


Nuclear cGAS: guard or prisoner?

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Abstract

cGAS, an innate immune sensor of cellular stress, recognizes double-stranded DNA mislocalized in the cytosol upon infection, mitochondrial stress, DNA damage, or malignancy. Early models suggested that cytosolic localization of cGAS prevents autoreactivity to nuclear and mitochondrial self-DNA, but this paradigm has shifted in light of recent findings of cGAS as a predominantly nuclear protein tightly bound to chromatin. This has raised the question how nuclear cGAS is kept inactive while being surrounded by chromatin, and what function nuclear localization of cGAS may serve in the first place? Cryo-EM structures have revealed that cGAS interacts with nucleosomes, the minimal units of chromatin, mainly via histones H2A/H2B, and that these protein–protein interactions block cGAS from DNA binding and thus prevent autoreactivity. Here, we discuss the biological implications of nuclear cGAS and its interaction with chromatin, including various mechanisms for nuclear cGAS inhibition, release of chromatin-bound cGAS, regulation of different cGAS pools in the cell, and chromatin structure/chromatin protein effects on cGAS activation leading to cGAS-induced autoimmunity.

Keywords chromatin; cyclic GMP–AMP synthase; DNA sensing; innate immunity; nucleosome

Subject Categories Chromatin, Transcription & Genomics; Immunology; Structural Biology

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A cytosolic DNA sensor in the nucleus

Imagine you are a principal of a medieval village and need to place a guard to listen for approaching villains at night. Would you place the guard at a very quiet spot near the outer wall, or rather in the vibrant and noisy inn at the center of the village? We all know the answer, but if it comes to the vertebrate's cell innate immune system, cGAS, the guard sensing foreign DNA, is strangely situated where all the noise is, i.e., bound to chromatin in the nucleus. Upon sensing DNA, cGAS (for “cyclic GMP–AMP synthase”) catalyzes the production of 2'3'-cGAMP from the common nucleotide–triphosphate precursors ATP and GTP (Ablasser *et al.*, 2013; Gao *et al.*,

2013; Sun *et al.*, 2013). 2'3'-cGAMP, a highly stable small molecule with a unique mixed-phosphodiester linkage, can be transferred between cells and is recognized by the transmembrane receptor protein Stimulator of Interferon Genes (STING STING) (Ablasser *et al.*, 2013; Zhang *et al.*, 2013) (Fig 1). Oligomers formed by activated STING trigger pleiotropic downstream events, including activation of IRF3- and NF- κ B-dependent signaling cascades for production of type I interferons and pro-inflammatory cytokines, autophagy, and lysosomal cell death (Ishikawa & Barber, 2008; Zhong *et al.*, 2008; Sun *et al.*, 2009; Wu *et al.*, 2013). For more details on cGAS–STING downstream signaling, please refer to (Hopfner & Hornung, 2020).

cGAS is a nucleotidyltransferase that consists of an unstructured flexible N-terminal domain of about 160 amino acids length, followed by a bilobal C-terminal catalytic domain of the Mab21 fold containing a zinc-binding dimerization motif (zinc-thumb, Fig 2A). cGAS is activated by DNA-dependent dimerization and multimerization (Fig 2B). Here, two DNA binding sites at its catalytic domain, denoted A and B, cooperate to assemble cGAS dimers on two sandwiched DNA molecules. Formation of higher-order oligomers concentrates cGAS and DNA and stabilizes the active dimer states. Human cGAS additionally possesses a third DNA binding site C, which is involved in providing additional DNA contacts to stabilize higher-order cGAS–DNA structures, including liquid-phase condensates (Civril *et al.*, 2013; Li *et al.*, 2013; Zhang *et al.*, 2014; Du & Chen, 2018; Xie *et al.*, 2019) (Fig 1).

cGAS efficiently recognizes dsDNA of a minimal length of > 40 bp, making pathogenic foreign DNA as well as endogenous nuclear or mitochondrial DNA potent cGAS agonists (Hopfner & Hornung, 2020). When self-DNA leaks into the cytosol during cellular stress (such as mitochondrial stress, DNA damage, mitotic arrest, or senescence), or is present in form of cytosolic micronuclei, cGAS is activated leading to a state of sterile inflammation (Härtlova *et al.*, 2015; West *et al.*, 2015; de Oliveira Mann & Kranzusch, 2017; Glück *et al.*, 2017; Harding *et al.*, 2017; Mackenzie *et al.*, 2017; Yang *et al.*, 2017; Ablasser & Chen, 2019; Zierhut *et al.*, 2019). In a healthy cell, however, cGAS is kept inactive even in cellular events that directly expose it to self-DNA, such as mitosis, when cGAS associates with chromatin directly after nuclear envelope breakdown or remains in the form of post-mitotic persistent nuclear cGAS pools bound to chromatin (Yang *et al.*, 2017; Zierhut *et al.*, 2019).

The question how cGAS autoreactivity to, e.g., genomic DNA is prevented arose as soon as cGAS was discovered as the cytosolic

