Received: 8 March 2023 | Accepted: 24 April 2023

DOI: 10.1002/bies.202300045

BioEssays

REVIEW ESSAY

Prospects & Overviews

Harnessing the cooperation between DNA-PK and cGAS in cancer therapies

The cooperation between DNA-PK and cGAS shapes tumour immunogenicity

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Abstract

The cyclic GMP-AMP synthase–stimulator of interferon genes (cGAS-STING) pathway is central for the initiation of anti-tumoural immune responses. Enormous effort has been made to optimise the design and administration of STING agonists to stimulate tumour immunogenicity. However, in certain contexts the cGAS-STING axis fuels tumourigenesis. Here, we review recent findings on the regulation of cGAS expression and activity. We particularly focus our attention on the DNA-dependent protein kinase (DNA-PK) complex, that recently emerged as an activator of inflammatory responses in tumour cells. We propose that stratification analyses on cGAS and DNA-PK expression/activation status should be carried out to predict treatment efficacy. We herein also provide insights into non-canonical functions borne by cGAS and cGAMP, highlighting how they may influence tumourigenesis. All these parameters should be taken into consideration concertedly to choose strategies aiming to effectively boost tumour immunogenicity.

KEYWORDS
anti-tumour immunity, cancer, cGAMP, cGAS, DNA-PK, inflammation, DNA repair

INTRODUCTION

Abnormal localisation of nucleic acids, such as the accumulation of DNA in the cytosol, is sensed as a danger signal and triggers the initiation of immune responses. Cytosolic nucleic acids may arise following pathogen infection, genotoxic or mitochondrial stress, and are detected by a range of cytosolic sensors that elicit the production of inflammatory mediators, including cytokines and chemokines. Those inflammatory mediators in turn promote immune cell activation and the establishment of an anti-viral state. This ability to trigger inflammatory responses in the presence of cytosolic nucleic acids is shared by most cells and represents one of the first lines of host defence.

The cyclic GMP-AMP (cGAMP) synthase (cGAS) is a central pattern recognition receptor for cytosolic nucleic acids that possesses highest affinity for dsDNAs,[1,2] but is also able to detect ssDNAs and RNA:DNA hybrids.[2,3] The interaction of cGAS with cytosolic nucleic acids leads to the production of the 2′3′-cGAMP second messenger, a cyclic dinucleotide that bears internal 2′- and 3′-hydroxy-monophosphate linkages between AMP and GMP.[1,2] The major characterised role of cGAMP is to activate the endoplasmic reticulum (ER)

Abbreviation: cGAMP, cyclic GMP-AMP; cGAS, cyclic GMP-AMP (cGAMP) synthase; DNA-PK, DNA-dependent protein kinase; DSB, double stand break; ENPP1, ectonucleotide pyrophosphatase/phosphodiesterase 1; HR, homologous recombination; HSV-1, Herpes simplex virus 1; IFN, interferon; IRF3, interferon regulatory factor 3; NF-kB, nuclear factor-kappa B; NHEJ, non-homologous end joining; PARP1, poly(ADP-ribose) polymerase 1; PDL-1, programmed death-ligand 1; STING, stimulator of interferon genes; TBK1, tank binding kinase 1.

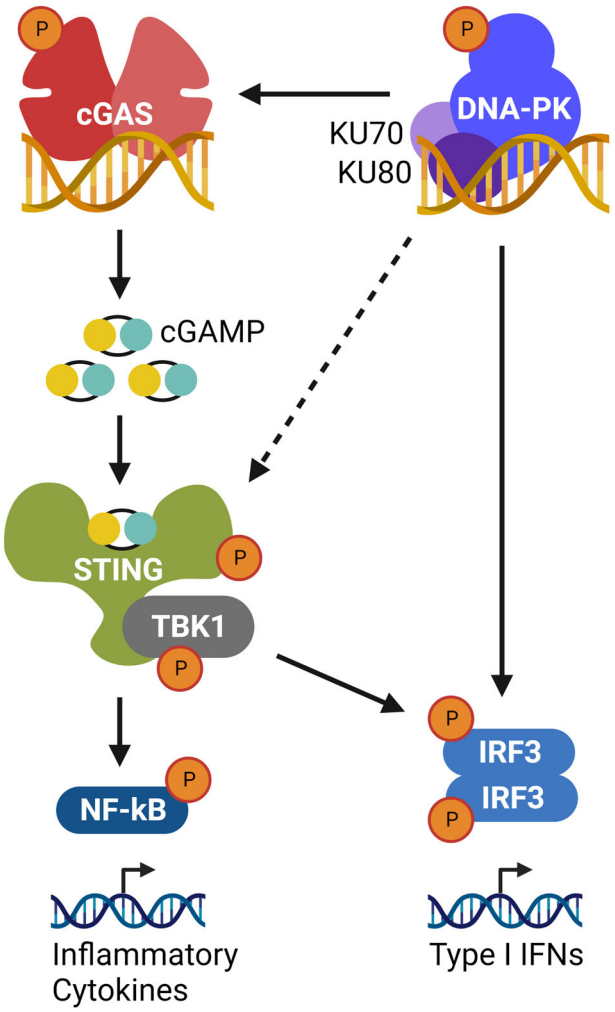

FIGURE 1 Cyclic GMP-AMP (cGAMP) synthase (cGAS) and DNA-dependent protein kinase (DNA-PK) sensing of dsDNA. Upon DNA binding, cGAS catalyses the production of the cGAMP second messenger. The interaction of cGAMP with stimulator of Interferon genes (STING) promotes the recruitment and activation of the tank binding kinase 1 (TBK1) kinase. Subsequently, TBK1 phosphorylates and activates the interferon regulatory factor 3 (IRF3) and nuclear factor-kappa B (NF-κB) transcription factors, promoting the transcription of type I interferons (IFNs) and inflammatory cytokines. DNA-PK participates to the response to dsDNA and promotes type I IFN responses and cytokine production by directly activating STING and/or IRF3, independently of cGAS. DNA-PK can also modulate cGAS activity. Created with BioRender.com.

resident protein stimulator of interferon genes (STING).[4] cGAMP interacts with STING, inducing STING oligomerisation, trafficking towards the Golgi apparatus, and the recruitment of the proteins required for the activation of stereotypical inflammatory responses comprising a type I interferon (IFN) signature. Indeed, STING activation promotes the recruitment of the tank binding kinase 1 (TBK1) together with transcription factors, such as nuclear factor-kappa B (NF-kB) and interferon regulatory factor 3 (IRF3).[4–7] The latter, upon phosphorylating activation, will translocate into the nucleus to induce a subset of inflammatory genes, including type I IFNs (Figure 1).

