGAMP. Associated with this conformational switch, α-helices 2 and 3 of the LBD (LBDα2 and LBDα3) tilt downwards, which is suggested to generate a surface geometry that promotes the lateral oligomerization of several STING molecules in a side-by-side configuration (Fig. 4d). This oligomerization constitutes an important prerequisite for STING gaining its signalling competence and — similar to cGAS oligomerization/condensation — has cooperative/switch-like properties. Oligomerization was found to be promoted by disulfide bond formation of Cys148 in the connector helix (Fig. 4b), suggesting that STING activation could be nearly irreversible.

STING variants with mutations in the oligomerization interface are not only incapable of activating TBK1 (see below) but are also unable to exit the ER, suggesting that oligomerization dictates translocation to the Golgi compartment[126,135]. At the same time, ligand binding also seems to make the CTT of STING better accessible to TBK1 — the STING oligomer formation positions '*cis*' TBK1 molecules in close proximity to '*trans*' TBK1 molecules of the adjacent

dimer, in a way that the activation loop of the '*trans*' TBK1 molecules is accessible to the catalytic centre of the kinase domain of the '*cis*' molecule. This allows TBK1 to in *trans* phosphorylate and activate TBK1 molecules in close proximity (Fig. 4d). Active TBK1 then exerts catalytic activity towards the critical serine residue within the pLxIS motif[134] (Ser366) of the CTT (Fig. 4d). Current structural data imply that TBK1 is only able to phosphorylate the CTT of STING of an adjacent STING dimer, rather than the CTTs of its own dimer. Upon phosphorylation, this motif serves as a docking site for IRF3, which is then brought into close proximity to the catalytically active TBK1 molecules[126,132].

As mentioned above, STING exits the ER towards the Golgi already upon oligomerization (Figs 4d,5). This process depends on canonical COPII coat complex-dependent anterograde transport[136], but it remains unclear what signal within STING is sensed to initiate this transport. STING travels via the ER–Golgi intermediate compartment (ERGIC) to the Golgi. Studies employing pharmacological inhibitors or microbial effector proteins suggest that STING activates IRF3 as early as the ERGIC[137]. In fact, blocking ER exit of STING by pharmacological means completely abolishes IRF3 activation. These results would suggest that STING oligomer formation is either linked to ER exit or that additional factors beyond oligomer formation are required for IRF3 activation that are only met at the ERGIC. Indeed, another interesting feature of STING activation is the fact that palmitoylation at cysteine residues close to transmembrane domain 3 is required to activate STING[138,139]. It was shown that palmitoylation facilitates STING cluster formation at the Golgi, which is required for maximal signal transduction. However, preventing or blocking palmitoylation does not interfere with the exit of STING from the ER. This would suggest that palmitoylation does not impact on structurally determined side-by-side oligomerization of STING that is induced by the conformational switch upon ligand binding. To this end, the exact nature of the palmitoylation-induced STING cluster formation and its role in signal transduction remains to be clarified. It should be noted that numerous additional post-translational modifications (Table 2), as well as interactions with modulatory proteins (Table 1), have been described to modulate various steps in STING-dependent signal transduction. For example, different types of ubiquitylation have been shown to facilitate its interaction with TBK1 or to impact on its half-life (Table 2). With new structural insight into the activation of STING and its interaction with TBK1, it will be worthwhile to address the molecular impact and epistatic relationship of these modifications to the individual steps of STING activation.

NF-κB and MAPK pathway activation by STING

Human and murine STING activation also triggers NF-κB and MAPK pathway activation, albeit to a lower extent compared with other PRR cascades[140]. In fact, unlike other PRR systems that assemble TRAF (tumour necrosis factor receptor-associated factor)-dependent signalosomes to recruit the TAK (transforming growth factor-β-activated kinase) and the IKK (inhibitor of NF-κB kinase) complex to activate MAPK and NF-κB, respectively, mammalian STING appears to employ an unconventional signalling route to the activation of these transcription factors (Fig. 5b). The exact mechanisms of STING-dependent NF-κB and MAPK activation remain unresolved, in that conflicting models have been proposed. On the one hand, it has been shown that TBK1 functions upstream of NF-κB activation[141,142]. On the other hand, evidence has been provided that the CTT is not required for this function[140,143], which would also rule out a role for TBK1. The latter model would be in line with studies on fly STING that lacks the CTT. Here, the immune deficiency signal transduction pathway that is orthologous to the vertebrate NF-κB cascade is triggered upon STING activation despite the absence of a

CTT[144,145]. Hence, it is possible that STING-dependent NF-κB activation is governed by a signal from within the LBD of oligomerized STING. Interestingly, ray-finned fish have evolved a C-terminal extension of the STING CTT, which serves to recruit TRAF6 (ref.[140]). This additional module results in a far stronger NF-κB activation as compared with mammalian STING species. Finally, it has been observed that STING can result in NF-κB activation downstream of DNA damage signalling independently of cGAS. This response was shown to assemble an unconventional STING signalling complex, resulting in TRAF6-dependent NF-κB activation[146]. How STING activates the MAPK pathway remains to be elucidated.

STING-triggered autophagy

As outlined above, two modules within the STING CTT are critically required to activate IRF3: the TBK1-binding motif is important for TBK1 recruitment and activation, whereas the pLxIS motif — upon phosphorylation by TBK1 — recruits IRF3 for its subsequent phosphorylation[134]. Interestingly, these modules within the CTT evolved only in the vertebrate lineage, alongside the type I interferon system[18], raising the question of the function of STING in these non-vertebrate species. In fact, one important STING-dependent signalling output is autophagy[147], which operates independently of its CTT and, as such, in the absence of TBK1 and IRF3 activation[136]. At the same time, blocking IKK activation does not impair autophagy[136]. Upon ER exit, STING triggers autophagy from the ERGIC, as seen by LC3 (also known as microtubule-associated proteins 1A/1B light chain 3B) lipidation and autophagosome formation (Fig. 4c). The canonical autophagy pathway is initiated by the activation of the Unc-51-like kinase 1 (ULK1) complex that activates components of the class III PI3K (PI3KC3) complex I by phosphorylation. This complex then triggers the local formation of phosphatidylinositol 3-phosphate (PtdIns3P) residues, forming the so-called phagophore. These residues serve as docking sites for PtdIns3P effector proteins that then recruit and activate the ubiquitin-like conjugation machineries that lead to the growth and functionalization of autophagic membranes[148]. In the context of STING, the canonical autophagy initiation machinery (ULK1 and PI3KC3 complexes) is not involved in STING-dependent autophagy, but further downstream components, such as the PtdIns3P effector protein WIPI2 and ATG5, are shared[136]. Clear mechanistic insight into the contribution of STING to autophagy initiation is missing, yet a conserved motif located in STING's LBDα4 seems to be important for this function. Induction of autophagy has also been reported for STING from the sea anemone, which lacks the CTT, indicating that autophagy appears to be an ancient, possibly primordial, function of STING. In line with these observations, fly STING, which also lacks the CTT, still functions to restrict microbial pathogens. Although this relies on transcriptional responses downstream of the immune deficiency pathway[144,145] (see above), autophagy induction has also been shown to be of importance[149]. Also, in mammals, STING-dependent autophagy has been shown to play a protective role in the clearance of microbial pathogens such as *Mycobacterium tuberculosis*[147], certain Gram-positive bacteria[150] and also herpes simplex virus 1 infection[151]. Intriguingly, in the context of herpes simplex virus 1 infection, autophagy but not type I interferon induction seems to constitute the prime effector function of STING in controlling viral infection[151].