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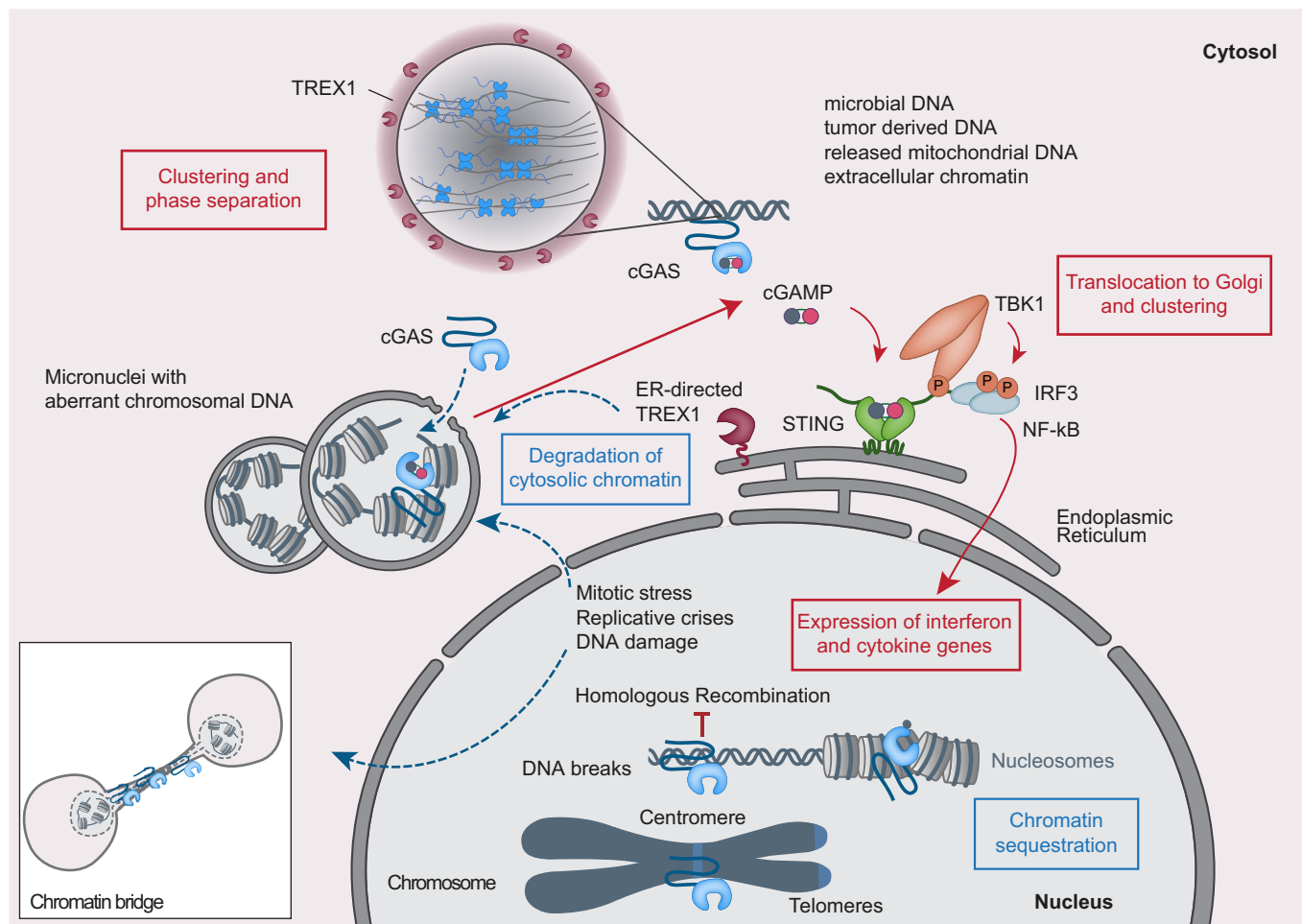


Figure 1. Simplified scheme of the cGAS–STING pathway and sources of nuclear and external DNA.

Cytosolic DNA recognition leads to formation of active cGAS via clustering and formation of large liquid–liquid phase-separated cGAS–DNA condensates excluding the ER-directed exonuclease TREX1. Nuclear cGAS is sequestered at chromatin in an inactive state. Active cGAS produces cyclic GMP–AMP (cGAMP), which binds to STING. STING relocates to the perinuclear Golgi and forms a clustered platform on which the TBK1 kinase phosphorylates the transcription factor IRF3. Phosphorylated IRF3 enters the nucleus and along with NF- κ B triggers expression of type I interferon and pro-inflammatory cytokine genes.

DNA sensor upstream of STING. First described as a cytosolic protein (Sun *et al.*, 2013), cGAS was soon found to actually be present in both cytosol and nucleus (Orzalli *et al.*, 2015). However, the lack of tools to study endogenous cGAS, combined with the lack of understanding of physiological roles of cGAS in the nucleus, kept the field in a state of controversy. In the meantime, nuclear cGAS has been implicated in a plethora of related functions, from canonical roles such as recognition of herpesviral DNA or HIV infection within the nucleus to non-canonical roles in genome surveillance and inhibition of DNA repair (Orzalli *et al.*, 2015; Lahaye *et al.*, 2018; Liu *et al.*, 2018; Jiang *et al.*, 2019). Recent findings indicate that cGAS is even sequestered in the nucleus through highly salt-resistant interactions with chromatin (Volkman *et al.*, 2019). These tight interactions involving DNA binding site B in cGAS are important for maintaining nuclear cGAS inactive and avoiding autoreactivity in the cell. In cells, cGAS can bind to nucleosomes with high affinity and without being activated (Lahaye *et al.*, 2018; Zierhut *et al.*, 2019). However, cGAS can be activated when incubated *in vitro*

with synthetic or extracted cellular chromatin, indicating that nucleosomal linker DNA may act as activating ligand *in vitro* and suggesting that additional cellular mechanisms are in place to keep cGAS suppressed in the nucleus (Mackenzie *et al.*, 2017).

Nuclear cGAS is inhibited by the acidic patch of the nucleosome

A recent collection of six publications, reporting a total of eleven cryo-electron microscopy structures of cGAS bound to the nucleosome, revealed how nuclear cGAS is tethered to and kept inactive by chromatin (Boyer *et al.*, 2020; Cao *et al.*, 2020; Kujirai *et al.*, 2020; Michalski *et al.*, 2020; Pathare *et al.*, 2020; Zhao *et al.*, 2020). These studies, using human nucleosome core particles (NCPs) reconstituted on the synthetic “Widom 601” DNA-positioning sequence, were facilitated by the earlier demonstration that the structurally more accessible cGAS catalytic domain is sufficient for nuclear