To date, cGAS is the only known mammalian protein that produces cGAMP in response to cytosolic DNA and thus was long regarded as an essential activator of STING. However, several studies have challenged this reductionist view, demonstrating that cGAS-independent pathways, converging or not onto STING, may be sufficient, or participate to, driving type I IFN responses. This is notably the case of DEAD-Box Helicase 41 (DDX41),[8] interferon gamma inducible protein (IFI16)[9] and the DNA-dependent protein kinase (DNA-PK).[10,11] DNA-PK is a holoenzyme comprised of the KU70 and KU80 proteins, essential for the recruitment of the DNA-PK catalytic subunit (DNA-PKcs) to free ends of dsDNAs.[12,13] The major function of DNA-PK is in the repair of double stand breaks (DSBs), genomic DNA lesions, by facilitating non-homologous end joining (NHEJ).[14] This activity of DNA-PK is also required in physiological processes such as variability diversity joining (VDJ) somatic recombination.[15]

Recent years have seen growing recognition of the role of DNA-PK in the detection of pathological cytosolic nucleic acid species. The DNA-PK complex has been shown to induce IFN expression through a mechanism that is not completely clear, but dependent on STING.[16–18] However, DNA-PK can also directly phosphorylate IRF3,[19] bypassing the requirement for STING, thus driving inflammatory responses to cytosolic DNAs (Figure 1) (reviewed in ref.[20]). Moreover, the DNA-PK complex interacts with the cGAS-STING pathway in both anti-viral and anti-tumoural immunity. In the present review, we first examine recent novel mechanistic insights into the regulation of cGAS expression and activation, bearing in mind that cGAS status is a key factor for tumour immunogenicity. We in particular focus our attention on DNA-PK-driven cGAS regulation. Next, we discuss the non-canonical functions of cGAS and cGAMP and how they may also affect tumour progression.

THE CGAS-STING AXIS IN THE CONTROL OF TUMOUR IMMUNOGENICITY

The impact of cGAS-STING pathway activation on tumourigenesis has been extensively reviewed,[21,22] highlighting that the outcome depends on poorly resolved parameters.

The cGAS-STING pathway in anti-tumoural immunity

In cancer cells, cytosolic nucleic acids may arise from genomic instability, leading to chromatin fragments that are a substrate for cGAS. In that context, it has been shown that those cytosolic DNAs can induce cGAS-STING pathway activation and cell cycle arrest.[23,24] Reactivation of endogenous retroviruses may also be a source of cytosolic DNA substrates for cGAS, promoting type I IFN production.[25] Such activation of cGAS in tumour cells is commonly anticipated to increase their immunogenicity, enhancing the recruitment of immune cells that are ultimately expected to promote tumour clearance. It has also been proposed that dying tumour cells can release self-DNA fragments in the tumour microenvironment, which can be engulfed by