STING trafficking to the lysosome and cell death induction

STING activation can trigger cell death in various ways. As such, along with its IRF3-dependent induction of antiviral genes, STING induces the expression of numerous pro-apoptotic and pro-necroptotic molecules that facilitate respective programmed cell death pathways (reviewed previously[152]). Moreover, in certain cell types, STING engagement can also induce LCD, a lytic

cell death programme (primarily characterized in human myeloid cells), which operates independently of classical programmed cell death pathways. This functionality was found to require translocation of STING to the lysosome[153]. Here, lysosomal STING accumulation triggers lysosomal membrane permeabilization, the subsequent release of lysosomal hydrolases and, thereby, cell death[153] (Fig. 5d). It is possible that this type of cell death is at play in cells from patients with SAVI (STING-associated vasculopathy with onset in infancy), who harbour gain-of-function mutations in the *STING1* gene[154]. In these patients, a constitutively active STING molecule triggers continuous antiviral gene expression, but also cell-intrinsic death of lymphocytes and myeloid cells. Indeed, in murine models of SAVI, a large part of disease progression and cell death occurs independently of IRF3 and antiviral gene expression[155,156,157]. In myeloid cells that succumb to STING-induced LCD, a secondary inflammatory response is triggered through the activation of the NLRP3 inflammasome mediated by the loss of plasma membrane integrity and cellular K^+ efflux, followed by the release of inflammatory cytokines by the dying cell. This pathway constitutes a general, non-specific response pattern of myeloid cells that is activated upon membrane perturbation[158]. Of note, this NLRP3-dependent inflammatory response has not evolved to specifically detect cell death downstream of STING and is also not operational in primordial organisms. However, it is conceivable that STING-induced LCD and associated secondary inflammation in general constitutes a 'last resort' response pattern that functions to prevent microbial propagation, as it has been ascribed to autophagy. Yet, to substantiate this concept, it would be important to study systems in which autophagy can be dissected from LCD. In cells that do not succumb to LCD, an alternative outcome of trafficking STING to the lysosome is its lysosomal degradation[153,159]. Indeed, the half-life of STING is largely controlled by the lysosomal machinery, rather than by proteasomal degradation or autophagy[136,159]. A functional consequence of lysosomal degradation of STING is a shutdown of its signalling. Consequently, preventing lysosomal trafficking or lysosomal activity results in augmented STING levels and also increased antiviral gene expression[136,159]. Imaging studies suggest that STING translocates to the lysosome after exiting the Golgi through the late endosome route[136,159], although specific determinants of this transport mechanism remain unclear. As for ER export in general (see above), STING translocation to the lysosome occurs independently of TBK1 and its CTT being phosphorylated. Two conserved regions within the LBDα3 have been implied in dictating translocation of STING to the lysosome[159], yet additional studies are required to explore the underlying mechanism.

Conclusions and perspectives

The mechanism of intracellular DNA detection has remained one of the most enigmatic fields in innate immunity. With the discovery of the cGAS–STING axis, we now have a good understanding of what the underlying components are and how they sense pathogens. However, it is now becoming more and more clear that the cGAS–STING axis also responds to a wide range of endogenous nucleic acids implicated in cellular stress and damage, and that its signalling outputs reach far beyond IRF3 activation. Moreover, cGAS appears predominantly as a nuclear protein in steady state with additional non-catalytic functions, raising the question of how cGAS is able to discriminate self from non-self or harmless from dangerous, respectively. Recent data provided a new perspective that robust cGAS activation occurs in oligomers or even may require higher-order assemblies in the form of liquid-like

phase-separated condensates. These insights have challenged the conventional picture of cGAS functioning as a cytosolic sensor for DNA with a simple ligand to receptor stoichiometry, but more work is needed to fully understand the role and composition of the condensate structures. Similarly, active STING oligomerizes to serve as a platform for subsequent signal transduction and its signalling capacity goes beyond the activation of IRF3. Such oligomerization and clustering-based mechanisms could ensure a cooperative 'switch-like' response of signalling as a function of increasing concentrations of agonistic ligand, with a high signal-to-noise response and specificity towards DNA that allows clustering, such as long DNA. On the level of cGAS–DNA interactions, we still do not understand the differences between the binding of cGAS to different ligands. One interesting aspect to study here is how cGAS interacts with different types of chromatin, in particular whether cGAMP is produced by nuclear cGAS and under which circumstances, and what features make chromatin in micronuclei a cGAS agonist. Furthermore, although certain hypotheses have been put forward regarding the mechanism of distinguishing self from non-self/damage by cGAS, including subcellular compartmentalization of cGAS, the mechanisms involved in this process remain elusive. Here, new insights could potentially be obtained through better understanding of the structural nature and composition of condensates and the role of the N-terminal domain in macromolecular interactions, which has eluded more detailed analyses owing to its unstructured nature. Finally, we also lack an organismic and evolutionary perspective of the 'non-canonical' — beyond cGAMP production and induction of interferon signalling — functions of cGAS–STING and whether they serve important physiological roles. These new findings could spur the generation of novel strategies to modulate cGAS–STING that go beyond targeting of the catalytic function of cGAS or the ligand binding pocket of STING, which could be used to either stimulate or inhibit cGAS–STING in the context of various pathologies, including cancer and diseases driven by pathologic inflammation (Box 2). In the context of cancer, it would be particularly interesting to study the role of cGAS as a blocker of DNA repair via homologous recombination, which could potentially fuel cancer-associated genomic alterations or, on the contrary, lead to deleterious loss of genomic integrity and cancer cell death[160]. It will also be of interest to further illuminate the role of STING in autophagy induction, which, similarly to DNA repair suppression, has dual roles in cancer[148]. At the same time, further studies should address the mechanisms and regulation of STING targeting the lysosome, understanding of which could give further control over cGAS–STING signalling (by switching it off) or could be used for the induction of LCD.

Box 1 - Non-self recognition by the innate immune system

The conceptual framework for how the innate immune system detects the presence of non-self is based on the pioneering work by Janeway[177]. Here, it was postulated and later experimentally validated that the host has evolved so-called pattern recognition receptors (PRRs) to sense the presence of pathogen-derived molecular patterns (PAMPs) as non-self. PAMPs are considered invariant molecules that are important for the microbial life cycle and specific to the microbial pathogen, but not present within the host. However, subsequent studies have shown that various PRRs can also be activated by endogenous, host-derived molecules under conditions of cell or tissue damage. In analogy to the PAMP terminology, these molecules are typically referred to as damage-associated molecular patterns (DAMPs)[178]. DAMPs can be formed or released in the context of microorganism-inflicted damage, and thereby indirectly alert the host to microbial infection. At the same time, DAMPs can also be generated under non-infectious damage, thus contributing to acute or chronic sterile inflammatory conditions. Which molecules act as DAMPs and what is the molecular basis of their detection by PRRs? On the one hand, it has been shown that endogenous molecules can be rendered agonistic for PRRs by certain damage-associated modifications (for example, oxidized lipids). On the other hand, DAMPs can also constitute unmodified self-molecules that pass a certain concentration threshold or gain access to certain PRRs that are usually shielded away from these molecules under steady-state conditions. Especially, host-derived nucleic acids are found in the latter category[179]. Endogenous nucleic acids are typically indistinguishable from microbial nucleic acids and, as such, respective PRRs are often positioned into compartments that are devoid of potential self-ligands. For example, Toll-like receptor 9 (TLR9) is located within the endolysosomal compartment to avoid the detection of extracellular self-DNA. Indeed, relocalizing this receptor to the cell surface results in a fatal autoinflammatory disease. Analogous to TLR9, it has been suggested that the positioning of cyclic GMP–AMP synthase (cGAS) within the cytoplasm functions to avoid self-DNA, which is abundantly present within mitochondria and the nucleus, but not the cytoplasm. However, more recent data (as discussed in this article) indicate that this model is too simplistic. Indeed, accumulating evidence suggests that cGAS is also present in the nucleus. Here, additional safeguard mechanisms ensure that recognition of nuclear self-DNA does not occur. These mechanisms seem to operate at the level of ligand availability, as well as regulation of the catalytic activity of cGAS by certain nuclear factors, but the underlying mechanisms have not been resolved.

