**Article**

**cGAS-like receptors sense RNA and control 3′2′-cGAMP signalling in Drosophila**

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Cyclic GMP–AMP synthase (cGAS) is a cytosolic DNA sensor that produces the second messenger cG[2′–5′]pA[3′–5′]p (2′3′-cGAMP) and controls activation of innate immunity in mammalian cells1–3. Animal genomes typically encode multiple proteins with predicted homology to cGAS4–10, but the function of these uncharacterized enzymes is unknown. Here we show that cGAS-like receptors (cGLRs) are innate immune sensors that are capable of recognizing divergent molecular patterns and catalysing synthesis of distinct nucleotide second messenger signals. Crystal structures of human and insect cGLRs reveal a nucleotidyltransferase signalling core shared with cGAS and a diversified primary ligand-binding surface modified with notable insertions and deletions. We demonstrate that surface remodelling of cGLRs enables altered ligand specificity and used a forward biochemical screen to identify cGLR1 as a double-stranded RNA sensor in the model organism Drosophila melanogaster. We show that RNA recognition activates Drosophila cGLR1 to synthesise the novel product cG[3′–5′]pA[2′–5′]p (3′2′-cGAMP). A crystal structure of Drosophila stimulator of interferon genes (dSTING) in complex with 3′2′-cGAMP explains selective isomer recognition, and 3′2′-cGAMP induces an enhanced antiviral state in vivo that protects from viral infection. Similar to radiation of Toll-like receptors in pathogen immunity, our results establish cGLRs as a diverse family of metazoan pattern recognition receptors.

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enzymes from the species Lucilia cuprina, Drosophila eugenalis, Drosophila erecta and Drosophila simulans (Extended Data Figs. 2b–f, 3a). Similar to Tc-cGLR, each active Diptera enzyme specifically responded to dsRNA, indicating that cGLR-based recognition of RNA is conserved across diverse insect species (Fig. 2b, Extended Data Fig. 4a).

The D. simulans enzyme identified in our screen shares 91% sequence identity with the protein product of the D. melanogaster gene CG12970 (GenBank NP_788360.2). Analysis of recombinant D. melanogaster CG12970 protein revealed that it also synthesizes a nucleotide product specifically in the presence of dsRNA and we therefore named this gene Tc-cGLR1 (Fig. 2c). To understand how dsRNA engages cGLR1, we modelled Dm-cGLR1 and Tc-cGLR1 structures as a DNA:cGAS complex (Protein Data Bank (PDB): 6CTA) reveals that cGLRs have a conserved architecture with a nucleotidyltransferase signalling core and a shared primary ligand-binding surface (dashed lines). The purple and green boxes indicate cutaways in b. Zoomed-in cutaways highlighting structural insertions and deletions unique to each cGLR. hMB21D2 and Tc-cGLR lack the Zn-ribbon motif present in cGAS (left) and hMB21D2 contains a C-terminal α-helix extension that contacts the central ‘spine’ helix (right). Alterations in the predicted ligand-binding surfaces suggest individual cGLRs are remodelled to recognize different molecular patterns.

To understand how Drosophila cGLR1 engages dsRNA, we modelled interactions using the Tc-cGLR and human cGAS–DNA structures as a template and observed that charge-swap mutations to the conserved basic ligand-binding surface disrupted product synthesis in vitro and STING signalling in cells (Fig. 2e, Extended Data Fig. 3c–f). Together, these data demonstrate that insect cGLRs and human cGAS use a shared mechanism of ligand detection and reveal that Dm-cGLR1 can function as a foreign RNA sensor.

A role in sensing long dsRNA suggests that the function of Dm-cGLR1 is to control a downstream immune response in Drosophila. In human cells, cGAS synthesizes the nucleotide second messenger 2′,3′-cGAMP, which contains a non-canonical 2′–5′ phosphodiester linkage that is required for potent activation of immune signalling. To determine how Dm-cGLR1 controls cellular signalling, we purified the cGAS product for direct comparison to 2′,3′-cGAMP. The Dm-cGLR1 product exhibited a C18 chromatography migration profile distinct from 2′,3′-cGAMP and all previously known naturally occurring cyclic dinucleotide (CDN) signals (Fig. 3a, Extended Data Fig. 6a). Production of this nucleotide signal was conserved in Diptera with Ds-cGLR1, Lc-cGLR and Deu-cGLR reactions, each synthesizing the same major reaction product (Extended Data Fig. 6a). Using nucleobase-specific labelling and nuclease digestion of the Dm-cGLR1 product, we observed a 3′–5′ linkage connected to an adenosine phosphate and a protected 2′–5′ linkage connected to a guanosine phosphate, indicating a mixed-linkage cyclic GMP–AMP species (Fig. 3b). We verified these findings with comparative high-performance liquid chromatography and tandem mass spectrometry profiling against a chemically synthesized standard, and confirmed that the shared Diptera cGLR
product is the novel isomer 3′2′-cGAMP (Fig. 3a, b). Using direct delivery of CDNs to permeabilized cells, we confirmed that dSTING preferentially responds to 3′2′-cGAMP in the cellular environment (Extended Data Fig. 8d). To define the mechanism of selective 3′2′-cGAMP recognition, we next determined a 2.0 Å crystal structure of dSTING bound to 3′2′-cGAMP 5 (Extended Data Fig. 8e). Each nucleobase of 3′2′-cGAMP is stacked between dSTING Y164 and R234, and the 2′–5′ phosphodiester linkage is positioned to extend outwards from the ligand-binding pocket by the deletion of a single lid residue and the formation of a salt bridge with E257 (Extended Data Fig. 8f). In human STING, high-affinity recognition of 3′2′-cGAMP requires readout of the 2′–5′ phosphodiester linkage by R232 in the β-strand lid1. In dSTING, the equivalent R229 makes no contact with either phosphodiester bond. Instead, R229 is repositioned to extend outwards from the ligand-binding pocket by the deletion of a single lid residue and the formation of a salt bridge with E257. The dSTING–3′2′-cGAMP structure reveals a tightly ‘closed’ conformation with dSTING protomers positioned 36 Å apart, similar to the closed conformation of human STING bound to 2′3′-cGAMP (Extended Data Fig. 8e). Each nucleobase of 2′3′-cGAMP is stacked between dSTING Y164 and R234, and E257 specifically coordinates the 3′2′-cGAMP guanosine N2 position (Extended Data Fig. 8f). In human STING, high-affinity recognition of 3′2′-cGAMP requires readout of the 2′–5′ phosphodiester linkage by R232 in the β-strand lid1. In dSTING, the equivalent R229 makes no contact with either phosphodiester bond. Instead, R229 is repositioned to extend outwards from the ligand-binding pocket by the deletion of a single lid residue and the formation of a salt bridge with E257. The dSTING–3′2′-cGAMP structure reveals a tightly ‘closed’ conformation with dSTING protomers positioned 36 Å apart, similar to the closed conformation of human STING bound to 2′3′-cGAMP (Extended Data Fig. 8e).
dSTING–3′2′-cGAMP structure are widely conserved in Diptera, and together explain a mechanism for how specific 3′2′-cGAMP-dependent signalling drives the activation of dSTING.