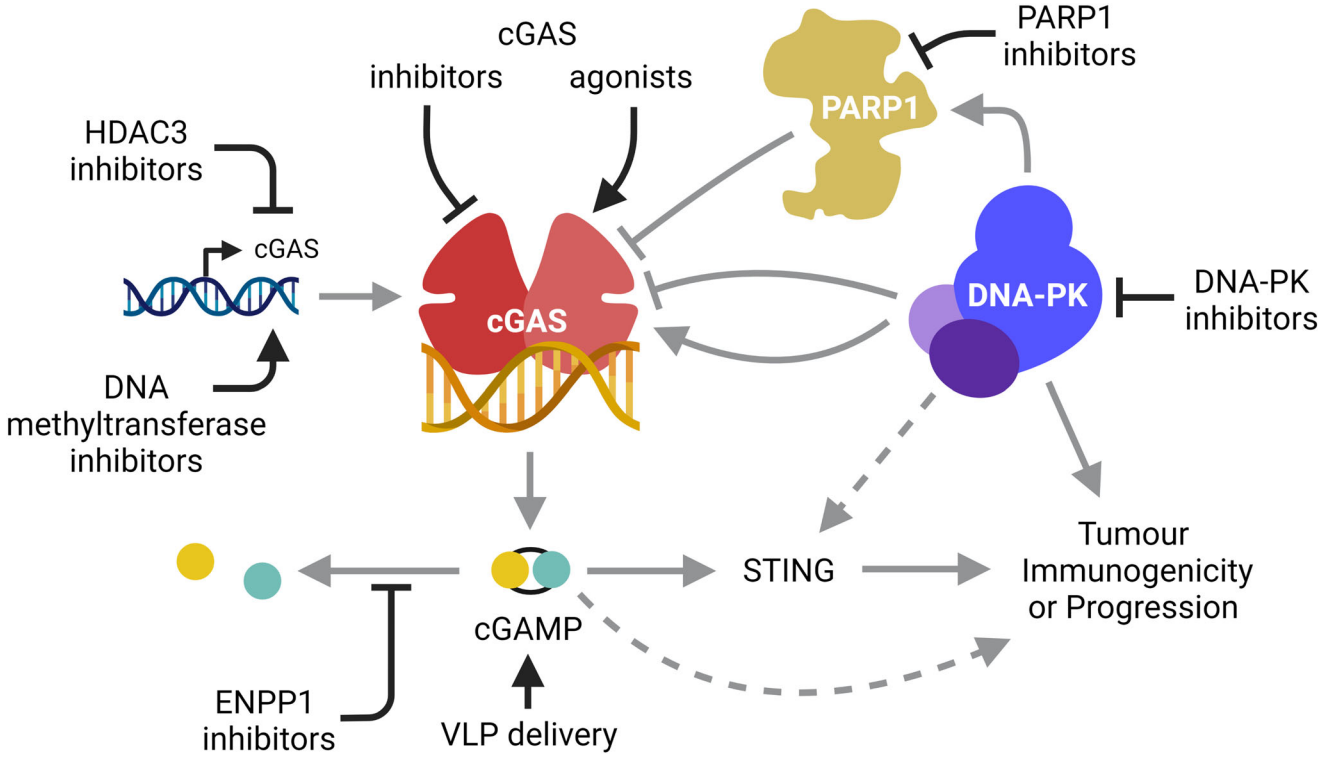

FIGURE 2 Targeting the cyclic GMP-AMP synthase–stimulator of interferon genes (cGAS-STING) and DNA-dependent protein kinase (DNA-PK) pathways for cancer treatment. Targeting the cGAS-STING pathway to boost tumour immunogenicity has been proposed as a valuable strategy for cancer treatment. Several methods have been proposed, including the use of HDAC3 and DNA methyltransferase inhibitions that can modulate cGAS expression. cGAS agonists or inhibitors are under development and some STING agonists are currently under clinical trials. cGAMP levels may also be increased in tumours by direct delivery through virus-like particles, or in the tumour microenvironment using ectonucleotide pyrophosphatase/phosphodiesterase 1 (ENPP1) inhibitors. In addition, DNA-PKcs inhibitors have entered clinical trials for cancer treatment. The anti-tumoural effect of this treatment may be the result of increased cell death, due to defective DSB repair, but also of inhibition of pro-tumoural inflammation. Finally, numerous poly(ADP-ribose) polymerase 1 (PARP1) inhibitors are either approved or in clinical trials for cancer treatment and, together with promoting cell death, they might modulate cGAS activity in tumours and in their microenvironment. Created with BioRender.com.

macrophages and dendritic cells, leading to activation of the cGAS-STING pathway,[26] in turn promoting the recruitment of cytotoxic $CD8^+$ T cells at the tumour site.[27,28] Additionally, the hypoxic nature of some solid tumours may also promote mitochondrial stress, that would favour the accumulation of immunogenic cytosolic DNAs in all cell types present within the microenvironment, thus modulating the global immune response.[29] Finally, regardless of the cell types in which cGAS is activated, the produced cGAMP has been shown to serve as an immune-transmitter (reviewed in ref.[30]). Several mechanisms have been involved in ensuring cGAMP export and uptake. More precisely, cGAMP can be transferred through gap junctions from cancer cells to tumour-associated antigen presenting cells, subsequently promoting T cell infiltration,[31] or to astrocytes thus promoting brain metastasis.[32] In addition, cGAMP can be actively exported from cells by ATP binding cassette subfamily C member 1 (ABCC1), which limits cell intrinsic STING signalling in vitro and in vivo.[33] Extracellular cGAMP can be uptaken by neighbouring cells via the importers solute carrier family 19 member 1 (SLC19A1) and SLC46A2[34,35] . Transport of cGAMP has also been shown to be mediated by the leucine rich repeat containing 8 (LRRC8) volume gated anion channels (VRAC), increasing STING-mediated IFN responses in the microenvironment.[36,37] The ATP-gated P2X purinergic receptor 7 (P2 × 7R) can also mediate the import of tumour-derived extracellular cGAMP into tumour associated macrophages in a context of high local ATP concentration, which occurs during tissue damage.[38] Finally, cGAMP can be encapsulated in virus-like particles that serve as a vehicle for delivery of cGAMP to bystander cells[39,40] (Figure 2). The key role of extracellular cGAMP in regulating inflammatory responses in the tumour microenvironment is also supported by studies demonstrating that the ectonucleotide pyrophosphatase/phosphodiesterase 1 (ENPP1), which promotes cGAMP cleavage,[41] is regulated during tumourigenesis. Tumours expressing ENPP1 evade inflammatory responses by preventing the transfer of cGAMP from cancer cells to immune cells. Consequently, loss of ENPP1 reduced tumour metastasis and restored tumour immune cell infiltration[42] (Figure 2).

In all these contexts, cGAS activation primarily induces anti-tumour responses,[43–45] thus positioning the cGAS-STING axis as a major pathway that may be therapeutically highjacked to promote anti-tumour responses and tumour clearance (Figure 2). In support, chemotherapy and radiotherapy both induce cGAS-STING pathway

activation through the accumulation of DNA breaks and cytosolic DNA fragments, thereby potentiating anti-tumoural responses[26,46,47] and inducing immunogenic cell death. As a potential drawback, chemotherapy, irradiation and STING agonists all induce the expression of the programmed cell death protein 1 (PD-1) and programmed death-ligand 1 (PDL-1) immune checkpoints.[48–50] This can be overcome by combining chemotherapy or STING agonists with checkpoint inhibitors, which is a promising therapeutic strategy to promote tumour regression.[51,52]

The cGAS-STING pathway and its pro-tumoural roles

The benefits of cGAS-STING activating therapeutics are contrasted by other evidences that demonstrate promotion of tumourigenesis following activation of this signalling axis. For example, chronic cGAS-STING pathway activation in chromosomally unstable cancer cells induces NF-κB activation and thus tumour supporting inflammatory cytokine production,[53,54] ultimately promoting metastatic dissemination[32,54] and chemoresistance.[55,56] Furthermore, STING activation in T cells can lead to T cell death, which can impair the establishment of anti-tumour adaptive immune responses.[57,58]

Importantly, downregulation of cGAS and/or STING has been reported in several cancers and has been proposed to serve as an immune escape strategy.[59,60] However, high expression of cGAS and/or STING has also been shown to predict poor outcome for cancer patients.[61] Tumour grade and origin, as well as the diversity of cells present in the tumour microenvironment and expression of immune checkpoints are parameters that likely contribute to dictate tumour immunogenicity,[62] supporting that further studies are required to avoid drawbacks of using STING agonists in clinics.[57]