Box 2 - cGAS–STING signalling in disease and therapy

Cyclic GMP–AMP synthase (cGAS)–stimulator of interferon genes (STING) is primarily involved in the response to microbial infection. Hence, activation of this signalling pathway could be explored as a viable strategy to combat infections, in particular as several viral pathogens are able to circumvent activation of cGAS–STING signalling[180]. However, as described throughout the main text, beyond recognition of pathogens, cGAS–STING also mounts an inflammatory response to certain self-DNA molecules, and, thus, regulation of this pathway could be a viable strategy to modulate sterile inflammation for therapeutic benefit. There are two primary disease scenarios that could benefit from modulation: activation or inhibition of cGAS–STING, namely cancer and diseases with pathogenesis attributed to increased inflammatory

responses. In the following, we briefly outline these scenarios. For a detailed overview, please refer to two other review articles[181,182].

The first indications for STING agonism exerting therapeutic effects in cancer stem from studies predating the discovery of this signalling cascade. The two tricyclic compounds DMXAA (5,6-dimethylxanthenone 4-acetic acid)[183] and CMA (10-carboxymethyl-9-acridanone)[184] were identified as molecules that exerted potent antitumour and antiviral activity, respectively. Later, it turned out that both these compounds serve as direct STING agonists. However, these compounds only activate rodent, but not human, STING[185,186], explaining the failure of DMXAA in clinical trials for cancer therapy[187]. Nevertheless, in line with the promising preclinical data on DMXAA in mouse models, STING agonism using cyclic GMP–AMP (cGAMP) or stabilized derivatives thereof was tested and found to be efficacious in various murine cancer models[67,188,189,190]. The beneficial role of cGAS–STING signalling was furthermore supported by the notion that spontaneous or treatment-associated antitumour responses were enhanced or facilitated by the engagement of cGAS–STING signalling, even in the absence of exogenous agonists[16,67,190,191]. Moreover, activation of cGAS–STING by cancer-associated agonistic DNA in transformed, senescent cells is important for their immune clearance in mice[82], and suppression of cGAS–STING activation has been observed in several cancers and cancer cell lines, including those that are telomerase negative and rely on the alternative lengthening of telomeres pathway[89]. Next to the protective role of the cGAS–STING axis in cancer, there are some reports on the pro-tumorigenic or pro-metastatic activity of STING agonism[192,193,194]. Nevertheless, STING agonism is being currently pursued as a therapy in various immuno-oncology settings[195].

A lot of sterile inflammatory conditions are triggered or perpetuated by inadvertent activation of pattern recognition receptors through the endogenous damage-associated molecular patterns. Given that cGAS cannot discriminate self from non-self DNA at the structural level, avoidance of self-DNA recognition needs to be counteracted by several means. Among others, cellular segregation of cGAS away from its ligand via compartmentalization of activatable cGAS to the cytosol and the plasma membrane, the sequestration of cGAS in the inactive form in the nucleus as well as the activity of DNA degrading enzymes that keep potential self-DNA substrates that enter the cytoplasm below a certain threshold seem to play key roles[179]. However, if these mechanisms are breached or overwhelmed by the abundant supply of self-DNA, for example in the context of persistent or increased DNA damage or cell death, cGAS–STING signalling can be potently activated. For example, loss of function mutations in the endolysosomal DNA endonuclease DNase II or the cytosolic DNA exonuclease TREX1, as observed in patients with Aicardi–Goutières syndrome, result in overactivation of cGAS–STING signalling due to increased availability of the DNA ligand[12,13,14,15]. As a consequence, constitutive STING activation and associated effector functions, such as type I interferon production, can result in cell or tissue damage. These disease entities are commonly referred to as type I interferonopathies[196], which also encompass diseases with other, related pathomechanisms. A similar scenario is observed for gain-of-function mutations in STING, as documented in patients with SAVI (STING-associated vasculopathy with onset in infancy), which result in a ligand-independent activation of its signalling[154]. Heterozygous mutations in TREX1 lead to less severe and heterogeneous phenotypes, such as systemic lupus erythematosus[197] or familial chilblain lupus erythematosus[198]. At the same time, a sizeable proportion of patients with systemic lupus erythematosus (15%) display increased cGAMP levels despite carrying wild-type alleles for TREX1 (ref.[199]). This argues for an as yet unknown or polygenic cause of systemic lupus erythematosus involving the cGAS–STING axis. At the

same time, aberrant activation of STING signalling has been implied in diverse chronic disease settings, such as macular degeneration[200], non-alcoholic steatohepatitis[201] or Parkinson disease[202]. Moreover, acute tissue damage, as seen in the context of myocardial infarction, has been shown to involve cGAS–STING activation with adverse outcomes[203]. Based on these considerations, efforts are being undertaken to develop compounds that antagonize the cGAS–STING signalling cascade at various levels: DNA binding of cGAS, catalytic activity of cGAS, cGAMP binding to STING and the use of allosteric modulators of STING, such as inhibitors of palmitoylation[204].

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Figure legends

Fig. 1: Overview of canonical cGAS–STING signalling.

Cyclic GMP–AMP synthase (cGAS) consists of a two-lobed catalytic domain and an extended amino-terminal (N-terminal) domain. It recognizes and is activated by DNA ligands (canonically single-stranded or double-stranded molecules longer than 40 bp) and assembles into a dimer, in which both cGAS protomers are bound to their own DNA ligand, with the DNA strands sandwiched between the two cGAS protomers. Each protomer has two principal DNA binding sites, A and B. In the dimer, the DNA molecules are bound to site A of one cGAS protomer and to site B of the respective other protomer. The crystal structure of this arrangement is shown in the right-hand box (PDB ID 6CTA). DNA binding sites A and B are annotated in this structure. This dimerization is required for cGAS to acquire a stable active conformation. Active cGAS produces the cyclic dinucleotide 2′,3′-cyclic GMP–AMP (2′,3′-cGAMP). The box on the right-hand side shows cGAMP as a stick model with green carbon atoms, blue nitrogen atoms, red oxygen atoms and white phosphorus atoms (PDB ID 4KSY). cGAMP binds to stimulator of interferon genes (STING), leading to TANK-binding kinase 1 (TBK1)-dependent phosphorylation (P) of interferon regulatory factor 3 (IRF3). The active IRF3 dimer translocates to the nucleus and activates transcription of type I interferon genes. cGAS–STING signalling leads also to the expression of genes for pro-inflammatory cytokines through NF-κB (dashed lines). Left insets list the main sources of DNA serving as cGAS ligands, downstream pathways of cGAS–STING signalling and implications of cGAS–STING signalling in diseases. ER, endoplasmic reticulum.

Fig. 2: Cellular localization and DNA ligands of cGAS.

Cyclic GMP–AMP synthase (cGAS) is found in the cytosol where it encounters its agonist DNA substrates (highlighted in red), mostly comprising long DNAs. However, other DNAs, including Y-form DNA (comprising a double-stranded DNA (dsDNA)–single-stranded DNA junction) from retroviruses are also able to activate cGAS. Agonistic DNA can be derived from a plethora of sources (shown in blue boxes), including exogenous sources (DNA viruses, retroviruses, intracellular bacteria) and endogenous sources (stressed mitochondria, which release mitochondrial DNA (mtDNA) and chromatin fragments in the cytosol and micronuclei resulting from mitotic or replicative crises and DNA damage). A prominent pool of cGAS is sequestered at the plasma membrane via binding to phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P2) on the inner plasma membrane leaflet. This sequestration could help keep cGAS away from endogenous DNA, at the same time allowing cGAS to detect more rapidly or efficiently DNA from viruses entering the cell (dashed arrows). In steady state, cGAS is also sequestered in the nucleus by binding to chromatin. Chromatin-bound cGAS is inactive. Nevertheless, it has been reported for HIV-2 that its DNA sensing by cGAS occurs in the nucleus, raising the possibility of nuclear sensing of viruses by cGAS. After binding its agonist DNA, cGAS is activated, which leads to enzymatic production of 2′,3′-cyclic GMP–AMP (2′,3′-cGAMP), which activates stimulator of interferon genes (STING) downstream signalling (Fig. 5). cGAMP can also be released to the extracellular space, for example by dying cells, where it can be degraded by phosphodiesterases, such as ENPP1. Nevertheless, some extracellular cGAMP can also enter other cells in the microenvironment either via endocytosis or uptake via transporters, such as SLC19A1, inducing paracrine signalling. Finally, cGAMP can spread to bystander cells via gap

junctions or by being incorporated into viral capsids. In addition to its role in activating STING, nuclear cGAS has also been implicated in inhibiting DNA repair via homologous recombination, but how certain types of DNA damage induce activation of cGAS, whereas non-damaged chromatin does not, remains to be established. ecDNA, extrachromosomal DNA; ER, endoplasmic reticulum; IL-1R, interleukin-1 receptor. MIMP, mitochondrial inner membrane permeabilization; MOMP, mitochondrial outer membrane permeabilization.