To determine how Dm-cGLR – 3′2′-cGAMP – dSTING signalling controls immune responses in vivo, we next injected 3′2′-cGAMP into D. melanogaster to directly monitor the dSTING response. 3′2′-cGAMP transiently induced the expression of Sting and three other Sting-regulated genes (Srg) in a dose-dependent manner (Fig. 4a, Extended Data Fig. 9). Notably, 3′2′-cGAMP-dependent signalling through dSTING was significantly more potent than the response triggered by injection of the bacterial CDN signal 3′3′-c-di-GMP (Fig. 4a, Extended Data Fig. 9e–k). Genetic mutations to Sting and the NF-kB homologue Relish ablated 3′2′-cGAMP-induced responses, demonstrating that signalling operates through a conserved dSTING–NF-kB pathway (Fig. 4a, Extended Data Fig. 9e–k). We challenged flies with viral infection and observed that 3′2′-cGAMP markedly suppressed the replication of two unrelated pathogen, and vesicular stomatitis virus (VSV) (Fig. 4b, c, Extended Data Fig. 10a, b). 3′2′-cGAMP activation of antiviral immunity was strictly dependent on Sting and resulted in a response that significantly delayed pathogen-mediated mortality (Fig. 4b, c, Extended Data Fig. 10a, b). Direct comparison of the protective effects against Drosophila virus infection showed that the endogenous signal 3′2′-cGAMP exhibited greater antiviral potency than 2′3′-cGAMP. 3′2′-cGAMP more robustly suppressed RNA viral loads and extended animal survival (Fig. 4d, Extended data Fig. 10c, d), revealing that the dSTING antiviral signalling axis is preferentially activated by 3′2′-cGAMP in vivo. Together, these results demonstrate that 3′2′-cGAMP is an antiviral nucleotide second messenger in D. melanogaster and establish a CDN–STING–NF-kB axis that protects animals from viral replication.

Along with cGAS recognition of dsDNA, the discovery of animal cGLR dsRNA sensors establishes a diverse class of pattern recognition receptors conserved throughout metazoans. Divergent structural homologues of cGAS in humans and insects demonstrate that cGLRs constitute a rapidly evolving family of proteins in which remodelling as endogenous second messengers and highlights the evolutionary plasticity of cGLR signalling. Our structural analysis also reveals that the human cGLR MB21D2 is competent for synthesis of nucleotide second messengers and has a remodelled ligand-binding groove that is probably adapted for detection of an unknown stimulus. Together with the known high frequency of hMB21D2 mutations in cancer,