CGAS EXPRESSION IN TUMOURIGENESIS

cGAS activation and cGAMP signalling are tightly regulated to allow a fast and tailored response to pathological cytosolic DNAs, while avoiding spurious activation. Chronic cGAS activation and cGAMP production leads to pathologies marked by dysregulated type I IFN signalling and immunopathology.[63] Several reviews address the regulation of the cGAS-STING signalling axis.[64,65] Such mechanisms include the regulation of cGAS expression levels and activity, the stability and trafficking of cGAMP, and the excitability of STING. While all relevant to the activation of anti-tumour responses following cGAS activation, we here focus on the parameters regulating the expression of cGAS, that is a central bottleneck determining the responsiveness to cGAS-targeting therapies.

cGAS is expressed in nearly all human tissues and can be detected in most cells[66] (https://www.proteinatlas.org/ENSG00000164430-CGAS), but the majority of cell types show low to moderate *cGAS* mRNA levels as compared to cells of the immune system, such as macrophages, monocytes, dendritic cells, granulocytes, T and B cells, or glial cells.[67] In the *cGAS* promoter, specificity protein 1 (Sp1) and cAMP response element-binding protein (CREB) binding sites, and interferon-sensitive response elements (ISREs) have been identified.[68,69] Both Sp1 and CREB are frequently deregulated in cancer cells[70,71] but their link to cGAS expression in cancer is unexplored. On the other hand, presence of ISREs could explain the inducibility of cGAS expression by IFNs.[69] Interestingly, a recent publication highlights cGAS upregulation though viral E6 protein in human papillomavirus infected keratinocytes, suggesting that tumorigenic viral infections modulate cGAS signalling in precancerous and cancerous cells, an aspect that merits further investigation.[72]

Epigenetic control of cGAS and/or STING expression has also been explored. The histone deacetylase HDAC3 promotes cGAS expression in microglia by deacetylation of the p65 NF-κB subunit, suggesting that cGAS expression is controlled by acetylation.[73] It seems likely that further cell type specific enhancer or promoter elements regulate *cGAS* transcription. In addition, cGAS and STING expression in melanoma and colorectal carcinoma were shown to be controlled by promoter methylation.[60,74] Consequently, reversal of cGAS and STING promoter methylation in melanoma cell lines by treatment with the 5-aza-2′-deoxycytidine (5-AZA-dC) demethylating agent enhanced responsiveness towards dsDNA, antigenicity and activation of tumour infiltrating lymphocytes.[74] Finally, the transcriptional silencing of cGAS in hypoxia through miR-25 and miR-93, which target the epigenetic regulator nuclear receptor coactivator 3 (NCOA3) and modulate *cGAS* expression indirectly, adds another level of transcriptional control and might be relevant for downregulation of cGAS in solid tumours, which are known to be highly hypoxic.[75]

In sum, even though recent studies shed light on the transcriptional control of cGAS, there are still considerable gaps in our understanding, that at present preclude efficient treatment implementation. For instance, a thorough understanding of the determinants of methylation-mediated regulation of cGAS expression could indicate HDAC or DNA methyltransferase inhibitors (both approved anti-cancer therapies) as treatment options for poorly immunogenic cancers (Figure 2), as was proposed for STING.[76,77]

DNA-PK: A REGULATOR OF CGAS ACTIVATION?

DNA-PK can operate as a STING- and cGAS-independent dsDNA sensor in human cells.[10,11] In addition, we and others showed STING- and cGAS-dependent enhancement of dsDNA sensing by DNA-PK activation.[11,18] Mechanistically, dsDNA transfection or genotoxic stress-associated accumulation of dsDNA in the cytosol lead to cytosolic re-localisation and DNA binding of the DNA-PK complex, followed by DNA-PKcs activation as attested by S2056-autophosphorylation.[11] In conditions in which both cGAS and DNA-PK are expressed, DNA-PKcs-dependent phosphorylation of cGAS at residue S435 strongly enhances cGAS-mediated production of cGAMP and downstream signalling in response to dsDNA transfection and genotoxic stress[11] (Figure 3). DNA-PKcs-dependent cGAS phosphorylation may be counteracted by the protein phosphatase 6C (PPP6C) that has been shown to regulate the S435 site.[78] While the catalytic