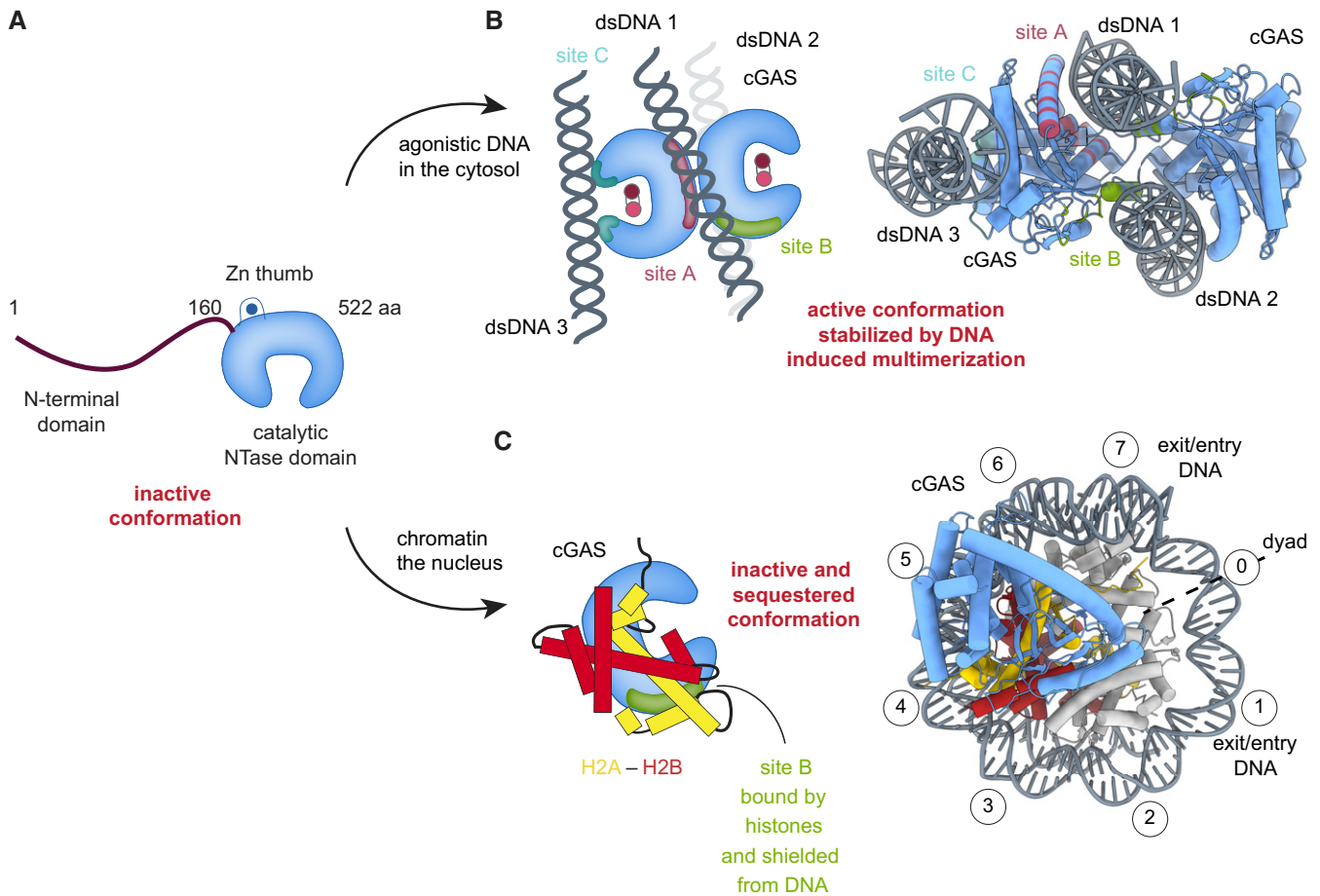


Figure 2. Active and chromatin-sequestered, inhibited states of cGAS.

(A) Schematics of cGAS domain architecture. (B) cGAS is activated by agonistic DNA through a process that involves dimer formation, clustering, and induction of an active conformation with restructured active site. DNA interacts with sites A and B, and in human cGAS also with site C (PDB codes 4LEZ, 6EDB). (C) Structure of the mouse cGAS catalytic domain (blue) bound to an NCP (gray with yellow/red H2A/H2B) in disc view (PDB code 7A08). Nucleosomal superhelix locations (SHL) of DNA wrapped around the histone core are shown as encircled numbers. Nucleosomal histones binding to DNA binding site B shields it from DNA interaction and sterically prevents formation of active cGAS dimers.

localization and tethering to chromatin (Volkman *et al*, 2019), allowing removal of the ~150 aa unstructured N-terminal stretch known to induce aggregation and liquid–liquid phase condensation.

cGAS turned out to be tightly anchored to a nucleosome surface known as the “acidic patch”, a conserved region at the interface between histones H2A–H2B serving as interaction site for many chromatin-bound proteins (Fig 2C) (McGinty & Tan, 2016). A highly conserved arginine residue (R241^{mcGAS}/R255^{hcGAS}) in vertebrate cGAS—the so-called arginine anchor—tightly binds to the canonical H2A acidic-patch residues (E61, D90, E92; Fig 3). Further, cGAS–NCP interactions include equally well-conserved residues located in two described tethering loops, containing the arginine anchor R241^{mcGAS}/R255^{hcGAS} as well as R222^{mcGAS}/R236^{hcGAS}, and a second interface located at cGAS DNA binding site B, where some DNA-contacting residues have been repurposed for protein–protein interactions with histones (Figs 2B and C, and 4B). The extent of the interaction surface between cGAS and the nucleosome’s histone core, approximately 880 Å², explains how nucleosomes can bind cGAS with low nanomolar affinity, surpassing cGAS’ affinity for agonistic dsDNA by 10- to 100-fold. In fact, NCPs are able to inhibit

cGAS *in vitro* even in the presence of free agonistic DNA. The mechanism of cGAS inhibition by chromatin becomes clear when comparing the available structures of active DNA-bound cGAS dimer with NCP-bound cGAS (Fig 2B), revealing at least two elements simultaneously contributing to inhibition: DNA binding site B is blocked by its interactions with histones H2A–H2B, and binding of free DNA to site A is sterically hindered by nearby nucleosomal DNA at nucleosomal superhelix location (SHL) 6/7, preventing formation of the active cGAS dimer. As DNA binding to both site A and B is required for cGAS dimerization and subsequent conformational changes into its catalytic active state, cGAS is hereby kept in an inactive conformation bound to chromatin.

While the new reports all agree on cGAS sequestration being mainly mediated by the acidic patch, they also reveal significant species-specific differences involving additional interaction surfaces between cGAS and the nucleosomal DNA. Six mouse cGAS–NCP and five human cGAS–NCP structural analyses yielded complexes with stoichiometries of either 1:1, 2:1, 1:2, or 2:2 (Fig 4A). Each NCP has two acidic patches related by a twofold symmetry, and since cGAS binding to the acidic patch does not induce apparent conformational

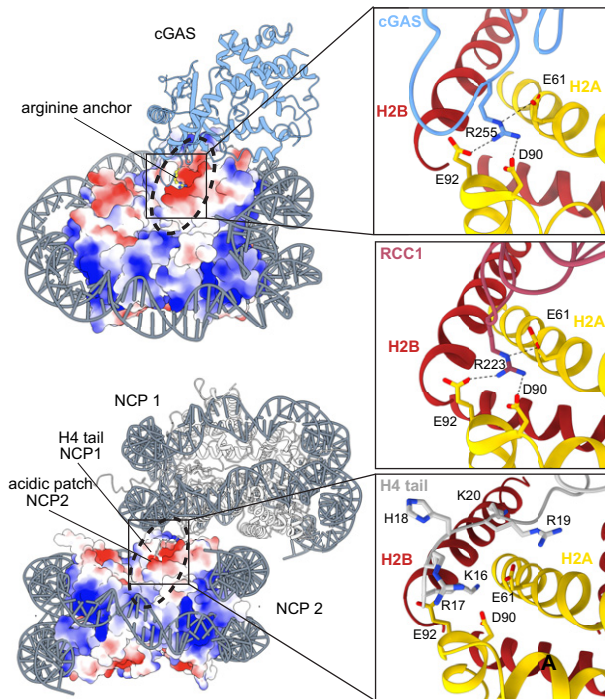


Figure 3. Recognition of the nucleosome acidic patch by cGAS and other binders via an arginine anchor.