Fig. 3: Mechanism of cGAS activation.

a) Structure of ligand-free (apo) human cyclic GMP–AMP synthase (cGAS) (in 'side' view, PDB ID 4KM5) (left). DNA-bound cGAS dimer in the active conformation (PDB ID 6CTA). DNA binding and dimer formation activate cGAS by ordering of an active site activation loop and closure of the active site channel. Of note, the amino-terminal (N-terminal) domain (indicated on apo cGAS) has not yet been resolved in any structure (middle). Oligomeric states showing how binding to longer DNA in a ladder configuration (PDB ID 5N6I) and DNA binding at the newly discovered site C (PDB ID 6EDB) can lead to reinforced dimers and more condensed structures (right; see part c for cartoon models). b) 'Front' view of cGAS showing the three DNA binding sites A, B and C (PDB ID 6EDB). C) Model for the allosteric/cooperative activation of cGAS by clustering. Binding of cGAS to non-agonistic (short) DNA does not robustly lead to formation of an active dimer state. cGAS also binds to nucleosomes, which does not lead to an active state but inactive cGAS sequestration; the mode of binding of cGAS to chromatin is as yet unknown. In the presence of longer DNA, however, different means of clustering oligomerization and arrangement of DNA (formation of ladder-like structures of cGAS dimers along DNA, interaction with DNA via N-terminal charged patches, mesh-like interactions with DNA via site C, DNA shaping via DNA-bending proteins) lead to more stable, active dimer structures and promote the formation of large liquid–liquid phase-separated condensates. In these condensates, cGAS is highly concentrated, which likely further stabilizes the active dimeric state and promotes its catalytic activity.

Fig. 4: Mechanism of STING activation.

a) Cartoon of the membrane-bound stimulator of interferon genes (STING) dimer with annotated structural domains and elements. B) Cryo-electron microscopy structure of the ligand-free chicken STING dimer (left, PDB ID 6NT6) and the 2′,3′-cyclic GMP–AMP (cGAMP)-bound STING dimer (PDB ID 6NT7). 2′,3′-cGAMP binding results in closing of the lid elements and a rotation of the ligand-binding domains (LBDs) with respect to the transmembrane domains (each formed by four transmembrane helices 1–4), generating a surface geometry for oligomerization. Cys148 of human STING (green stars) is implicated in disulfide bond formation that stabilizes oligomers. c) Sequence region in the C-terminal tail (CTT; not resolved in the structural studies) harbouring the site that upon phosphorylation by TANK-binding kinase 1 (TBK1) binds interferon regulatory factor 3 (IRF3). d) Cartoon model depicting the events from 2′,3′-cGAMP binding to IRF3 activation. 2′,3′-cGAMP binds to the ligand-binding pocket formed by the LBDs of the STING dimer. This is associated with a 180° clockwise rotation of its LBDs in relation to the transmembrane region, and this conformation change is suggested to generate a surface geometry that promotes lateral STING oligomerization. STING dimers associate — via their CTTs — with TBK1. Ligand binding and STING oligomerization appear to promote in trans phosphorylation of TBK1. Active TBK1 then phosphorylates the STING CTT (this occurs on an adjacent dimer of STING, rather than the dimer to which TBK1 is

bound). CTT phosphorylation (P) creates a docking site for IRF3, which is subsequently phosphorylated by TBK1. IRF3 then dimerizes, gaining transcriptional activity. C-terminal, carboxy-terminal; ER, endoplasmic reticulum; ERGIC, ER–Golgi intermediate compartment.

Fig. 5: Effector functions of STING.

Stimulator of interferon genes (STING) functions as a dimer, which is formed at the endoplasmic reticulum (ER). Upon 2′,3′-cyclic GMP–AMP (2′,3′-cGAMP) binding, STING undergoes a conformational shift that results in the closure of its lid structure, a rotation of its ligand-binding domains (LBDs) in relation to its transmembrane domains (Fig. 4a,b), which favours oligomerization of active STING dimers in a side-to-side configuration. STING oligomers exit the ER towards the ER–Golgi intermediate compartment (ERGIC). a) As early as the ERGIC, STING oligomers facilitate TANK-binding kinase 1 (TBK1). Active TBK1 then phosphorylates Ser366 of the STING C-terminal tail (CTT), which serves as a docking site for interferon regulatory factor 3 (IRF3). IRF3 is then phosphorylated by TBK1, resulting in the formation of an active transcription factor complex (Fig. 4d). b) Activation of NF-κB is either governed downstream of TBK1 activation, which requires the CTT of STING, or emanates from a yet to be identified signal from LBDs in STING in its active configuration. c) At the ERGIC, STING has been implicated in autophagy induction. Specifically, the STING-containing ERGIC has been shown to serve as a membrane source for autophagosome biogenesis, with cGAMP stimulation promoting lipidation of the key autophagosome biogenesis factor, LC3. This activity of STING is independent of its CTT or TBK1 activity, but the exact mechanism and contribution of STING to autophagy induction remain to be determined. d) After being routed through the Golgi, STING is trafficked through late endosomes to the lysosome. The exact topology of STING at or within the lysosome needs to be determined. Possible locations are at the outer lysosomal membrane or within multivesicular bodies (MVBs). In certain cell types (for example, human myeloid cells), lysosomal accumulation of STING leads to lysosomal membrane permeabilization, which subsequently results in a lytic form of cell death known as lysosome-dependent cell death and a secondary inflammatory response. Nevertheless, the primary role of directing STING to the lysosome seems to be its degradation, which limits the active pool of STING molecules.

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Competing interests

The authors declare no competing interests.

Related links

PDB ID 6CTA: https://www.rcsb.org/structure/6CTA

PDB ID 4KSY: https://www.rcsb.org/structure/4KSY

PDB ID 4KM5: https://www.rcsb.org/structure/4KM5

PDB ID 5N6I: https://www.rcsb.org/structure/5N6I

PDB ID 6EDB: https://www.rcsb.org/structure/6EDB

PDB ID 6NT6: https://www.rcsb.org/structure/6NT6

PDB ID 6NT7: https://www.rcsb.org/structure/6NT7

Glossary

Innate immune system

A heterogeneous system of molecules, signal transducers and cells that has evolved to detect invading microbes, and elicits a first line of antimicrobial defence and activates the adaptive immune system.

Pattern recognition receptors

(PPRs). Germline-encoded receptors of the innate immune system. They detect pathogen or danger/damage-associated molecular patterns and elicit cellular defence reactions.

Pathogen and damage-associated molecular patterns

(PAMPs and DAMPs). Molecules that signal the presence of pathogens (that is, PAMPs) or danger/damage (that is, DAMPs). They are recognized by pattern recognition receptors.

Adaptive immune system

A branch of the immune system that comprises B and T lymphocytes. It elicits a highly specific response to antigens through highly variable, clonally expressed B cell receptors/antibodies or T cell receptors.

Nucleotidyl transferase

(NTase). A class of enzymes that transfer nucleoside phosphates onto an acceptor, typically a hydroxyl group.

Cyclic dinucleotide

Two nucleoside phosphates, joined in a circular dinucleotide through two phosphodiester linkages. Cyclic dinucleotides are found in prokaryotes and higher eukaryotes, and typically have second messenger functions to regulate diverse activities, such as bacterial biofilm and planktonic lifestyles (c-di-GMP), osmolyte homeostasis (c-di-AMP) and eukaryotic innate immune signalling (cyclic GMP–AMP).