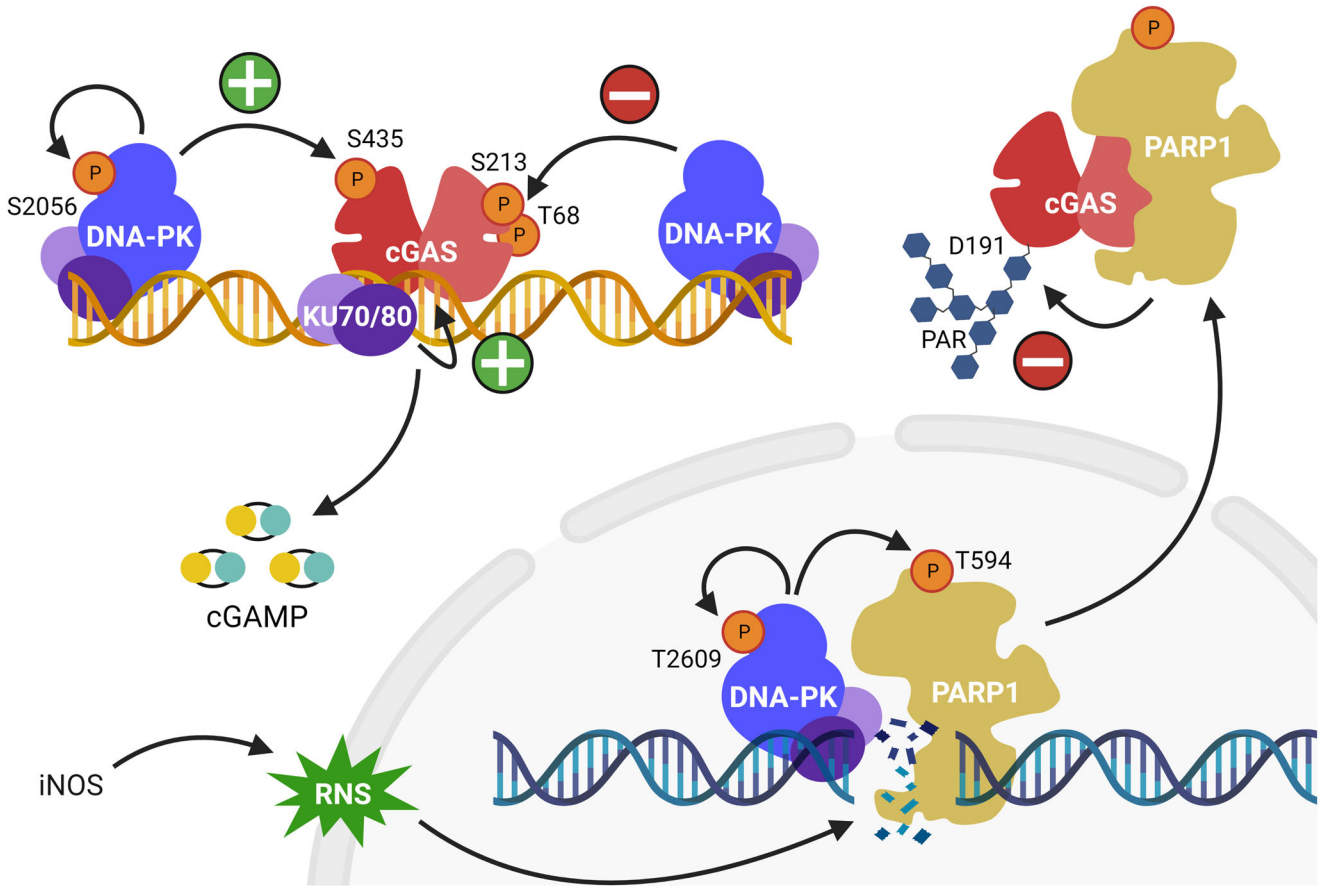

FIGURE 3 DNA-dependent protein kinase (DNA-PK) modulates cyclic GMP-AMP (cGAMP) synthase (cGAS)-dependent sensing of dsDNA. The DNA-PK complex, comprised of the catalytic subunit DNA-PKcs and DNA-binding subunits KU70 and KU80, modulates cGAS activity in a context-dependent manner. Upon DNA binding, DNA-PK enhances cGAS activity through two major mechanisms: first, KU70 and KU80, interacting with cGAS, facilitate cGAS-dsDNA interaction and cGAS condensation; second DNA-PKcs phosphorylates cGAS at residue S435, strongly enhancing cGAS-dependent cGAMP production. In contrast, DNA-PKcs participates also in the inhibition of cGAS activity, either by promoting cGAS phosphorylation at residue S213 and T68 or by phosphorylating poly(ADP-ribose) polymerase 1 (PARP1) at residue T594. PARP1 is in turn re-localised to the cytosol and, by PARylating cGAS, reduces its affinity for dsDNA. Created with BioRender.com.

activity of DNA-PKcs is required for S435 phospho-dependent cGAS priming, KU70 and KU80 were shown to increase the affinity of cGAS to DNA, as well as cGAS condensation, independently of DNA-PKcs[79] (Figure 3). DNA-PK-cGAS cooperation may be particularly important for cGAS-STING-mediated sensing of endogenous DNA in absence of infection and at early time points.[11]

In contrast, several reports indicate that DNA-PK can counteract cGAS-mediated dsDNA sensing in response to Herpes simplex virus 1 (HSV-1) DNA virus infection and at late time points after stimulation.[80,81] In that context, DNA-PKcs has been proposed to phosphorylate cGAS at residues T68 and S213 and to thereby inhibit cGAS-STING signalling[80] (Figure 3). Catalytic DNA-PKcs activity is required for inhibition of DNA sensing and promotion of viral replication.[80] What exactly regulates the choice of cGAS phosphorylation sites for modification by DNA-PKcs and thus the outcome of cGAS-DNA-PK interaction necessitates further investigation. In addition, how DNA-PKcs-dependent cGAS phosphorylation is integrated with other phospho-regulatory circuits that control cell cycle progression,[82,83] remains an open question.

Recent evidence highlights an alternative mechanism of cGAS inhibition through DNA-PK.[81] Wang et al. proposed that in the context of HSV-1 infection, DNA-damage is induced by an increase of reactive nitrogen species (RNS), which are produced by inducible nitric oxide synthase (iNOS). DNA-damage signalling triggered T2609-phosphorylation of DNA-PKcs, subsequently leading to downstream phosphorylation of poly(ADP-ribose) polymerase 1 (PARP1), mediating cytoplasmic re-localisation of PARP1[81] (Figure 3). Nuclear PARP1 is implicated in DNA damage repair, chromatin remodelling, replication fork stabilisation and translational control by modification of a range of target proteins through PARylation and has emerged as an important target in cancer therapy.[84,85] In HSV-1 infection however, cytosolic PARP1 modulates cGAS by PARylation at residue D191, thereby reducing cGAS binding affinity to dsDNA and thus interfering with the cGAS-STING dependent downstream immune response[81] (Figure 3). Importantly, this mechanism may also play a role in diverse inflammatory contexts, as well as etoposide-induced DNA damage.[81] How this translates to self-tolerance towards endogenous DNA in benign stress and if there is also a role of PARP1 in

modulating nuclear cGAS-dependent sensing are exciting new avenues to pursue.