Top: Surface electrostatics representation of the cGAS–nucleosome complex (PDB code 7A08) and close-ups of different arginine anchor interactors with the acidic patch at the interface between histones H2A–H2B (yellow, red). cGAS (blue, PDB code 7A08), RCC1 (pink, PDB code 3MVD). Bottom: Surface electrostatics representation of two stacked NCPs and close-up of H4 tail interactions from NCP1 with NCP2 (gray, PDB code 1A0I).

changes to the histone core, each NCP can bind two cGAS monomers to form a 2:1 complex. Notably, structures of human cGAS–nucleosome complexes showed preferential 2:2 stoichiometry, with two cGAS protomers sandwiched between two nucleosomes (Fig 4B). Here, the two cGAS protomers connect two NCPs, resulting in multimers of stacked NCPs. On the other hand, 1:2 stoichiometry in human cGAS–NCP structures can be attributed to one of two cGAS monomers missing in some of the particles of such cGAS–NCP stacks, due to its flexibility. Stacking of NCPs by the human cGAS protein is achieved by the previously described interactions of cGAS DNA binding site B to the acidic patch and SHL5.5 of the proximal NCP, with additional interactions of cGAS DNA binding site C to nucleosomal DNA at position SHL3 and SHL4 of the distal NCP. The interaction with the distal NCP is mainly mediated by residues in the less conserved KRKR-loop (K285^{hcGAS}, K299^{hcGAS}, R300^{hcGAS}, K301^{hcGAS}, and R302^{hcGAS}) of human cGAS, with potential additional contributions of residues on the KKH-loop (K427^{hcGAS}, K248^{hcGAS}, and H429^{hcGAS}). These positively charged residues are only partially conserved in mouse cGAS, explaining the species-specific structural differences. Interestingly, residues on a nearby conserved β-hairpin loop in human cGAS (K365^{hcGAS}, E366^{hcGAS}, G367^{hcGAS}, N368^{hcGAS}, and G369^{hcGAS}) are in close proximity to the C-terminal tail of histone H2B of the distal NCP (Fig 4B). It may thus be worth testing if histone tails, and their post-translational modifications, also play a role in cGAS–NCP interactions, and whether this might be species-

specific. In addition, the question arises whether cGAS-induced NCP stacking, along with its ability to compact DNA into a higher-order state (Jiang *et al*, 2019), affects chromatin structure *in vivo*, and whether tethered cGAS can also regulate or impact on functional properties of chromatin.

Implications of chromatin structure for cGAS and autoimmunity

Hierarchical packaging of eukaryotic DNA into chromatin plays a central role in the regulation of DNA-associated cellular processes, such as transcriptional regulation or the DNA damage response. The ‘primary structure’ of chromatin results from the organization of the genome into nucleosomes, where 147–165 bp of nucleosomal DNA are wrapped around the histone octamer consisting of two copies each of the four core histones (H2A, H2B, H3, and H4). This so-called beads-on-a-string first basic layer of organization does, however, not reflect the actual state of chromatin under physiological conditions. While the exact local three-dimensional organization of chromatin in the cell remains an area of intense research and might be subject to regulation and modulation, a paradigm in the field for chromatin folding and condensation suggests that with increasing salt concentrations, an array of nucleosomes folds into a 10 nm-diameter filament, followed by formation of a 30 nm-diameter fiber-like structure (Hansen, 2002; Krietenstein & Rando, 2020). These two states of intra-nucleosomal interactions can be regulated *in vitro* by ionic strength and are mainly mediated by interactions of the H4 N-terminal tail from one nucleosome with the acidic patch of the adjacent nucleosome (Fig 3) (Arya & Schlick, 2009). This implies that inhibition of cGAS by the nucleosome acidic patch should be sensitive to the chromatin organization state, i.e., that the level of chromatin condensation should dictate the availability and accessibility of cGAS-binding acidic patch surfaces on chromatin.

Chromatin can be further divided into at least two very distinct states of organization. Euchromatin represents the “expressed” state, being less condensed and present at transcriptionally active genes that are marked by histone H3 and H4 acetylation and H3K4 methylation (H3K4me). In contrast, heterochromatin, which represents the “repressed” state and is required for proper nuclear architecture, is highly condensed and concentrated in pericentromeric and telomeric regions, which in turn are enriched in repetitive DNA sequences such as satellite DNA and transposons (Allshire & Madhani, 2018). Hallmarks of heterochromatin structure are histone H3 trimethylation modifications (H3K27me3 and H3K9me3) and the associated heterochromatin protein 1 (HP1), which forms a dimer bridging two H3K9-methylated nucleosomes and thus further increases compaction. Condensation of heterochromatin is fundamental for maintaining genome stability as the compact structure of heterochromatin restricts or controls activities such as transcription, replication, recombination, and DNA damage response signaling, especially in regions with highly repetitive sequences (Janssen *et al*, 2018).

Mapping the chromatin binding sites of overexpressed GFP-NLS-cGAS and transgenically expressed GFP-cGAS in human and murine dendritic cells, respectively, revealed that nuclear cGAS is enriched on centromeric satellite and LINE DNA repeat elements (Gentili