Cellular senescence

A state that is generally characterized by a permanent cell cycle arrest in the context of ageing or tumour suppression. It can be promoted by multiple factors, including oxidative stress, DNA damage, mitochondrial dysfunction and the unfolded protein response.

Aicardi–Goutières syndrome

A rare, genetically determined progressive encephalopathy with autoimmune features that is caused by mutations in various genes involved in nucleic acid metabolism.

Systemic lupus erythematosus

A systemic, chronic, autoimmune disease that affects connective tissue of the kidneys, heart, lungs, brain, blood and skin.

Liquid–liquid phase separation

In cell biology, the separation of macromolecules (proteins, nucleic acids) into two liquid-like phases with high and low concentrations of the macromolecule.

Major histocompatibility complex class II

(MHC II). Surface molecules, typically found on antigen presenting cells such as dendritic cells, macrophages and B cells, that display peptides derived from extracellular proteins for interaction with immune cells.

Dendritic cells

Specialized antigen-presenting cells in a heterogeneous group that act as a link between the innate and adaptive immune system. They take up and process antigens and induce naive T lymphocyte activation and effector cell differentiation.

Extracellular vesicles

Secreted vesicles produced by many cell types, including tumour cells that can carry a wide variety of cellular components, including DNA, RNA, proteins and other molecules. They include two main subpopulations known as microvesicles and exosomes that differ in their mode of biogenesis (plasma membrane versus endosomal origin).

Natural killer cells

Cytotoxic effector lymphocytes of the innate immune system that play critical roles in antitumour and antimicrobial defence.

BAK/BAX macropores

Pores in the outer membrane of mitochondria, formed by the BAK and BAX proteins to trigger cytochrome *c* release and apoptosis.

Effector caspases

Proteinases activated through cleavage by initiator caspases (caspase cascade) that then proteolytically cleave many target proteins to execute apoptosis.

Mitochondrial stress

Pathophysiological conditions leading to mitochondrial DNA stress, mitochondrial unfolded protein response and stress signalling pathways.

Nucleosomal arrays

Sequences of regularly spaced nucleosomes along the DNA, typically found at gene bodies of eukaryotic chromosomes.

Nucleosome core particles

Histone protein octamers together with ~147 bp of tightly wrapped DNA, which is protected from nuclease digestion.

Linker DNA

DNA connecting two adjacent nucleosome core particles. This can range in length in different species, cell types and loci.

Nuclear envelope blebbing

The formation of membrane bulges at the nuclear envelope. At these sites, the membrane separates from the underlying lamina, allowing the chromatin to herniate and protrude into the bleb. In the event of membrane rupture, blebbing results in exchanging material between cytosol and nucleoplasm.

Telomeres

Specialized protective end structures of linear chromosomes, consisting of a repetitive DNA sequence and associated proteins.

Senescence-associated cell cycle arrest

The shortening of telomeres leading to a prolonged DNA damage response and check-point activation to trigger a permanent cell cycle arrest, a hallmark of senescent cells.

Dicentric chromosomes

Chromosomes containing two centromeres formed through genome rearrangements.

Alternative lengthening of telomeres

(ALT). The telomerase-independent, recombination-dependent mode of extension of telomeres in cancer cells.

Autophagy

A regulated system in which the cell degrades unwanted cellular components by incorporation into autophagosomes followed by fusion with lysosomes.

γ-H2AX

The phosphorylation of histone 2A variant X at Ser139, leading to nucleosomes containing γ-H2AX. This mark is an early cellular response to DNA double-strand breaks, which serves in the recruitment of other repair factors.

Poly-ADP ribose polymerase 1

(PARP1). An enzyme that binds and marks DNA breaks by adding poly-ADP-ribose (PAR) onto itself and other targets.

LINE1

(Long interspersed nuclear element 1). A class I transposable element, typically 6 kb long, present in the genome of humans and some other organisms. LINE1 elements comprise around 17% of the human genome.

Homologous recombination

A DNA double-strand break repair pathway in which a DNA end is resected, and the resulting single-strand is extended on a homologous template

Gap junctions

Specialized cell–cell channels formed by juxtaposed connexon pores of adjacent cells, allowing the cytoplasmic exchange of small molecules and ions.

TIMELESS

A protein implicated in the circadian rhythm, replication and enhancing homologous recombination through interactions with different proteins.

Non-homologous end-joining

A DNA double-strand break repair pathway in which two DNA ends are directly ligated, often after limited processing by nucleases and DNA polymerases, in a pathway that depends on DNA-dependent protein kinase and DNA ligase IV.

COPII coat complex

A protein complex in the secretory pathway composed of five proteins that coats membrane vesicles transporting material from the endoplasmic reticulum to the Golgi apparatus.

ER–Golgi intermediate compartment

(ERGIC). An organellar structure that mediates trafficking between the endoplasmic reticulum (ER) and the Golgi apparatus.

Palmitoylation

A lipid modification of proteins through covalent attachment of palmitic acid, regulating different properties of proteins, such as membrane interaction, stability and trafficking.

MAPK

A type of protein kinase that transduces extracellular signals, such as growth factors, cytokines and mitogens, to cellular programmes, such as growth, differentiation, inflammation and others.

LC3

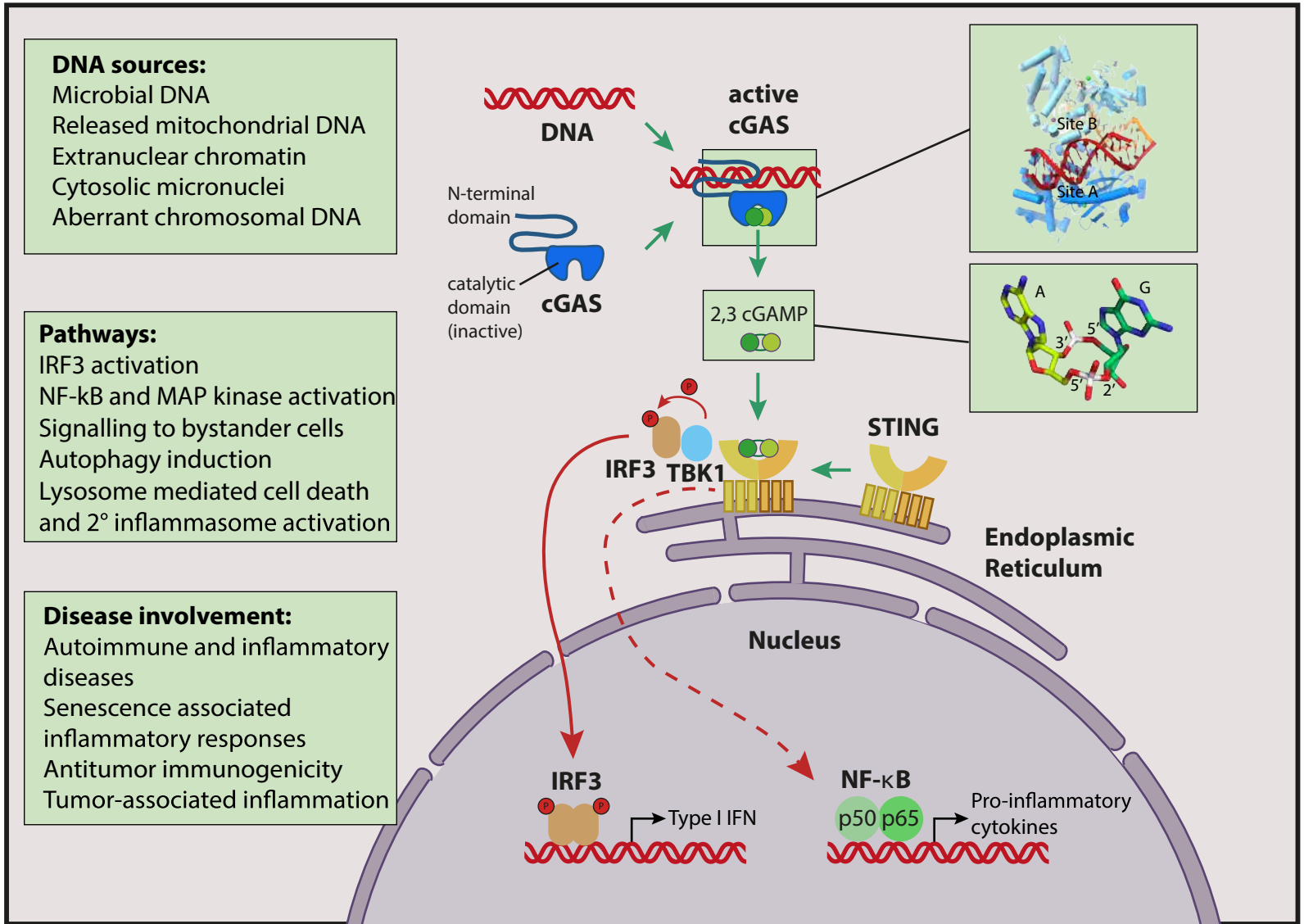
(Also known as microtubule-associated proteins 1A/1B light chain 3B). A protein that functions in substrate selection in autophagy and is used as a marker for autophagosomes.