**Fig. 3 | Discovery of 3′2′-cGAMP as a metazoan nucleotide second messenger.** a, High-performance liquid chromatography (HPLC) analysis of the Dm-cGLR1 reaction (orange) and comparison with synthetic standards (black or dashed lines) demonstrates that Dm-cGLR1 synthesizes 3′2′-cGAMP as the major product. A minor Dm-cGLR1 reaction product is 2′3′-c-di-AMP (see also Extended Data Fig. 6a). b, Thin-layer chromatography analysis of mouse cGAS and Dm-cGLR1 reactions labelled with either α-32P-ATP or α-32P-GTP (indicated as [α-32P]TPP) and treated as indicated. Pairwise labelling and nuclease PI digestion verify that cGAS and Dm-cGLR1 synthesize distinct cGAMP isomers with opposite phosphodiester linkage specificities. Representative of n = 3 independent experiments. High-resolution mass spectrometry confirms the major Diptera cGLR product as 3′2′-cGAMP (see also Extended Data Fig. 6b). c, HPLC quantification of insect cGLR nucleotide products. 3′2′-cGAMP is the dominant product of each identified Diptera cGLR (denoted by a black line), and 2′3′-cGAMP is the dominant product of cGAS and Tc-cGLR. Data are the mean quantified product of n = 3 independent experiments. d, Thermal denaturation assay showing that dSTING selectively recognizes 3′2′-cGAMP (see also Extended Data Fig. 8b, c). Representative of n = 3 independent experiments. e, Crystal structure of the dSTING–3′2′-cGAMP complex reveals a tightly closed homodimer and an ordered β-strand lid, indicating high-affinity engagement of the endogenous Drosophila second messenger 3′2′-cGAMP. f, Alignment and conservation of the stem helix and β-strand lid in human and insect STING proteins. Critical ligand-binding residues (blue dot) and adaptations specific to Diptera (red outline) are denoted. g, Superposition of the dSTING–3′2′-cGAMP (blue–orange) complex and the human STING–2′3′-cGAMP (grey–pink) (PDB: 4KSY) complex reveals that human STING readout of the 2′5′ phosphodiester bond by R232 is absent in dSTING (left). Human STING S162 (grey) contacts the free 3′ OH of the guanosine base in 2′3′-cGAMP (pink). dSTING N519 (blue) extends across the ligand-binding pocket to contact the free 3′ OH of the adenosine base in 3′2′-cGAMP (orange). (right).
these results support a more extensive role for cGLR signalling in human biology. The existence of multiple unique cGLRs encoded within a single species (Extended Data Fig. 2a) suggests a model in which the cGLR signalling scaffold is harnessed to detect several distinct stimuli. In support of this conclusion, Hartmann, Imler, Cai and colleagues have identified cGLR2 as a second functional cGLR in Drosophila and have demonstrated in vivo that cGLR1 and cGLR2 have discrete roles in Drosophila immunity29. Together, our results define cGLRs as receptors in animal cells that are capable of detecting diverse pathogen-associated molecular patterns and dictating response to the foreign environment.

**Online content**

Any methods, additional references, Nature Research reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at https://doi.org/10.1038/s41586-021-03743-5.

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Bioinformatics and dipteran cGLR sequence analysis

Building on previous analyses,8-10,30-31 animal cGAS homologues suitable for crystallography were identified using the amino acid sequences of human cGAS (hcGAS) and D. melanogaster CG7194 to seed a position-specific iterative BLAST (PSI-BLAST) search of the NCBI non-redundant protein database. The PSI-BLAST search was performed with an E-value cut-off of 0.005 for inclusion into the next search round, BLOSUM62 scoring matrix, gap costs settings existence 1 and extension 1, and using conditional compositional score matrix adjustment. Candidate homologues identified from this search included the uncharacterized human protein MB21D2 and the T. castaneum sequence XP_969398.1. Pairwise structural comparison between hMB21D2, Tc-cGLR and protein structures in the PDB was performed using DALI13, and Z-scores for homologues less than 90% identical to one another (PDB90) were plotted in GraphPad Prism. A Z-score of 15 for Tc-cGLR and 13 for hMB21D2 was selected as a lower cut-off to emphasize directly relevant homologues in analysis.

Following structure determination of hMB21D2 and T. castaneum XP_969398.1, predicted cGLRs were further identified in Diptera using PSI-BLAST searches seeded with either D. melanogaster CG7194 or the Tc-cGLR sequence, selecting in each round for proteins matching known cGLR domain organization and active-site residues. Diptera cGLR sequences were aligned using MAFFT (FFT-NS-i iterative refinement method)33 and used to construct a phylogenetic tree in Geneious Prime v2020.12.23 using the neighbour-joining method and Jukes–Cantor genetic distance model with no outgroup. Further manual analysis and curation of candidate cGLR sequences were performed based on alignments and predictive structural homology using HHpred34 and Phyre2.35 Sequences were selected for predicted structural homology to cGAS, including the presence of a conserved nucleotidyltransferase domain with a G(S/G) activation loop and a [E(D)/H][E/D] X50-90 [E/D] catalytic triad. Manual refinement was also used to exclude duplicate sequences, gene isoforms and proteins less than 250 or greater than 700 residues. NCBI available genomes from 42 species in Diptera are represented in the final tree, including 31 species in the genus Drosophila. Clustering of sequences in the final unrooted tree was used to define clades, with no more than 30% sequence identity shared between members of different clades. Further manual analysis of the tree was used to determine the number and distribution of predicted cGLRs by species (see Extended Data Fig. 2a). PROMAL3D36 was used for structure guided alignment of apo hcGAS (PDB: 4KMS)32, hMB21D2 and Tc-cGLR in Extended Data Fig. 1a. MAFFT (FFT-NS-i iterative refinement method)33 was used to align STING sequences in Extended Data Fig. 8a. Geneious Prime software was used to generate the sequence alignments in Fig. 3f and Extended Data Figs. 1a, 3a, 8a.