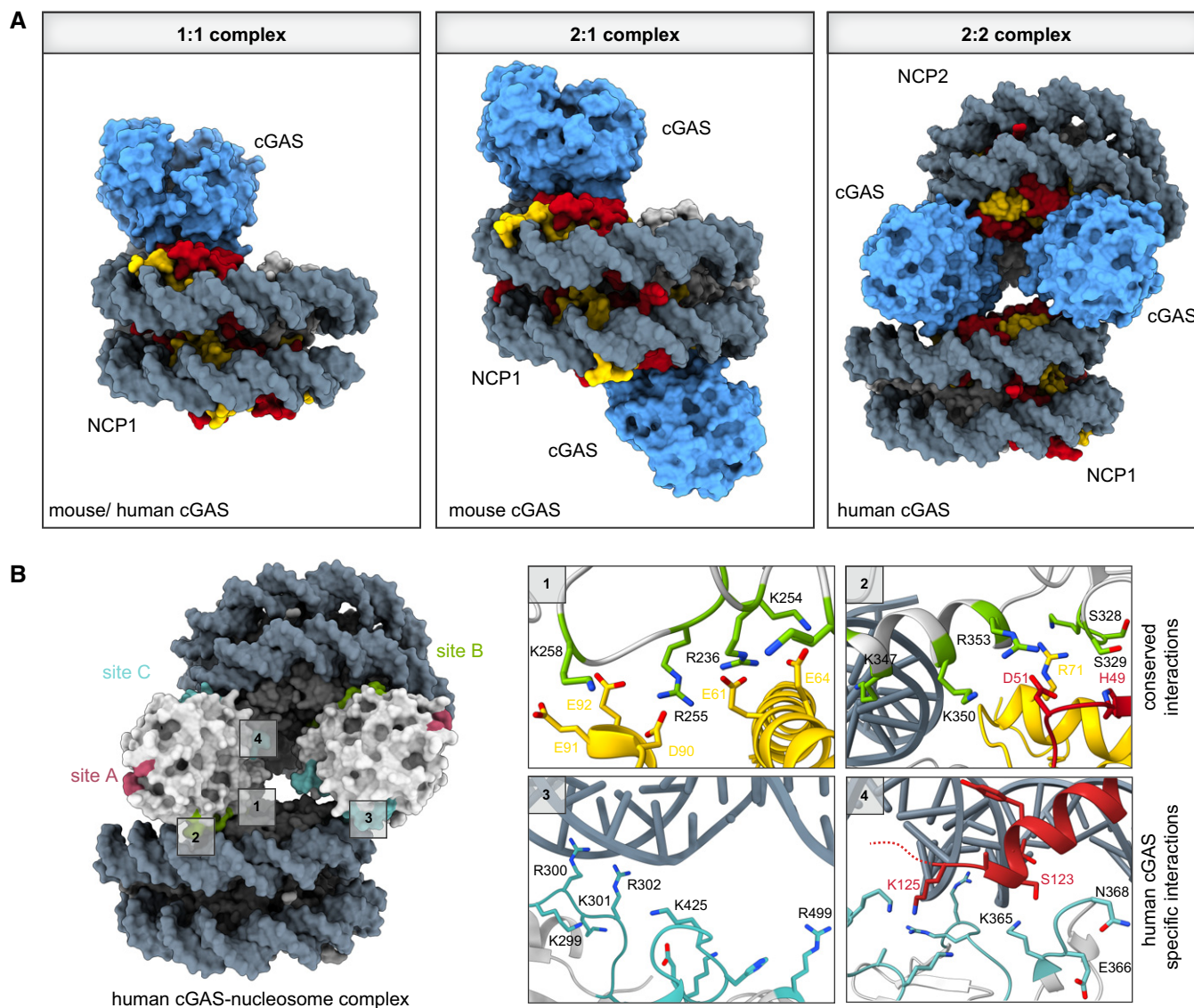


Figure 4. Stoichiometries of mouse and human cGAS–nucleosome complexes and their interaction sites.

(A) Comparison of cGAS–nucleosome complex stoichiometries from different cryo-electron microscopy structures. While mouse cGAS is found in 1:1 (PDB code 7A08) or 2:1 complexes with NCPs (PDB code 6XJD), human cGAS exhibits a preferred 2:2 stoichiometry (PDB code 7COM). (B) Close-ups of four distinct cGAS–nucleosome interaction interfaces involving DNA binding sites B and C (PDB code 7COM and 6Y5D). Human cGAS DNA binding site A (pink), site B (green), and site C (cyan) are depicted. Close-ups 1 and 2 show the conserved residues in site B involved in anchoring cGAS to the acidic patch, and close-ups 3 and 4 show the human-specific cGAS site C residues involved in interactions with nucleosomal DNA of NCP2.

et al, 2019). Interestingly, cGAS localization on chromatin does not correlate with open chromatin marks such as H3K27Ac but instead with repressive H3K9me enriched on pericentromeric heterochromatin, and with the centromeric histone H3 variant CENP-A, suggesting that cGAS preferably localizes to densely packed regions in heterochromatin. In addition, while overexpressed cGAS is distributed throughout the nucleus, endogenous cytosolic cGAS accessing the nuclear compartment in the course of mitotic nuclear envelope rupture forms peripheral cGAS foci. Which mechanisms control cGAS distribution in the nucleus remains to be determined, as well as how this distribution may change depending on the cell state, e.g., infection or cell cycle progression. Another interesting

question is how nuclear cGAS is kept away from active sites of transcription, including nucleosome-free/nucleosome-depleted regions (NFRs). NFRs are found in transcriptional regulatory regions such as promoters, enhancers, or terminators, while gene-coding regions generally have a higher and more regular nucleosome occupancy. Given the heterogeneous structure and organization of chromatin in the nucleus, and there is the counterintuitive notion that at least *in vitro*, compact but not open chromatin is a substrate stimulating cGAS activity (Mackenzie *et al*, 2017; Michalski *et al*, 2020), details of cGAS activation by chromatin states need clarification (Fig 5A–C). For instance, levels of nuclear cGAS might require tight regulation as to not exceed the number of freely available nucleosomal

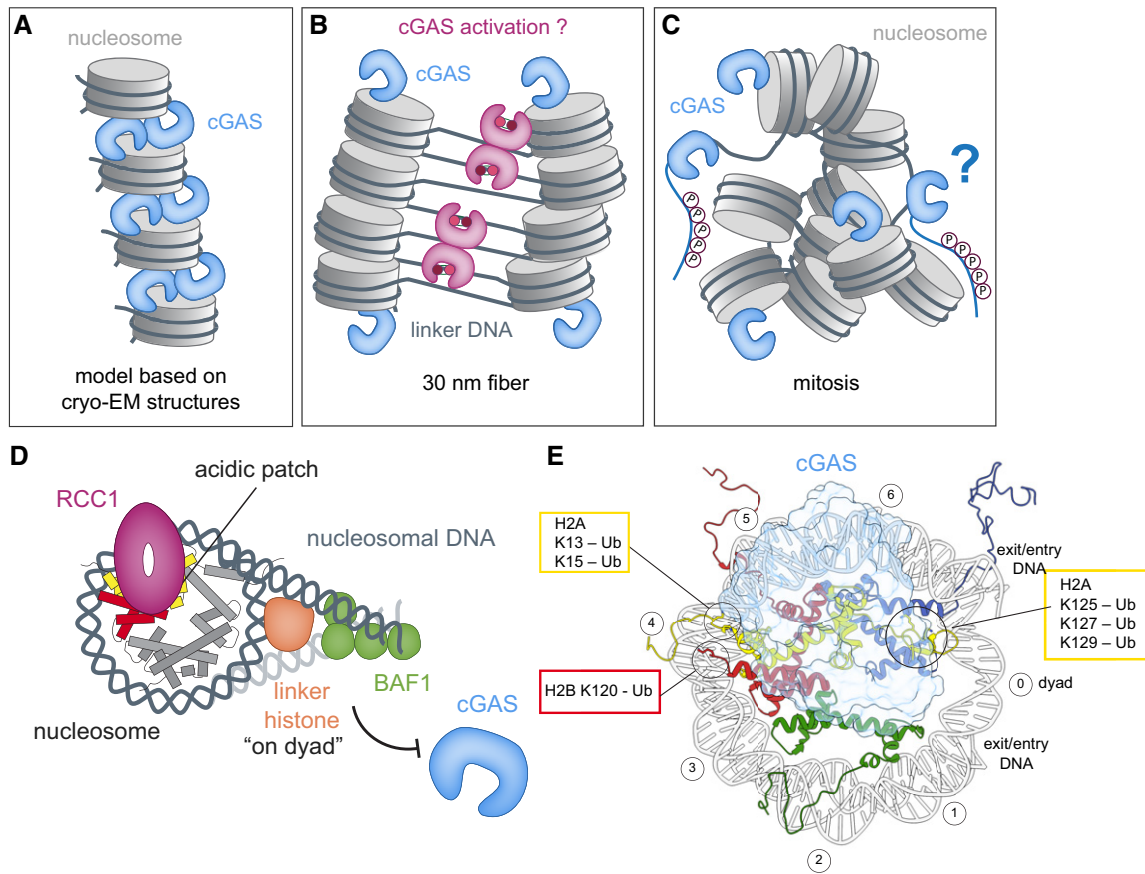


Figure 5. Effects of chromatin structure and chromatin bound proteins on cGAS activation.