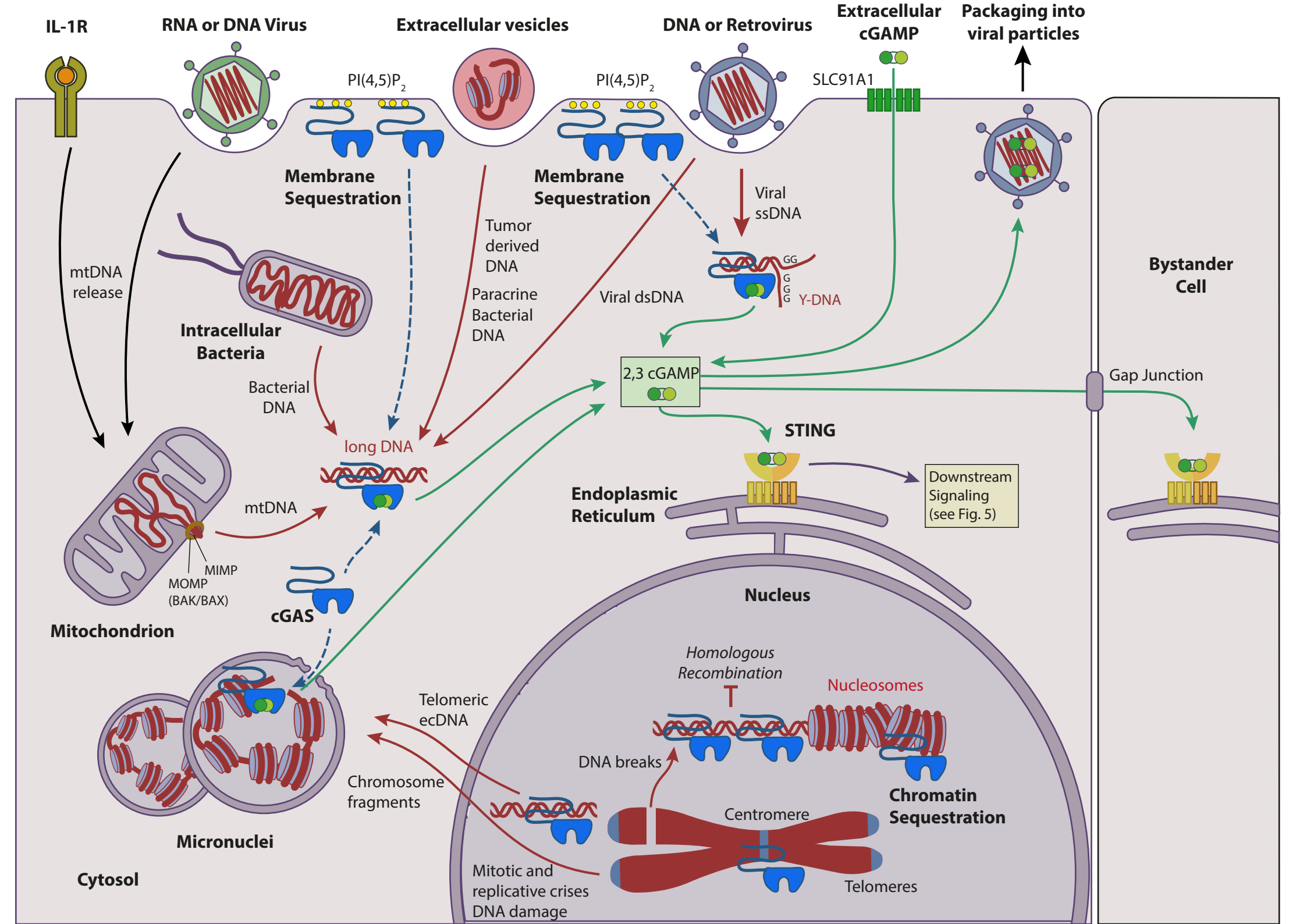
SAVI

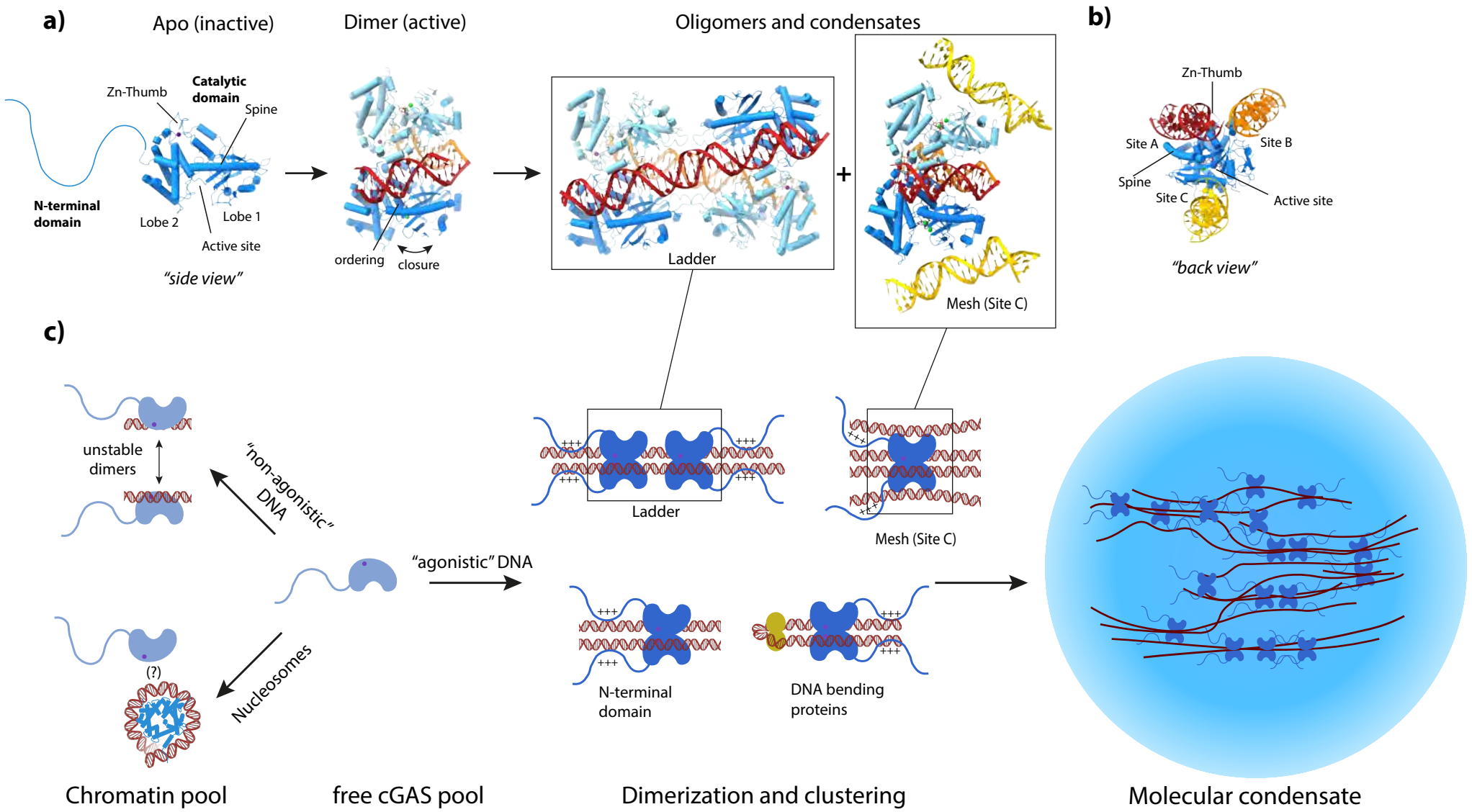
(STING-associated vasculopathy with onset in infancy). A rare autoinflammatory vasculopathy characterized by severe skin lesions and interstitial lung disease.