Interestingly, this is not the first report of PARP1-cGAS interconnection. Liu et al. proposed earlier that cGAS can modulate DNA damage repair outcomes following nuclear translocation by interaction with PARP1 via poly(ADP-ribose) PAR and recruitment to DNA damage sites dependent on histone family member X (H2AX) phosphorylation.[86] Once recruited to DNA-damage foci, cGAS binding to PARP1 was proposed to compete for the formation of homologous recombination (HR) promoting complex of PARP1 and PAR-binding protein timeless, and thereby inhibit HR-dependent repair.[86,87] Whether and how PARP1-dependent inhibition of cGAS sensing, and cGAS-dependent inhibition of PARP1-mediated repair processes are interconnected is currently unclear. Nuclear or cytosolic localisation of activated DNA-PK may be a key step determining the localisation and outcome of the PARP1-cGAS interaction.

Intriguingly, in the past years, PARP1 inhibitors have emerged as promising oncologic treatment option. Indeed, PARP1 inhibitors can induce tumour cell death in cancers presenting loss of function mutations in BReast CAncer 1 and 2 genes (BRCA1/2), characterised by HR defects.[88,89] They have also been shown to increase cancer sensitivity to irradiation when combined to DNA-PKcs inhibitors.[90] In addition, PARP1 inhibition increases tumour immunogenicity via the cGAS-STING pathway,[91,92] which may be explained by the interaction between PARP1 and cGAS[81] (Figure 2).

DNA-PKCS EXPRESSION IN CANCER

DNA-PKcs plays cellular functions, spanning from DNA double strand break (DSB) repair,[14] maintenance of chromosome stability by controlling telomere length and mitosis, apoptosis, inflammatory responses and metabolism.[93] DNA-PKcs is also a regulator of transcription factors implicated in carcinogenesis, such as the tumour suppressor p53,[94] as well as hypoxia-inducible factor-1 alpha (HIF1a), which controls genes required for the adaptation to hypoxic conditions.[95] Thus, DNA-PKcs activity influences multiple tumour-associated pathways,[93] making DNA-PKcs a promising target for anti-tumoural therapies.

Genetic alterations of *PRKDC*, the gene encoding DNA-PKcs, are found in numerous cancer types with a frequency above 10%.[96] Several somatic mutations are found in *PRKDC* in breast and pancreatic cancer, including one nonsense mutation (c.7825A) at the Thr2609 residue,[97] belonging to a cluster essential in regulating DNA-PKcs activity and NHEJ-mediated DSB repair. Single nucleotide polymorphism (SNP) variants were found in intron 8 of the *PRKDC* gene and were associated with bladder and hepatocellular carcinoma.[98,99]

PRKDC/DNA-PKcs levels differ depending on tumour types. Intriguingly, in most types of tumours, *PRKDC*/DNA-PKcs expression is higher in tumour as compared to healthy adjacent tissues. This is the case for instance of esophageal adenocarcinoma,[100] renal cell carcinoma,[101] nasopharyngeal carcinoma[102] and non-small cell lung cancer.[103] Moreover, *PRKDC*/DNA-PKcs expression positively correlates with tumour grade in glioma,[11] colorectal carcinoma and nasopharyngeal carcinoma.[102]

It is believed that under normal growth conditions DNA-PKcs acts as a tumour suppressor, for instance by modulating p53 functions, or by maintaining genome integrity.[93] Indeed, low *PRKDC* expression or expression of defective alleles may promote accumulation of DNA lesions and thus genome instability, driving carcinogenesis. In contrast, in established tumours and during malignant progression, DNA-PKcs acts as a tumour promoter, since it promotes resistance to genotoxic therapy, sustains cell proliferation, promotes vascularisation by increasing the secretion of vascular endothelial growth factor (VEGF) and increases metastasis-related proteins such as metalloproteinases that allow tissue infiltration and metastasis.[104]

Chemotherapy and radiotherapy are conventional cancer treatments that rely on the induction of lethal DSBs in tumour cells. Since NHEJ is one of the key pathways implicated in DSB repair, DNA-PKcs expression in tumour is likely a key parameter to take into consideration when choosing therapeutic strategies. Indeed, there is evidence that chemotherapy efficiency is lower in patients with high DNA-PKcs levels[105] and that DNA-PKcs levels are higher in surviving tumour cells after radiotherapy, suggesting that DNA-PKcs-high cells selectively survive or that cancer cells upregulate DNA-PKcs expression following irradiation to evade genotoxicity.[106] For these reasons, current therapeutic strategies aim to combine chemotherapy or radiotherapy with the use of DNA-PKcs inhibitors that allows re-sensitisation of cells to the treatment.[107] Currently, at least eight clinical trials are ongoing with the aim of using DNA-PKcs inhibitor in combination with chemotherapy, irradiation or immunotherapy (NCT03724890; NCT02316197; NCT05687136; NCT02516813; NCT01421524; NCT04555577; NCT01353625; NCT05002140) (Figure 2).

Moreover, in non-cancerous cells, when cell cycle checkpoints are active, DNA-PKcs inhibition coupled to DSB induction leads to reduced micronuclei formation and thereby limits type I IFN responses.[108] In contrast, in p53-deficient cancer cells, the combination of radiotherapy with DNA-PKcs inhibitors promoted severe chromosomal abnormalities and accelerated micronuclei formation, ultimately inducing cGAS-STING dependent inflammatory responses.[109] However, in this context, expression of PD-L1 in irradiated cancer was also increased. Trivalent combination with the bifunctional TGFβ "trap"/anti-PD-L1 cancer immunotherapy has been proposed as a valuable alternative for treatment of p53-deficient/mutant solid tumours, rescuing $CD8^+$ T cell recruitment and improving survival in mice.[109] Intriguingly, RNA expression analyses of solid tumour samples from cohorts with patients treated with immune checkpoint inhibitors revealed that tumour samples presenting with *PRKDC* mutations show higher expression of chemokines, $CD8^+$ T cells, NK cells compared to tumours with wild type *PRKDC*, again associated with better patient survival.[110] Thus, together, these findings suggest that *PRKDC* mutations may be a stratification marker for immunotherapy and combination with immune modulators, including STING agonists.