(A–C) Schematics of cGAS (blue) bound to nucleosomes (gray) based on cryo-EM structure models (A), 30 nm-fiber condensed-chromatin model (B), and oligomers during mitosis (C). Accessibility of acidic patches determines whether cGAS is sequestered away from free linker DNA. During mitosis, cGAS is additionally inhibited through hyperphosphorylation of its N-terminal region. (D) Chromatin-binding factors, such as RCC1, BAF1, and linker histones, alter chromatin structure and compete with cGAS for binding sites. (E) Relation of important histone ubiquitylation sites on the nucleosome (disc view) to the cGAS-binding site B.

binding sites (considering that there are also numerous other acidic-patch binders present; Fig 3). Consistent with this, induced chromatin decondensation/decompaction through histone deacetylase inhibition or cellular *HMGN5* overexpression was found to suppress activation of experimentally elevated nuclear cGAS levels (Guey *et al*, 2020).

The stoichiometry of available chromatin sites for cGAS sequestration is particularly imbalanced during cell division. At the onset of vertebrate mitosis, breakdown of the nuclear envelope allows the recruitment of both the nuclear as well as cytosolic pool of cGAS onto chromosomes, disabling any potential regulatory mechanisms based on cellular cGAS compartmentalization (Zierhut *et al*, 2019). In addition, chromosomes undergo global compaction during mitotic entry resembling the described *in vitro* state of liquid-like phase condensates (Batty & Gerlich, 2019) (Fig 5C). This is partly due to free Mg^{2+} ion levels rising during mitosis to concentrations that are sufficient to promote chromatin condensation (and thus subsequent cGAS activation) *in vitro* (Maeshima *et al*, 2018). Other mechanisms contributing to mitotic chromatin compaction involve chromatin binding factors, interactions of flexible histone tails with DNA, and mitosis-associated changes in histone tail post-translational modifications such as deacetylation. Addressing this

conundrum, a mechanism for cGAS inhibition during mitosis involves cell cycle-dependent hyperphosphorylation of its N-terminal region (Zhong *et al*, 2020; Li *et al*, 2021). The evenly distributed negative charges of phosphorylated serine and threonine residues along the N-terminal regions of cGAS specifically interfere with its recognition of nuclear DNA and selectively suppress its activity during mitosis. Moreover, there are additional controls of STING downstream signaling during cell division, such as mitotic Golgi vesiculation, which may further inhibit STING activation by limiting its trafficking after cGAMP binding (Uhlorn *et al*, 2020).

The opposite effect is observed when cGAS is activated by chromatin for instance in micronuclei. Micronuclei are aberrant nuclear structures containing chromatin fragments surrounded by a nuclear envelope. They form after mitosis as result of a range of cell division defects, such as mitotic errors, impaired chromosome segregation, or DNA damage. These events lead to the appearance of so-called chromatin bridges and lagging chromosomes, which then recruit their own nuclear envelope to generate micronuclei. cGAS is recruited to micronuclei upon rupture of their nuclear envelope (Bartsch *et al*, 2017; Dou *et al*, 2017; Glück *et al*, 2017; Harding *et al*, 2017; Mackenzie *et al*, 2017; Yang *et al*, 2017; Bakhoun *et al*, 2018), resulting in the exposure of micronuclear chromatin to the

cytosolic compartment and in recruitment and activation of the cytosolic cGAS pool. One reason for the activation of cGAS by micronuclear chromatin might be that the limited amount of accessible nucleosome acidic patches they contain are insufficient to sequester the whole cytosolic pool of cGAS, allowing free cGAS activation by nearby free chromosomal DNA. In this context, the DNA 3'-5' exonuclease TREX1 was found as a critical regulator of cytosolic DNA sensing in chromosomally unstable cells, degrading micronuclear DNA when bound to the endoplasmic reticulum (ER) (Mohr *et al*, 2021). In contrast to the degradation of micronuclear DNA by ER-associated TREX1, cytosolic immunostimulatory DNA bound to cGAS forms phase-separated liquid–liquid droplets that limit its degradation by TREX1 (Zhou *et al*, 2021). Other possible mechanisms differentiating cGAS-activating potential of micronuclear chromatin vs intact-nuclei chromatin would be potential aberrant chromatin structures in micronuclei recognized by cGAS, or yet to be identified mechanisms or factors for cGAS inhibition present in nuclei but absent from micronuclei. Nevertheless, there is so far no direct biochemical evidence that aberrant chromatin in micronuclei cannot just recruit, but also activate cGAS—recent work in fact implicates co-occurring chromatin bridges, rather than micronuclei, as the actual source of cGAS activation after drug-induced mitotic errors (preprint: Flynn *et al*, 2021). Another example of cytosolic chromatin activating cGAS is neutrophil extracellular traps (NETs), which are released by neutrophils during a specific form of regulated cell death called NETosis. These structures, composed of chromatin and antimicrobial molecules, are phagocytosed by macrophages and translocate into the cytoplasm, where they activate cGAS (Apel *et al*, 2021). As in the case of micronuclei, NETs expose aberrant chromatin structures to an excess of cytosolic cGAS, again possibly exhibiting too few acidic patches to inhibit cGAS. Indeed, fragmentation of NETs is required for cGAS activation *in vitro*, whereas digestion of their extranucleosomal DNA results in cGAS inactivation, suggesting that decompaction of chromatin exposing extranucleosomal DNA is required for cGAS activation in the cytosol.

What other mechanisms for cGAS inhibition exist in the nucleus?