Protein expression and purification

Recombinant cGLR and dSTING proteins were expressed and purified using methods previously optimized for hcGAS.33 Animal cGLR and dSTING sequences were codon-optimized for expression in Escherichia coli and cloned from synthetic constructs (GeneArt or Integrated DNA Technologies) into a custom pET16 expression vector with an N-terminal 6× His–MBP fusion tag or an N-terminal 6× His–SUMO fusion. The full-length coding sequence was used except for hcGAS 157–522, mouse cGAS 147–607, hMB21D2 S29–F491, Ds-cGLR19–393 and D. eugracilis STING 150–340 as specified. The N terminus of D. eugracilis STING 150–340 was fused to the full-length coding sequence of T4 lysozyme connected by a Gly-Ser linker sequence. Briefly, transformed BL21-CodonPlus(DE3)-RIL E. coli (Agilent) were grown in MDG media overnight before inoculation of M9ZB media at an OD600 of 0.0475. M9ZB cultures were grown to OD600 of 2.5 (approximately 5 h at 37°C with shaking at 230 rpm) followed by cooling on ice for 20 min. Cultures were induced with 500 μM IPTG before incubation at 16°C overnight with shaking at 230 rpm. Cultures were pelleted the following day and either flash frozen in liquid nitrogen for storage at ~80°C or directly lysed for purification. Selenomethionine-substituted proteins for crystallography experiments were purified using a modified growth protocol as previously described33 For large-scale protein purification, proteins were expressed with a 6× His–SUMO2 (Tc-cGLR, Ds-cGLR, Deu-cGLR, Lc-cGLR and dSTING) or 6× His–MBP (Dm-cGLR1 and Der-cGLR1) fusion tag and grown as approximately 4–8×11 cultures in M9ZB media. Pellets were lysed by sonication in lysis buffer (20 mM HEPES pH 7.5, 400 mM NaCl, 30 mM imidazole, 10% glycerol and 1 mM dithiothreitol) and clarified by centrifugation at approximately 47,850g for 30 min at 4°C and subsequent filtration through glass wool. Recombinant protein was purified by gravity flowover Ni-NTA resin (Qiagen). Resin was washed with lysis buffer supplemented to 1 M NaCl and then eluted with 20 ml of lysis buffer supplemented to 300 mM imidazole. SUMO2 fusion proteins were cleaved by supplementing elution fractions with approximately 250 μg of human SENP2 protease (D364–L589 with M497A mutation) during overnight dialysis at 4°C against dialysis buffer (20 mM HEPES pH 7.5, 250 mM NaCl and 1 mM dithiothreitol). MBP-tagged fusion proteins were buffer exchanged into lysis buffer with 4% glycerol and no imidazol to optimize conditions for overnight cleavage by recombinant TEV protease at approximately 10°C. cGLR proteins were next purified by ion-exchange chromatography using 5 ml HiTrap Heparin HP columns (GE Healthcare) and eluted across a 150–1,000 mM NaCl gradient. Target protein fractions were pooled and further purified by size-exclusion chromatography using a 16/600 Superdex 75 column or 16/600 Superdex 200 column (Cytiva) and storage buffer (20 mM HEPES pH 7.5, 250 mM NaCl and 1 mM TCEP). Final proteins were concentrated to approximately 20–30 mg ml−1 and flash frozen with liquid nitrogen and stored at ~80°C for crystallography or supplemented with 10% glycerol before freezing for biochemistry experiments. Tc-cGLR and Ds-cGLR1 mutant proteins were purified from 11 M9ZB cultures using Ni-NTA affinity chromatography and overnight dialysis directly into storage buffer (20 mM HEPES pH 7.5, 250 mM sodium chloride, 10% glycerol and 1 mM TCEP) without SUMO2 tag cleavage.

For small-scale protein purification used in the Diptera cGLR screen, recombinant proteins were expressed with a 6× His–MBP fusion tag with the exception of hcGAS, mouse cGAS, Tc-cGLR, Deu-cGLR, Lc-cGLR and Ds-cGLR1, which were expressed with a 6× His–SUMO2 fusion tag. Small-scale cultures were grown in 20 ml of M9ZB media, lysed with sonication, and recombinant protein was purified as previously described. Briefly, protein was purified directly from lysates by centrifugation and flow-through over Ni-NTA resin (Qiagen) in 2 ml Mini Spin columns (Epoch Life Sciences). Following elution with elution buffer (20 mM HEPES pH 7.5, 400 mM NaCl, 300 mM imidazole, 10% glycerol and 1 mM dithiothreitol), proteins were buffer exchanged into storage buffer (20 mM HEPES pH 7.5, 250 mM NaCl, 10% glycerol and 1 mM TCEP). Fresh protein preparations were immediately used for in vitro nucleotide synthesis reactions.

Protein crystallization and structure determination

Crystals of native and selenomethionine-substituted hMB21D2 S29–F491, Tc-cGLR and T4 lysozyme-dSTING L150–340 were grown at 18°C using hanging-drop vapour diffusion. Optimized crystals were grown in EasyXtal 15-well trays (NeXtal Biotechnologies) with 350 μl of reservoir solution and 2 μl drops set with a ratio of 1 μl of protein solution and 1 μl of reservoir solution. hMB21D2 crystals were grown using the reservoir solution (1.2 M ammonium sulfate, 5 mM MgCl2, 100 mM MES pH 6.2) based on conditions previously identified by Wang and Huang37. For 1 day before cryoprotection with reservoir solution supplemented with 30% glycerol and freezing in liquid nitrogen. Tc-cGLR crystals were grown using the reservoir solution (0.3 M potassium thiocyanate and 10–16% PEG-3350) for 5–16 days before cryoprotection with reservoir
solution supplemented with 15% ethylene glycol and freezing in liquid nitrogen. Apo T4 lysozyme–dSTING crystals were grown using the reservoir solution (0.2 M sodium citrate, 0.1 M Tris-HCl and 22% PEG-3350) 7 days before cryoprotection with reservoir solution supplemented with 15% ethylene and freezing in liquid nitrogen. T4 lysozyme–dSTING–3′2′-cGAMP crystals were grown using the reservoir solution (0.1–0.2 M sodium acetate pH 4.8, 0.2 M ammonium formate and 20–22% PEG-3350) supplemented with 250 μM 3′2′-cGAMP (Biolog) for 10 days before cryoprotection with reservoir solution supplemented to 35% PEG-3350 and freezing in liquid nitrogen. Growth of single HM21D2 and Tc-cGLR crystals was further optimized with streak seeding. X-ray diffraction data were collected at the Advanced Photon Source beamlines 24-ID-C and 24-ID-E and at the Advanced Light Source beamlines 5.0.1 and 8.2.2. Data were processed with XDS and Aimless\(^{16}\) using the SSRl autoxds script (A. Gonzales, SSRl, Stanford, CA, USA). Experimental phase information for all proteins was determined using data collected from selenomethionine-substituted crystals. Anomalous sites were identified, and an initial map was generated with AutoSol within PHENIX\(^{16}\). Structural modelling was completed in Coot\(^{16}\) and refined with PHENIX. Final structures were refined to stereochemistry statistics for the Ramachandran plot (favoured/allowed), rotamer outliers and MolProbity score as follows: HM21D2, 97.72%/2.28%, 0.71% and 1.27; 7c-cGLR, 98.17%/1.57%, 0.28% and 1.02; dSTING apo, 98.00%/2.00%, 0.33% and 1.30; and dSTING–3′2′-cGAMP, 97.06%/2.86%, 1.72% and 1.63. See Supplementary Table 1 and the ‘Data availability’ section for deposited PDB codes. All structure figures were generated with PyMOL. 2.3.0.