However, an inflammatory tumour microenvironment is not always beneficial. For instance, in glioblastoma, tumour-associated

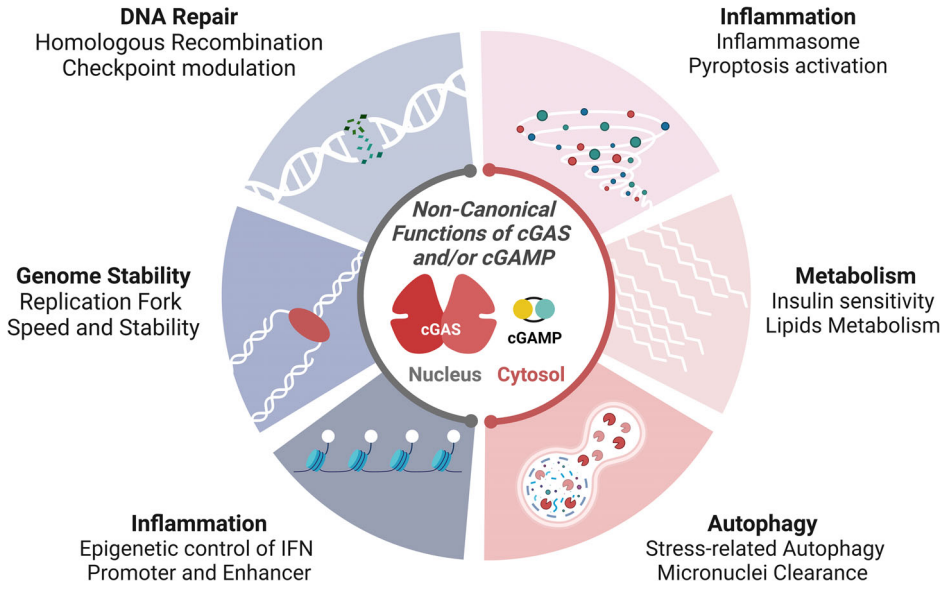

FIGURE 4 Non-canonical functions of cyclic GMP-AMP (cGAMP) synthase (cGAS) and/or cGAMP on cytosolic or nuclear cellular processes. cGAS and cGAMP are central in innate immune responses that have been recently involved in cellular processes in the cytosol (right/red part) and in the nucleus (left/grey part). In both cytosol and nucleus, cGAS and cGAMP can promote inflammatory responses independently of stimulator of interferon genes (STING). In the cytosol cGAMP acts as a metabolic regulator, while cGAS is involved in regulating autophagy. Finally, in the nucleus cGAS and cGAMP have a role in DNA repair and in genome stability. Created with BioRender.com.

macrophages are generally indicators of poor outcome[111] and inhibition of macrophage recruitment improves patient survival.[112] Interestingly, glioma patient data meta-analysis and immunohistochemistry analysis showed a positive correlation between cGAS and *PRKDC*/DNA-PKcs expression at the mRNA and protein level, which was positively correlated with tumour grade and was of bad prognosis.[11] In patients with higher cGAS and DNA-PKcs expression, an increase in chemokines and macrophage markers was also observed.[11] Therefore, as exemplified for glioblastoma, one could speculate that in a context in which DNA-PKcs drives cGAS-dependent tumour-promoting inflammation, the use of DNA-PKcs inhibitors may be a promising therapeutic approach (Figure 2).

NON-CANONICAL FUNCTIONS OF CGAS/CGAMP

Besides their well-known role in inflammatory responses, actors of the cGAS-STING pathway bear non-canonical functions (Figure 4), which may also modulate tumourigenesis. While they have to be carefully considered in cGAS/STING targeting therapies, these non-canonical functions may also be harnessed to promote tissue-specific responses. Below, we discuss some of these functions in the cytosol or nucleus, and how they may contribute to tumour fate.

Non-canonical functions of cGAS/cGAMP in the cytosol

The cGAS-STING pathway interacts with other inflammatory pathways such as the inflammasome (Figure 4),[113] thus it likely contributes to the identified role of inflammasome components in cancer initiation and progression.[114,115] A key tumour-related non-canonical aspect of the cGAS-cGAMP-STING pathway is its capacity to suppress endothelial cell proliferation in response to the bacterial endotoxin lipopolysaccharide (LPS), independently of IRF3 or NF-κB,[116] while inducing senescence and fibrosis.[117] Together with dysregulation of endothelial cell proliferation, this could modulate cancer progression, a process in which angiogenesis and inflammation are known facilitators.

cGAS and cGAMP were also implicated in a crosstalk with metabolic processes. For example, in obesity the activation of the cGAS-STING pathway fuels detrimental low-grade inflammation.[118] However, treatment with cGAMP improves systemic glucose homeostasis and insulin sensitivity, while ameliorating diet-induced proinflammatory responses in liver and adipose tissues.[119] Indeed, unlike in immune cells, exogenous cGAMP was shown to exert anti-inflammatory effects in hepatocytes and adipocytes, ameliorating their function (Figure 4). In support of the protective role of cGAS in metabolic cells, during liver ischemic/reperfusion (I/R) injury, cGAS prevents hepatocyte apoptosis in a STING-independent manner, notably through inhibition of autophagy.[120] While the role of autophagy in cancer remains ambiguous,[121] one may speculate that elevated cGAS expression in late-stage cancer may provide resistance to metabolic, hypoxic and therapeutic stress (Figure 4). In addition, cGAMP directly activates the fatty acid desaturase 2 (FADS2),[122] an enzyme that catalyses the rate limiting step in polyunsaturated fatty acid (PUFA) desaturation, thereby modulating glucose and lipid metabolism. As a consequence, in cells presenting low STING levels, such as hepatocytes,[123] the cGAS-cGAMP-FADS2 axis could be a major signalling pathway favouring pro-metastatic metabolic rewiring[124] (Figure 4).