Chromatin organization and structure are affected by several chromatin-bound proteins. Well-known influencers of chromatin structure dynamics are linker histones such as H1 and H5, which stabilize interactions of additional 20-bp linker DNA with the nucleosome periphery to form the chromatosome (Fig 5D). Linker histones are known to drive further compaction of chromatin by stabilizing folded and oligomeric states *in vitro* and by promoting heterochromatin formation *in vivo* (Dorigo *et al*, 2004; Fan *et al*, 2005; Lu *et al*, 2009; Song *et al*, 2014). Linker histones may affect cGAS inhibition by chromatin on the one hand via further compaction of chromatin, reducing the number of accessible acidic patches for cGAS sequestration, and on the other hand through hindrance of cGAS activation by restricting access to linker DNA. In any case, binding of linker histones to nucleosomal DNA inhibits cGAS activity *in vitro* more strongly than chromatin lacking linker histones (Uggetti *et al*, 2020). Roles of histones in limiting cGAS activation in the nucleus are further observed in patients with the type I

interferonopathy Aicardi–Goutières syndrome (AGS). Here, biallelic mutations in *LSM11* and *RNU7-1*, encoding components of the replication-dependent histone pre-mRNA-processing complex, result in misprocessing of canonical histone transcripts in this subset of AGS patients and thus specifically disturb linker histone stoichiometries, while maintaining core histone levels (Uggetti *et al*, 2020). The altered levels of linker histones lead to cGAS redistribution within the nucleus, including more frequent nuclear membrane herniations containing increased cGAS concentrations, followed by an enhanced cGAS-dependent type I interferon signature. Taken together, histones play a key role in suppressing autoreactivity to nuclear self-DNA by cGAS sequestration via the histone H2A–H2B interface and the additional requirement for linker histones bound to chromatin to avoid autoimmunity. Further investigation is required to fully understand how changes in nuclear linker histone stoichiometries and distinct linker histone variants affect nuclear cGAS distribution and how cGAS localization in turn relates to its activation in the nucleus.

Linker histones are not the only proteins restricting accessibility of cGAS to nuclear DNA, as the barrier-to-autointegration factor 1 (BAF1) can also dynamically outcompete cGAS for DNA binding upon acute loss of nuclear envelope integrity (Guey *et al*, 2020; Ma *et al*, 2020) (Fig 5D). BAF1 is a small (10 kDa) chromatin-bound protein essential for mitosis, nuclear assembly, and the DNA damage response (Haraguchi *et al*, 2001; Margalit *et al*, 2005; Haraguchi *et al*, 2008; Jamin & Wiebe, 2015). BAF1 depletion in cells results in increased nuclear envelope ruptures, leading to the recruitment of cytosolic cGAS, formation of cGAS foci at the nuclear entry site, and subsequently a robust interferon response. Thus, BAF1 binding to DNA at sites of nuclear envelope rupture provides an additional mechanism to avoid cGAS autoreactivity toward nuclear self-DNA, especially when the cytosolic cGAS pool gains access to nuclear DNA.

In addition to protein–protein interactions, RNA can also outcompete dsDNA for binding to cGAS, as protection of quiescent hematopoietic stem cells from exhaustion due to cGAS activity depends on the presence of a circular RNA (termed *cia-cGAS*) in the cell nucleus (Xia *et al*, 2018). In this case, cGAS inhibition relies on the high nuclear abundance and stronger cGAS-binding affinity of *cia-cGAS* RNA compared to self-DNA, preventing cGAS activation by nuclear DNA and subsequent immune response induction. Finally, possible post-translational modifications of cGAS or histones are another aspect to consider regarding regulation of cGAS activity in the nucleus. Notably, a so-far unidentified post-translational modification specific for a portion of chromatin-bound cGAS and disappearing upon DNA stimulation could represent one such mechanism (Michalski *et al*, 2020), but further investigation is required to identify this modification and understand its role in cGAS regulation.

How is cGAS released from chromatin?

The tight interaction of cGAS with chromatin raises the question to what extent regulated dissociation is necessary in the context of, e.g., cellular infections. While the cGAS–nucleosome complex is, with a dissociation half-life of about 70 s, quite dynamic for allowing rapid redistribution onto free DNA, the latter cannot efficiently compete and release cGAS from the acidic patch of the nucleosome (Michalski *et al*, 2020). Nucleo-cytosolic distribution of cGAS might

be sufficient to allow sensing of cytosolic DNA, but recognition of agonistic nuclear DNA in the context of homologous recombination or infections presumably needs protein cofactors or post-translational modifications on either histones or cGAS to prevent nucleosomal competition. For instance, some of the described sumoylation and ubiquitination sites in cGAS (e.g., K335^{mcGAS}) (Cui *et al*, 2017) have the potential to prevent nucleosome tethering by steric hindrance and may play a role in regulating chromatin binding of cGAS.

Histone tails are well known for their functions in nucleosome dynamics and recruitment of co-factors to chromatin. Deletion of N-terminal tails from all four core histones did not affect cGAS binding to the nucleosome or its inhibition, suggesting that sequestration by the acidic patch is the dominant mechanism of inhibition (Boyer *et al*, 2020). However, histone tails and possible modifications thereof can play a major role in the regulation of chromatin-bound cGAS, such as the facilitation of cGAS release. Modifications in close proximity of the cGAS-nucleosome binding site, or modifications on spatially close histone tails, could potentially displace cGAS or prevent its sequestration by the acidic patch. Comparing the available structures of ubiquitinated nucleosomes, possible cGAS-regulating histone modifications include ubiquitination of histone H2A at sites K13, K15 or K125, K127, K129, and H2B ubiquitination on K120 (Fig 5E) (Wilson *et al*, 2016; Anderson *et al*, 2019; Worden *et al*, 2019). These histone modifications are known to be important in the DNA damage response, where each of them has different physiological consequences, such as promoting non-homologous end joining (NHEJ) or homologous recombination (HR) (Uckelmann & Sixma, 2017). Ubiquitination of H2BK120, for instance, stimulates chromatin relaxation, which in turn facilitates recruitment of factors required for DNA damage checkpoint activation and DNA repair initiation (Schwertman *et al*, 2016). Another modification is S139 phosphorylation of variant histone H2A.X (creating the γ H2A.X mark), which was found to interact with cGAS and to facilitate its recruitment to the site of DNA damage (Liu *et al*, 2018). However, further experimental evidence is required to understand how cGAS activity and its localization on chromatin is regulated by these modifications upon DNA damage.

The release of cGAS from chromatin could also be directed by competition with other chromatin co-factors interacting with the acidic patch. The acidic patch is a highly negatively charged narrow groove in the nucleosome core, known as its principal protein-docking region utilized by numerous nucleosome interactors such as regulator of chromosome condensation 1 (RCC1, Fig 3) (Makde *et al*, 2010), latency-associated nuclear antigen (LANA) (Barbera *et al*, 2006), interleukin-33 (IL-33) (Roussel *et al*, 2008), silent information regulator 3 (SIR3) (Armache *et al*, 2011) and high-mobility group nucleosomal binding domain 2 (HMGN2) (Kato *et al*, 2011). cGAS has to compete with the H4 tail and other proteins for the acidic patch, in particular since, e.g., RCC1 has a much higher abundance in the cell than cGAS (Hein *et al*, 2015).