**Nucleotide product synthesis analysis**

cGLR nucleotide synthesis activity was analysed by thin-layer chromatography (TLC) as previously described\(^{3}\). For the Diptera cGLR screen, recombinant protein preparations were incubated in 10 μl reactions containing 0.5 μl α-32P labelled NTPs (approximately 0.4 μCi each of ATP, CTP, GTP and UTP), 200 μM unlabelled NTPs, 10 mM MgCl\(_2\) and 1 mM MnCl\(_2\) in a final reaction buffer of 20 mM Tris-HCl pH 7.5, 100 mM KCl and 1 mM TCEP. Reactions were additionally supplemented with approximately 1 μg poly I:C or 5 μM ISD45 dsDNA as indicated. Reactions were incubated at 37 °C overnight and subsequently treated with 1 μl Quick CIP phosphatase (New England Biolabs) for 20 min at 37 °C to remove unreacted phosphate signal. Each reaction was diluted 1:10 in 100 mM sodium acetate pH 5.2, and 0.5 μl was spotted on a 20-cm × 20-cm PEI-cellulose TLC plate. Plates were run with 0.5× 20% ammonium formate as a running buffer. TLC images were visualized by TLC, enzymes were tested at 5 μM with 5 μM nucleic acid condensates to settle. Fluorescence microscopy images were acquired at 25 °C using a Leica TCS SP5 X (Leica Microsystems) mounted on an inverted microscope (DM6000; Leica Microsystems) with an oil immersion ×63/numerical aperture 1.4 objective lens (HCX PL APO; Leica Microsystems). AF488-labelled Ds-cGLR1 was then further purified on a PD-10 desalting column (GE Healthcare) eluted with storage buffer (20 mM HEPES-KOH pH 7.5, 250 mM KCl and 1 mM TCEP). Final AF488-labelled Ds-cGLR1 was concentrated to approximately 5 mg ml\(^{-1}\), flash frozen in liquid nitrogen and stored as aliquots at −80 °C. hcGAS and hcGAS NTase domain (D157–F522) proteins were prepared as previously described\(^{3}\).

To induce nucleotide condensation, Ds-cGLR1 (10 μM, containing 1 μM AF488-labelled Ds-cGLR1) was mixed with various lengths of RNA (10 μM each) in buffer (20 mM Tris-HCl pH 7.5, 1 mg ml\(^{-1}\) BSA and 1 mM TCEP) in the presence of various salt concentrations at 25 °C in a total reaction volume of 20 μl. The details of proteins, nucleic acids and salt concentrations are indicated in the figures. Ds-cGLR1–RNA reactions were placed in 384-well non-binding microplates (Greiner Bio-One) and incubated at 25 °C for 30 min before imaging to allow condensates to settle. Fluorescence microscopy images were acquired at 25 °C using a Leica TCS SP5 X (Leica Microsystems) mounted on an inverted microscope (DM6000; Leica Microsystems) with an oil immersion ×63/numerical aperture 1.4 objective lens (HCX PL APO; Leica Microsystems). AF488-labelled Ds-cGLR1, hcGAS and hcGAS NTase domain proteins were detected with excitement at 488 nm (emission at 500–530 nm). Microscopy images were processed with FIJI\(^{12}\) and contrast adjusted with a uniform threshold setup for each enzyme.

**Cellular STING signalling assays**

Human HEK293T cells were purchased directly from the American Type Culture Collection (ATCC) and were maintained in complete media (DMEM supplemented with penicillin, streptomycin and 10% FBS) at 37 °C. HEK293T cells were validated by the ATCC and were not tested for mycoplasma contamination. For all assays, 4.5 × 10\(^4\) cells were plated in 96-well plates. STING and cGLR activity assays were performed using the Dual-Luciferase Reporter Assay System (Promega) as previously described\(^{13}\), with modifications. Lipofectamine 2000 was used to transfect IFNβ-firefly luciferase and TK-Renilla luciferase reporters and 5 ng of pcDNA4–mouse STING or 15 ng of pcDNA4–dsSTING hybrid construct (human STING transmembrane domains fused to the D. eugracilis STING CDN-binding domain (L150–1340) appended with the human STING C-terminal tail). For cGLR signalling assays, 150 ng of Drosophila cGLR1, 30 ng with ,120 ng empty vector, or 150 ng empty vector were additionally transfected. The native coding sequence was used for each cGLR and STING pcDNA4 plasmid. Twenty-four to thirty hours after transfection, luciferase was measured using a GloMax microplate reader (Promega), and relative IFNβ expression was calculated