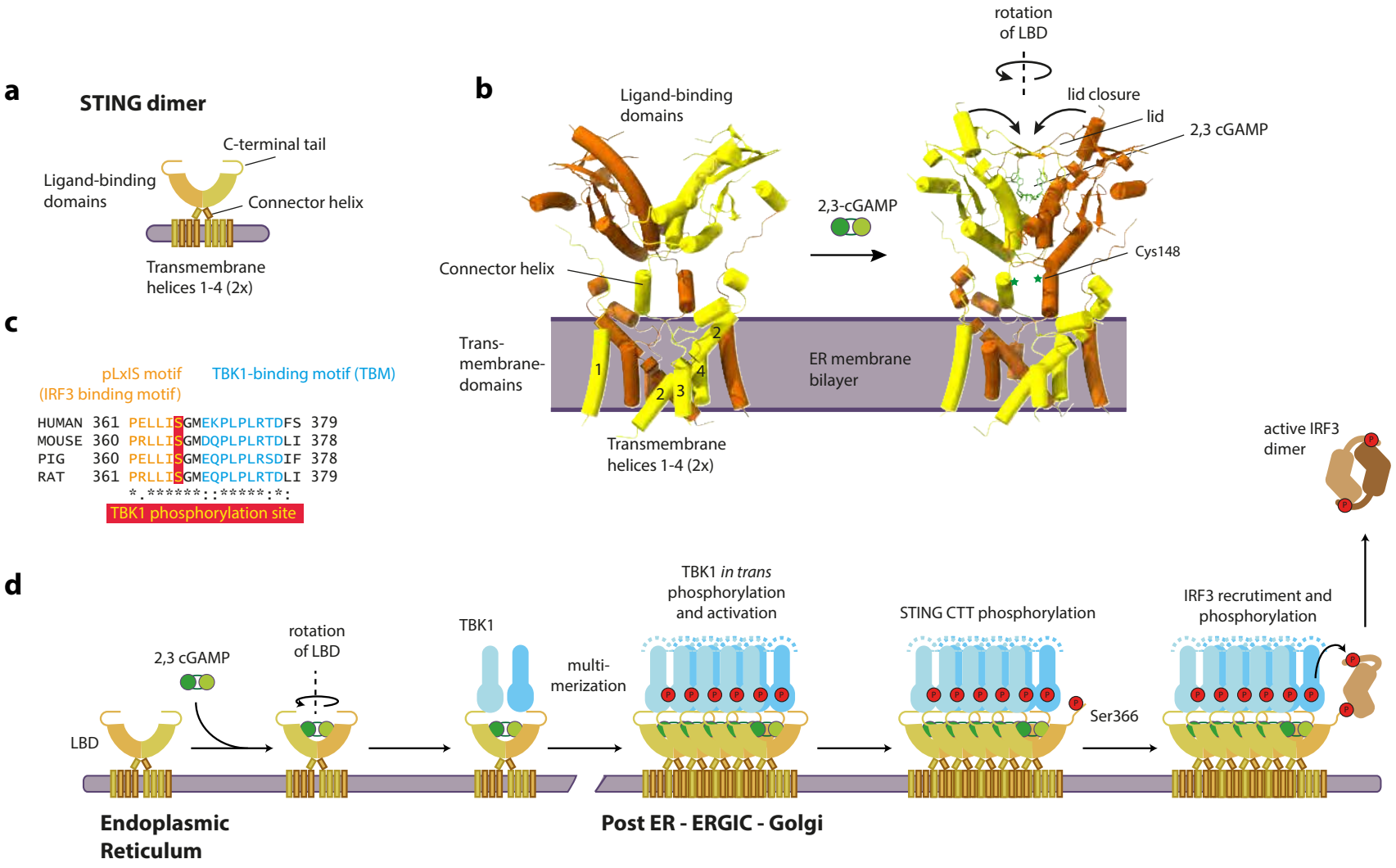
NLRP3 inflammasome

A multiprotein complex that initiates a pro-inflammatory cell death with the release of IL-1β in response to activation by NLRP3, which senses microbial and endogenous danger signals.


DNA sources:
Microbial DNA
Released mitochondrial DNA
Extranuclear chromatin
Cytosolic micronuclei
Aberrant chromosomal DNA
Pathways:
IRF3 activation
NF-kB and MAP kinase activation
Signalling to bystander cells
Autophagy induction
Lysosome mediated cell death
and 2° inflammasome activation
Disease involvement:
Autoimmune and inflammatory diseases
Senescence associated inflammatory responses
Antitumor immunogenicity
Tumor-associated inflammation
DNA
active cGAS
N-terminal domain
catalytic domain (inactive)
cGAS
2,3 cGAMP
Site B
Site A
A
G
3′
5′
5′
2′
STING
IRF3
TBK1
Endoplasmic Reticulum
Nucleus
IRF3
Type I IFN
NF-κB
p50 p65
Pro-inflammatory cytokines


IL-1R
RNA or DNA Virus
Extracellular vesicles
DNA or Retrovirus
Extracellular cGAMP
Packaging into viral particles
PI(4,5)P2
PI(4,5)P2
SLC91A1
Membrane Sequestration
Membrane Sequestration
Viral ssDNA
mtDNA release
Tumor derived DNA
Paracrine Bacterial DNA
Viral dsDNA
GG
G
G
G
Y-DNA
Intracellular Bacteria
Bacterial DNA
2,3 cGAMP
Gap Junction
Bystander Cell
long DNA
STING
Endoplasmic Reticulum
Downstream Signaling (see Fig. 5)
mtDNA
MIMP
MOMP
(BAK/BAX)
Mitochondrion
cGAS
Nucleus
Homologous Recombination
Nucleosomes
Telomeric ecDNA
DNA breaks
Chromosome fragments
Chromatin Sequestration
Centromere
Micronuclei
Mitotic and replicative crises DNA damage
Telomeres
Cytosol


a)
Apo (inactive)
Dimer (active)
Oligomers and condensates
Zn-Thumb
Catalytic domain
Spine
N-terminal domain
Lobe 2
Lobe 1
Active site
"side view"
ordering
closure
Ladder
+
Mesh (Site C)
b)
Zn-Thumb
Site A
Site B
Spine
Site C
Active site
"back view"
c)
unstable dimers
"non-agonistic" DNA
Ladder
Mesh (Site C)
"agonistic" DNA
(?)
Nucleosomes
N-terminal domain
DNA bending proteins
Chromatin pool
free cGAS pool
Dimerization and clustering
Molecular condensate
Stabilization and concentration of active cGAS dimers


a
STING dimer
C-terminal tail
Ligand-binding domains
Connector helix
Transmembrane helices 1-4 (2x)
b
Ligand-binding domains
rotation of LBD
lid closure
lid
2,3 cGAMP
2,3-cGAMP
Cys148
Connector helix
Trans-membrane-domains
ER membrane bilayer
Transmembrane helices 1-4 (2x)
c
pLxIS motif (IRF3 binding motif)
TBK1-binding motif (TBM)
HUMAN 361 PELLISGMEKPLPLRTDFS 379
MOUSE 360 PRLLISGMDQPLPLRTDLI 378
PIG 360 PELLISGMEQPLPLRSDIF 378
RAT 361 PRLLISGMEQPLPLRTDLI 379
*.******::*****:*:
TBK1 phosphorylation site
active IRF3 dimer
d
2,3 cGAMP
rotation of LBD
LBD
TBK1
multi-merization
TBK1 in trans phosphorylation and activation
STING CTT phosphorylation
Ser366
IRF3 recrutiment and phosphorylation
Endoplasmic Reticulum
Post ER - ERGIC - Golgi

Golgi exit

Late endosome

d. Lysosomal trafficking

Lysosomal membrane permeabilization

Release of lysosomal hydrolases

Lysosomal cell death

Trans Golgi

Lysosome

MVB?