How is cGAS localization regulated in the cell?

cGAS localization to both the cytosol and the nucleus has now been established in different cell types including fibroblasts and keratinocytes (Orzalli *et al*, 2015), primary immune cells (Gentili *et al*, 2019;

Jiang *et al*, 2019; Volkman *et al*, 2019), and cancer cells (Liu *et al*, 2018). However, distribution of these two cGAS protein pools in the cell differs depending on determinants such as cell-type, cell differentiation or cell density (Yang *et al*, 2017; Jiang *et al*, 2019; Volkman *et al*, 2019). In differentiated primary mouse macrophages for instance, almost 95% of cGAS protein in the cell is localized to the nucleus (Volkman *et al*, 2019). While the cytosolic cGAS protein pool is important for immune signaling against aberrant dsDNA in the cytosol, the nuclear cGAS pool is kept inactive, suggesting that cGAS enzymatic activity is an exclusive function of cytosolic cGAS. It remains unclear what the contributions are of nuclear vs the cytosolic pools of cGAS protein in activation of the cGAS–STING pathway during, e.g., infection. Is the cytosolic cGAS fraction at steady-state enough to elicit an immune response against the evading pathogen or must predominantly nuclear cGAS relocate to the cytosol in order to fight infection? This also raises the question of what potential upstream cellular stress signals influence cGAS localization.

Use of the cGAS arginine anchor mutant R255E^{hcGAS}, in order to release cGAS from its tight sequestration by chromatin in cells, has revealed that cGAS remains nuclear even when not bound to the nucleosomal acidic patch, indicating the requirement for an active mechanism of cGAS translocation to the cytosol (Michalski *et al*, 2020). Part of this localization could be the propensity of cGAS to bind genomic DNA, as a cGAS DNA-binding site mutant (C396A/C397A) has a reduced nuclear presence (Jiang *et al*, 2019). In addition, both the N-terminal domain of cGAS, containing amino acids 1–160, and the C-terminal catalytic domain 161–522 can spontaneously accumulate in the nucleus, suggesting that at least two independent nuclear localization signals are present in cGAS (Gentili *et al*, 2019). These nuclear localization signals are opposed by a recently discovered functional NES on the C-terminal catalytic domain (169–174) of cGAS, which is required for translocation of cGAS into the cytosol (Sun *et al*, 2021). Here, the cGAS NES is required for cGAS export to the cytosol upon DNA stimulation and for the subsequent interferon response, suggesting that the available cytosolic cGAS fraction prior to stimulation is insufficient to elicit a robust immune response to dsDNA (Sun *et al*, 2021).

Why is cGAS in the nucleus in the first place?

The biologically most interesting still unanswered question surrounding nuclear cGAS is why cGAS is kept there in the first place. One possible explanation is that the nucleus is merely a reservoir to keep cytosolic cGAS levels low, yet enabling the cell to replenish or increase cytosolic levels in a regulated fashion without the need for slow transcriptional programs. Alternatively, since most DNA viruses shown to be antagonized by the cGAS–STING pathway are known to replicate exclusively in the nucleus while shielding their genome from detection when in the cytosol, cGAS might have nuclear DNA-sensing functions as well. In this context, several studies have focused on how cGAS exerts its canonical functions as an antiviral pathogen recognition receptor in the nucleus, facilitated by other nuclear immune factors or auxiliary proteins (Orzalli *et al*, 2015; Lahaye *et al*, 2018). Interestingly, nuclear DNA viruses and retroviruses, such as HSV-1, organize their genomes into nucleosomes by appropriating eukaryotic histones and

hijacking the host nucleosome assembly machinery (Lieberman, 2008; Oh *et al.*, 2015). Some large DNA viruses even encode histone homologs themselves, and two recent cryo-EM structures of these viral nucleosome-like particles revealed their structural similarity to eukaryotic counterparts, including partial conservation of the acidic patch (preprint: Liu *et al.*, 2021; Valencia-Sánchez *et al.*, 2021). The HSV-1 viral genome is packaged into nucleosomes already 1 h after infection and subsequent nuclear entry (Kent *et al.*, 2004), indicating that wrapping of the viral genome into nucleosomes could represent a novel viral immune evasion mechanism. In this case, the virus uses inhibition of cGAS by the acidic patch as a strategy to overcome immune recognition in the nucleus when its genome first gets exposed for replication in the host.

On the other hand, evidence is now accumulating for additional, STING-independent non-canonical functions of cGAS in the nucleus, including roles in tumor progression by inhibiting DNA repair, or as decelerator of DNA replication forks suppressing replication-associated DNA-damage, and even in regulation of histone modifications in response to an RNA virus infection (Liu *et al.*, 2018; Jiang *et al.*, 2019; Chen *et al.*, 2020; Cui *et al.*, 2020). DNA damage-induced lesions such as DNA double-strand breaks (DSB) can be repaired via two major pathways: NHEJ, which ligates broken DNA ends regardless of homology, and HR, which uses an undamaged DNA template to repair the break while retaining the original sequence. Two independent studies recently found that nuclear cGAS selectively inhibits DSB repair by HR, thus exacerbating genome instability leading to tumorigenesis and cell death (Liu *et al.*, 2018; Jiang *et al.*, 2019). Both studies agree that the catalytic domain of cGAS is sufficient for inhibiting HR and that cGAS DNA-binding properties and nuclear localization are required, but not its catalytic activity. However, two alternative mechanisms for how cGAS inhibits HR have been proposed: (i) poly-ADP-ribose (PAR)-mediated interaction between cGAS and PARP1, which in turn impairs the interaction of PARP1 with the DSB repair factor Timeless (Liu *et al.*, 2018), and (ii) cGAS-dependent compaction of template DNA into higher-order, ladder-like, phase-separated structures that prevent RAD51-ssDNA filaments from invading the dsDNA template, therefore inhibiting D-loop formation underlying HR (Jiang *et al.*, 2019). Since the *in vitro* studies were done in absence of nucleosomes, it is yet unclear whether DNA ends per se could overcome nucleosomal inhibition of DNA binding by cGAS, requiring additional recruitment mechanisms to sites of DNA damage. Furthermore, since pathway choice between NHEJ and HR is substantially regulated at the level of chromatin, selective inhibition of HR by cGAS may also proceed via cGAS-nucleosome interactions. Further clarification is needed to understand the role of nuclear cGAS beyond its actions as an innate immune sensor.

Conclusion

Research on the biology of nuclear cGAS is just in the beginning. Surveillance of genomic instability or cellular stress by detecting changes in chromatin structure or inhibition of HR leading to the elimination of cells under acute genomic stress represent possible primordial functions of cGAS and could provide an explanation for its evolutionary conservation prior to the emergence of interferon-based immunity. Thus, cGAS might be strategically positioned as a guard in the village's noisy inn, and not only as sequestered

“prisoner”. There it could sense upcoming plots or detect intruders that mingle among the local villagers, i.e., cell stress, before they can cause major damage. Predominant localization of cGAS to the nucleus of the cell could indicate that this guard's function is to first keep the village safe from within and as a second task to protect it against threats from the outside.

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Conflict of interest

The authors declare that they have no conflict of interest.

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