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**Electrophoretic mobility shift assay**

Analysis of in vitro protein–nucleic acid complex formation was conducted as previously described\(^{4}\). Briefly, 1 μM 40-bp dsRNA or 45-bp dsDNA was incubated with Ds-cGLR1 or hcGAS NTase domain (D157–S22) at a concentration of 0.5, 1 or 2 μM. Complex formation was performed with the final reaction buffer (20 mM HEPES-NaOH pH 7.5, 75 mM KCl and 1 mM dithiothreitol). Reactions (20 μl) were incubated at 4 °C for 20 min before separation on a 2% agarose gel using 0.5× TB buffer (45 mM Tris and 45 mM boric acid) as a running buffer. The agarose gel was post-stained in 0.5× TB buffer supplemented with 10 μg ml\(^{-1}\) ethidium bromide with gentle shaking at 25 °C for 45 min. Complex formation was visualized using a ChemiDoc MP Imaging System (Bio-Rad).

**In vitro condensate formation assays**

In vitro condensate formation was analysed as previously described with minor modifications\(^{10,11}\). Briefly, Ds-cGLR1 was labelled with AlexaFluor-488 (AF488) carboxylic acid (succinimidyl ester) (Thermo Fisher Scientific) according to the manufacturer’s manuals using a molar ratio of 1:10 at 4 °C for 4 h. Excess free dye was removed by dialysis against buffer (20 mM HEPES-KOH pH 7.5, 250 mM KCl and 1 mM dithiothreitol) at 4 °C overnight, and AF488-labelled Ds-cGLR1 was then further purified on a PD-10 desalting column (GE Healthcare) eluted with storage buffer (20 mM HEPES-KOH pH 7.5, 250 mM KCl and 1 mM TCEP). Final AF488-labelled Ds-cGLR1 was concentrated to approximately 5 mg ml\(^{-1}\), flash frozen in liquid nitrogen and stored as aliquots at −80 °C. hcGAS and hcGAS NTase domain (D157–F522) proteins were prepared as previously described\(^{3}\).

To induce condensate formation, Ds-cGLR1 (10 μM, containing 1 μM AF488-labelled Ds-cGLR1) was mixed with various lengths of RNA (10 μM each) in buffer (20 mM Tris-HCl pH 7.5, 1 mg ml\(^{-1}\) BSA and 1 mM TCEP) in the presence of various salt concentrations at 25 °C in a total reaction volume of 20 μl. The details of proteins, nucleic acids and salt concentrations are indicated in the figures. Ds-cGLR1–RNA reactions were placed in 384-well non-binding microplates (Greiner Bio-One) and incubated at 25 °C for 30 min before imaging to allow condensates to settle. Fluorescence microscopy images were acquired at 25 °C using a Leica TCS SP5 X (Leica Microsystems) mounted on an inverted microscope (DM6000; Leica Microsystems) with an oil immersion ×63/numerical aperture 1.4 objective lens (HCX PL APO; Leica Microsystems). AF488-labelled Ds-cGLR1, hcGAS and hcGAS NTase domain proteins were detected with excitation at 488 nm (emission at 500–530 nm). Microscopy images were processed with FIJI\(^{12}\) and contrast adjusted with a uniform threshold setup for each enzyme.
NAH2PO4 pH 6.8 supplemented with 3% acetonitrile. and the cone voltage at 40 V. The source temperature and desolvation temperature was set at 2.5 kV in positive ionization mode. The capillary voltage was set at 37 °C for 1 h and then nucleotide product was recovered by filtering reactions through a 30-kDa cut-off concentrator (Amicon) to remove protein. Nucleotide products were separated on an Agilent 1200 Infinity Series LC system using a C18 column (Zorbax Bonus-RP 4.6 × 150 mm, 3.5 μm) at 40 °C. Products were eluted at a flow rate of 1 ml min⁻¹ with a buffer of 50 mM NaH₂PO₄ pH 6.8 supplemented with 3% acetonitrile.

To purify the Deu-cGLR product for mass spectrometry analysis, nucleotide synthesis reaction conditions were scaled as previously described for bacterial cGAS/DncV-like nucleotidyltransferase reactions. Briefly, a 10-ml reaction containing 528 nM Deu-cGLR enzyme, 25 μM ATP, 25 μM GTP, 10 μg poly I:C, 1 mM MnCl₂, 50 mM Tris-Cl pH 7.5, and approximately 25 mM KCl was incubated with gentle rotation for 36 h at 37 °C follow by Quick CIP (NEB) treatment for 6 h. The reaction was monitored using a 20 μl aliquot supplemented with α-²³P-labelled NTPs and to visualize product formation by TLC. Following incubation, the large-scale reaction was filtered through a 10-kDa concentrator (Amicon) and purified by anion-exchange chromatography using a 1-mL Sepharosecolumn (Cytiva) with water and eluting with a 0–2 M ammonium acetate gradient. Fractions corresponding to the main product 3′2′-cGAMP were differentiated from fractions corresponding to 2′3′-c-di-AMP by HPLC analysis. Products were further purified by size-exclusion chromatography using a Superdex 30 Increase 10/300 GL (Cytiva) with dH₂O as a running buffer. Peak fractions were eluted in 1-ml volumes, pooled and evaporated for storage before mass spectrometry analysis.