Finally, cGAS plays a role in micronuclei homeostasis. Micronuclei are considered markers of genome instability and are associated with cancer[54] and their recognition by cGAS triggers innate immune responses.[108,125] Intriguingly, cGAS also enables micronuclei clearance through autophagy.[126] cGAS overexpression could thus also promote cancer cell survival by dampening IFN-response after genotoxic stress by promoting autophagy of micronuclei (Figure 4).

Non-canonical functions of cGAS/cGAMP in the nucleus

In the past years a role for cGAS in controlling genome instability has been described, notably through HR inhibition (Figure 4).[86,127] cGAS-driven HR inhibition promotes genome instability either leading to cell death[127] or sustaining tumourigenesis.[86] In addition, cGAS-dependent cGAMP production controls DNA repair responses, notably through preventing PARylation of essential components of the HR pathway in a STING-dependent, but IFN signalling-independent manner.[128] Moreover, upon genotoxic stress, cGAS promotes autophosphorylation of the Ataxia-telangectasia mutated (ATM) DNA repair protein in a cGAMP-, STING- and TBK1-dependent manner, leading to G1 cell cycle arrest independently of IFN signalling.[128]

cGAS also controls replication fork dynamics (Figure 4). Indeed, cGAS binds to replication forks and controls their speed and stability in a STING- and cGAMP-independent manner. In both, non-transformed and cancer cells, the absence of cGAS leads to increased sensitivity to radiation and chemotherapy.[129] To the contrary, cGAS was shown to promote replication fork protection in non-cancer cells, through a mechanism requiring cGAMP and STING, but not IFN signalling.[130,131] Therefore, while cGAS may provide protection from genome instability in non-cancer cells, thereby suppressing cancer initiation, in conditions of replication stress, cGAS may promote cancer cell survival.

Intriguingly, a non-canonical function of nuclear cGAS in innate immunity, which is independent of cGAMP production, was recently uncovered in mice (Figure 4). Upon DNA and RNA virus infection, nuclear cGAS promotes transcription of type I IFN genes, by altering epigenetic regulation of promoter and enhancer regions,[132] opening new questions on its role in the regulation of genes implicated in senescence or proliferation.

CONCLUSION

Many current therapeutic strategies aim at reactivating tumour immunogenicity. A major target for achieving this goal is the cGAS-STING pathway. While a number of STING agonists have entered clinical trials,[133] no cGAS agonist, apart from G3-ended Y-form Short DNA (G3-YSD), a canonical nucleic acid ligand, is available to date. Knowing the non-canonical functions of cGAS and cGAMP, such compounds may have interesting properties and have distinct target and off-target profiles as compared to STING agonists.

cGAS inhibitors may be beneficial in contexts where cGAS signalling drives tumour progression[11,86] (Figure 2). Several small molecules have been reported as potential human cGAS inhibitors. These compounds, such as G150, display good efficacy in vitro. However, the in vivo potency appears as limited by their mechanism of action, which is based on competition with cGAS substrates ATP and GTP.[134] While cGAS was shown to be also a target of broad acting molecules, such as Aspirin and Suramin (reviewed in ref.[135]) no specific cGAS inhibitor has entered clinical trials. In the future, efforts will need to be made to develop specific, potent and safe inhibitors of cGAS in order to fulfill this unmeet clinical need.

There is as of today a clear need for stratification strategies to allow determining which patients would benefit from administration of cGAS and/or STING activators or inhibitors. Understanding whether differential regulation of cGAS and STING expression/activation and of their regulators occurs in cancer and non-cancer cells appears as a paramount endeavour.

The KU70, KU80 and DNA-PKcs components of the DNA-PK DNA repair complex have been described to be individually and concertedly involved in triggering dsDNA-dependent inflammatory responses in cancer cells with low cGAS expression. Furthermore, DNA-PK has also been demonstrated to boost the activity of cGAS, when co-expressed.[11,79] These findings have major implications for our understanding of the regulation of inflammatory responses in physiological and pathological contexts. Particularly in the context of tumour biology, where several tumours downregulate cGAS and/or STING, DNA-PK may allow by-passing the requirement for cGAS. In contrast, the synergy between cGAS and DNA-PK could also allow boosting inflammatory responses in poorly immunogenic tumours. The status of DNA-PK and cGAS expression and responsiveness in tumours and immune and non-immune cells from the tumour microenvironment are likely key factors to take into consideration to stratify patients and to develop and choose between tumour immunogenicity-boosting therapeutic strategies. Thus, the discovery of cGAS regulators and alternative cytosolic nucleic acid detection pathways is likely to open novel therapeutic windows for cancer patients.

AUTHOR CONTRIBUTIONS

Conceptualisation, writing – original draft and writing – review & editing: Clara Taffoni, Moritz Schüssler, Isabelle K. Vila and Nadine Laguette. *Data curation*: Clara Taffoni, Moritz Schüssler and Isabelle K. Vila. *Visualisation*: Moritz Schüssler and Isabelle K. Vila.

ACKNOWLEDGEMENTS

We acknowledge the SIRIC Montpellier Cancer (INCa_Inserm_DGOS_12553). Work in NL's laboratory is currently funded by LA LIGUE pour la recherche contre le cancer (ARNs therapeutics, AAPARN 2021.LCC/JuF), Agence Nationale de Recherche sur le SIDA et les Hépatites virales (ANRS: ECTZ117448), La Région Languedoc Roussillon, the CNRS and the Université de Montpellier (Plan d'investissements France 2030, LabUM EpiGenMed, I-SITE). Clara Taffoni is supported by the ANRS (ECTZ119088). Moritz

Schüssler is supported by La Ligue ARNs thérapeutiques (AAPARN 2021.LCC/JuF).

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Data sharing not applicable to this article as no datasets were generated or analysed during the current study.

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How to cite this article: Taffoni, C., Schüssler, M., Vila, I. K., & Laguette, N. (2023). Harnessing the cooperation between DNA-PK and cGAS in cancer therapies. *BioEssays*, e2300045. https://doi.org/10.1002/bies.202300045