STING degradation

Golgi

Cis Golgi

Fusion with lysosome

c. Autophagy induction

Formation of isolation membrane

LC3 lipidation

ERGIC

cGAMP

ligand binding

rotation

multi-merization

ER exit

ER

b. NF-kB activation

p50 p65

LBD-dependent?

CTT & TBK1 dependent?

Post ER - ERGIC - Golgi

a. IRF3 activation

TBK1 *in trans* phosphorylation and activation

STING CTT phosphorylation

IRF3 recrutiment and phosphorylation

active IRF3 complex

Ser366

Post ER - ERGIC - Golgi

Table 1 Accessory binding partners of cGAS and STING

Binding partner	Function	Interaction	Ref.
cGAS			
NONO	Associates with cGAS in the nucleus and facilitates sensing of HIV DNA	Direct interaction (no specific domain mapped)	60
PQBP1	Binds reverse-transcribed HIV-1 DNA and facilitates its detection by cGAS	Direct interaction through the WW domain within PQBP1	61
ZCCHC3	Enhances the binding of cGAS to dsDNA	Direct interaction with the NTase fold (amino acids 213–382) and the C-terminal fragment (amino acids 383–522) of cGAS	63
G3BP1	Enhances the binding of cGAS to dsDNA	Interaction with the N-terminal domain	62
STING			
STIM1	Retains STING at the ER under steady-state conditions	Direct interaction of STIM1 with transmembrane regions of STING	161
IFI16	Facilitates recruitment of TBK1 to STING and its phosphorylation of STING	Not tested	162
TOLLIP	Stabilizes STING on the ER under steady-state conditions	Direct interaction of the C-terminal region of TOLLIP with transmembrane regions of STING	163

cGAS, cyclic GMP–AMP synthase; C-terminal, carboxy-terminal; dsDNA, double-stranded DNA; ER, endoplasmic reticulum; G3BP1, GTPase-activating protein SH3 domain-binding protein 1; IFI16, interferon-γ inducible protein 16; NONO, non-POU domain-containing octamer binding protein; NTase, nucleotidyl transferase; N-terminal, amino-terminal; PQBP1, polyglutamine binding protein 1; STIM1, stromal interaction molecule 1; STING, stimulator of interferon genes; TBK1, TANK-binding kinase 1; TOLLIP, Toll interacting protein; WW, protein interaction domain containing a signature double tryptophane motif; ZCCHC3, CCHC-type zinc-finger protein 3.

Table 2 Regulation of cGAS and STING activity by post-translational modifications

Site	Location	Type of PTM	Enzyme	Function	Refs
cGAS					
Asp140, Asp157	N-terminal domain	Cleavage	Caspase 1	Suppresses type I interferon signalling	116
Tyr215	Site A	Phosphorylation	Bruton's tyrosine kinase	Reduces DNA binding and DNA damage-induced nuclear localization	37,38
Asp319	Active site metal coordinator	Cleavage	Caspase 3	Suppresses type I interferon signalling	110,164
Ser305	Active site	Phosphorylation	AKT	Inhibits catalytic activity	109
Lys347, Lys384, Lys394*	Site A (Lys384) cGAS dimer interface (Lys347, Lys394)	Sumoylation	Unknown	Inhibits cGAS	114
		Desumoylation	SENP7	Facilitates cGAS activity	
Lys231, Lys479	Site A (Lys231) Surface residue near active site (Lys479)	Sumoylation	TRIM38	Inhibits cGAS	113
		Desumoylation	SENP2	Facilitates cGAS activity	
Glu286*	Surface near site C (?)	Glutamylation	TTLL6	Inhibits DNA binding	165
		Deglutamylation	CCP6	Promotes DNA binding	
Glu314*	Surface near active site	Glutamylation	TTLL4	Inhibits synthase activity	165
		Deglutamylation	CCP5	Promotes synthase activity	
Lys384, Lys394, Lys414	Site A (Lys384) Dimerization surface (Lys394) Active site (Lys414)	Acetylation	Unknown	Inhibits activity	111
		Deacetylation	HDAC3	Promotes activity	
Lys414	Active site	Ubiquitylation (Lys48)	Unknown	Promotes proteasomal degradation	166
		Deubiquitylation	TRIM14, USP14	Increases cGAS protein stability	
Lys173, Lys384	Site A	Ubiquitylation (Lys27)	RNF185	Enhances catalytic activity	115

Site	Location	Type of PTM	Enzyme	Function	Refs
Lys137, Lys150, Lys224, Lys236	Connector region (Lys137, Lys150) LBD (Lys224, Lys236)	Ubiquitylation (Lys27)	AMFR	Promotes interaction with TBK1	167
Ubiquitylated lysines (Lys48)	Not tested	Deubiquitylation (Lys48)	USP27X	Enhances stability	168
STING					
Ser366	CTT	Phosphorylation	TBK1	Provides docking site for IRF3	169
Cys88, Cys91	TMD	Palmitoylation	DHHC3, DHHC7 and DHHC15	Promotes multimerization	138
Lys150	Connector region between TMD and LBD	Ubiquitylation (Lys48)	RNF5	Induces proteasomal degradation	170
Lys150	Connector region between TMD and LBD	Ubiquitylation (Lys63)	TRIM56	Promotes dimerization and multimerization	171
Lys150	Connector region between TMD and LBD	Ubiquitylation (Lys11)	RNF26	Regulates accessibility for Lys48-linked ubiquitylation	172
Lys20, Lys150, Lys224, Lys236	Cytosolic face of TMD (Lys20) Connector region (Lys150) LBD (Lys224, Lys236)	Ubiquitylation (Lys63)	TRIM32	Promotes interaction with TBK1	173
Lys224	LBD	Ubiquitylation (Lys63)	MUL1	Promotes phosphorylation by TBK1	174
Lys137, Lys150, Lys224, Lys236	Connector region (Lys137, Lys 150) LBD (Lys 224, Lys 236)	Ubiquitylation (Lys27)	AMFR	Promotes interaction with TBK1	167
Cys148	Connector region	Disulfide bond	NA	Promotes multimerization	135

Site	Location	Type of PTM	Enzyme	Function	Refs
Lys338	CTT near LBD attachment	Sumoylation	TRIM38	Promotes protein stability	113
		Desumoylation	SENP2	Decreases protein stability	
Lys150	Connector region between TMD and LBD	Deubiquitylation (Lys48)	CYLD	Promotes stability	175
Not tested	NA	Deubiquitylation (Lys27)	USP13	Reduces interaction with TBK1	176

All amino acid numberings are provided for the human protein. If studies in other species were conducted (designated*), the amino acid numbering of the homologous positions of the human protein is provided. AKT, RACα serine/threonine-protein kinase (also known as PKB); AMFR, autocrine motility factor receptor; CCP, cytosolic carboxypeptidase; cGAS, cyclic GMP–AMP synthase; CTT, C-terminal tail; CYLD, cylindromatosis; DHHC, named after Asp–His–His–Cys sequence motif; HDAC3, histone deacetylase 3; IRF3, interferon regulatory factor 3; LBD, ligand-binding domain; MUL1, mitochondrial E3 ubiquitin protein ligase 1; NA, not applicable; PTM, post-translational modification; RNF, RING finger protein; SENP, sentrin/SUMO-specific protease; STING, stimulator of interferon genes; TBK1, TANK-binding kinase 1; TMD, transmembrane domain; TRIM, tripartite motif proteins; TTLL, tubulin-tyrosine ligase-like; USP, ubiquitin specific peptidase.