Nucleotide mass spectrometry analysis and 3′2′-cGAMP identification

Purified nucleotide product samples were evaporated at 40 °C under a gentle nitrogen stream. The residual pellet was resuspended in 200 μl HPLC grade water (J.T. Baker), and 40 μl was then mixed with 40 μl of water containing 50 ng ml⁻¹ tenofovir as internal standard and transferred to measuring vials. Experiments for 3′2′-cGAMP identification were performed on an ACQUITY UPLC I-Class/Vion IMS-QTOF high-resolution LC−MS system (Waters Corporation). Reverse-phase chromatographic separation was carried out at 30 °C on a C18 column (Nucleodur Pyramid C18 50 × 3 mm, 3 μm Macherey Nagel) connected to a C18 security guard (Phenomenex) and a 2-μm column saver. Separation was achieved using a binary gradient of water containing 10 mM ammonium acetate and 0.1% acetic acid (solvent A) and methanol (solvent B). The analytes were eluted at a flow rate of 0.6 ml min⁻¹. The eluting programme was as follows: 0–4 min: 0% B, 4–7.3 min: 0–10% B. This composition of 10% B was held for 1 min, then the organic content was increased to 30% within 2.7 min. The column was then re-equilibrated to 0% B for 2 min. The total analysis run time was 13 min. High-resolution mass spectrometry data were collected on a Vion IMS-QTOF mass spectrometer equipped with an electrospray ionization source, operating in positive ionization mode. The capillary voltage was set at 2.5 kV and the cone voltage at 40 V. The source temperature and desolvation gas temperature was 150 °C and 600 °C, respectively. Analyte fragmentation was achieved using argon as the collision gas. Collision energy of 10 V was used to obtain a low collision energy spectrum. For high collision energy spectrum, the collision energy was ramped from 15 to 30 V. Data acquisition was controlled by the UNIFI 1.9.4.0 software (Waters). For 3′2′-cGAMP identification, the retention times, drift times and fragment spectra of a synthetic 3′2′-cGAMP standard (Biol) were collected as a reference and compared with those of the suspected 3′2′-cGAMP in the samples.

3′2′-cGAMP quantification

For quantification of 3′2′-cGAMP, chromatographic conditions were transferred to a API4000 mass spectrometer (Sciex) coupled to a Shimadzu HPLC system (Shimadzu). The analytes were ionized by means of electrospray ionization in positive mode applying an ion spray voltage of 3,000 V. Further electrospray ionization parameters were as follows: curtain gas (CUR): 30 psi; collision gas (CAD): 9; source temperature: 650 °C; gas 1: 60 psi and gas 2: 45 psi, respectively. Detection was performed in SRM mode, selecting first for the double-protonated parent ion of 3′2′-cGAMP and 3′3′-cGAMP (used in calibrator series). This resulted in the following mass transitions: 3′2′-cGAMP and 3′3′-cGAMP: m/z 338.2 → 152 (quantifier), m/z 338.2 → 136 (identifier). Tenofovir served as the internal standard (m/z 288 → 176).

For 3′2′-cGAMP semi-quantitative quantification from lysate samples in the Diptera cGLR screen, calibration curves were plotted as peak area ratios of 3′3′-cGAMP as an internal standard versus the nominal concentration of the calibrators. The calibration curve was calculated using quadratic regression and 1/x weighting.

Synthetic cyclic dinucleotide standards

Synthetic cyclic nucleotide standards used for HPLC analysis and mass spectrometry analysis were purchased from Biolog Life Science Institute: 3′-cGAMP (cat no. C 177), 2′3′-cGAMP (cat no. C 161), 3′2′-cGAMP (cat no. C 238), 2′3′-c-di-AMP (cat no. C 187) and 2′3′-c-di-GMP (cat no. C 182).

Nuclease P1 and poxin cleavage analysis

Nuclease P1 cleavage analysis was performed using Dm-cGLR1 reactions labelled with either α-³²P-ATP or α-³²P-GTP as previously described. Briefly, radiolabelled nucleotide products were incubated with nuclease P1 (80 μM; N8630, Sigma) in buffer (30 mM NaOAc pH 5.3, 5 mM ZnSO₄ and 50 mM NaCl) for 30 min in the presence of Quick CIP (NEB). Poxin cleavage reactions were carried out using purified insect viral AcNPV enzyme as previously described. For HPLC analysis of poxin cleavage, 100-μl reactions were performed using 100 μM synthetic 2′3′-cGAMP or 3′2′-cGAMP, 50 mM AcNPV poxin, 50 mM HEPES pH 7.5, 10 mM KCl and 1 mM TCEP. Reactions were incubated at 37 °C and at each specified time reactions were terminated by heat inactivation at 95 °C for 2 min before HPLC analysis as described above. For TLC analysis of poxin cleavage reactions, the reactions were performed using α-³²P-labelled 2′3′-cGAMP synthesized by mcGAS or 3′2′-cGAMP synthesized by Deu-cGLR in 5-μl reactions containing 2.5 μM nucleotide product and 1 μM AcNPV poxin, 50 mM HEPES pH 7.5, 10 mM KCl and 1 mM TCEP. Reactions were incubated at 37 °C and at each specified time reactions were terminated by heat inactivation at 80 °C for 5 min before PEI-cellulose TLC analysis as described above.

STING CDN thermal shift assay

A final concentration of 15 μM dSTING was mixed with 3× SYPRO orange dye and 100 μM synthetic CDN (Biol) (or a 40 nM to 100 μM concentration gradient as described in Extended Data Fig. 8c) in 20 mM HEPES-KOH pH 7.5 and 100 mM KCl. Samples were heated from 20 to 95 °C in a Bio-Rad CFX thermocycler with HEX channel fluorescence measurements taken every 0.5 °C. The derivative of each curve over time was calculated using GraphPad Prism and graphed as a percent maximum change in fluorescence or used to calculate the melting temperature.
D. melanogaster cyclic dinucleotide injection and signalling analysis

Fly stocks were raised on standard cornmeal agar medium at 25°C. All fly lines used in this study were Wolbachia free. \textit{w^{1118}, dSTING\textsuperscript{Control}} and \textit{dSTING\textsuperscript{Rxn}} stocks have been described previously\cite{33,34}. \textit{Relish20} flies isogenized to the DrosDel \textit{w^{1118}} isogenic background were a kind gift from L. Teixeira (Instituto Gulbenkian de Ciência)\cite{35}. Cyclic dinucleotides including 2′-cGAMP (Biolog), 2′,3′-cGAMP (Invivogen) and 3′,3′-c-di-GMP (Invivogen) were dissolved in 10 mM Tris-HCl pH 7.5 and diluted to the indicated concentrations. Adult flies (3–5 day old) were injected with 69 nl of cyclic dinucleotide solution or 10 mM Tris-HCl pH 7.5 (negative control) by intrathoracic injection using a Nanoject II apparatus (Drummond Scientific). Flies were collected 24 h later in pools of 6 individuals (3 males and 3 females) or 10 individuals (5 males and 5 females) and homogenized for RNA extraction and quantitative PCR with reverse transcription (RT–qPCR) analysis, as described\cite{36}. The sample size for all \textit{Drosophila} experiments was determined using previously published protocols\cite{37}. Flies were randomly selected for each experimental group and blinding was not performed.

D. melanogaster viral challenge assays

For 3′2′-cGAMP and virus co-injection, flies were injected with 69 nl of virus (DCV: 5 plaque-forming units (p.f.u.), vesicular stomatitis virus (VSV): 2,000 p.f.u.) in 10 mM Tris-HCl pH 7.5 or in a 0.9 mg ml\(^{-1}\) 3′2′-cGAMP solution. For titration experiments comparing cGAMP isomers, 69 nl of DCV (5 p.f.u.) in serial diluted concentrations of 2′3′-cGAMP or 3′2′-cGAMP were injected in the body cavity of the flies. Survival was monitored daily, and flies were collected in pools of 6 individuals (3 males and 3 females) or 10 individuals (5 males and 5 females) at the indicated time points to monitor the viral RNA load by RT–qPCR.

Statistical analyses

All statistical analyses were performed using GraphPad Prism 9.0.1. Error bars and sample size for each experiment are defined in the figure legends. Comparisons between groups for gene expression and viral loads were analysed by unpaired parametric t-test, two-tailed with no corrections; comparison between groups for survival curves following viral infection were analysed by log-rank test.

Reporting summary

Further information on research design is available in the Nature Research Reporting Summary linked to this paper.

Data availability

Coordinates and structure factors of hMB21D2, Tc-cGLR, dSTING and the dSTING–3′2′-cGAMP complex have been deposited in the PDB under the accession codes 7LT1, 7LT2, 7MWY and 7MW2. All other data are available in the paper or the supplementary materials. Source data are provided with this paper.

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Additional information

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41598-021-08734-3. Correspondence and requests for materials should be addressed to P.J.K. Peer review information Nature thanks Osmar Nureli and the other, anonymous, reviewer(s) for their contribution to the peer review of this work. Peer reviewer reports are available.

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Extended Data Fig. 1 | See next page for caption.
Extended Data Fig. 1 | Sequence and structural analysis of hMB21D2 and Tc-cGLR. a, Structure guided sequence alignment of the catalytic domain of hcGAS (PDB: 4KM5)\(^{12}\), hMB21D2 and Tc-cGLR. Strict secondary structure conservation further supports conserved structural homology despite primary sequence divergence. The [D/E]hD[X_{50–90}]D catalytic triad is highlighted with a red outline and the human Zn-ribbon insertion that is absent in other cGLRs is denoted with a red dashed outline. hMB21D2 contains an additional 61 residues that are not resolved in the crystal structure and are absent from the alignment. b, c, Zoomed-in cutaways of the hMB21D2 (b) and Tc-cGLR (c) crystal structures highlighting positioning of conserved catalytic residues in the nucleotidyltransferase active site. In hcGAS, the analogous residues coordinate two Mg\(^{2+}\) metal ions to control synthesis of 2′3′-cGAMP (inset, middle; PDB: 6CTA)\(^{14}\). The hMB21D2 structure is in an inactive state distinguished by misaligned catalytic residues and occlusion of the active site by an extended Gly-Gly activation loop, indicating that catalytic activation is probably controlled by a conformational rearrangement. d, e, TLC analysis of in vitro tests for potential activating ligands of hMB21D2. No nucleotide products were identified upon stimulation with 40-nt or 40-bp nucleic acid ligands (d) or ligands known to activate mammalian Toll-like receptors (e). Data shown are representative of \(n = 3\) independent experiments. f, Z-score structural similarity plot showing homology between hMB21D2 and Tc-cGLR with representative structures in the PDB (PDB90). Increasing Z-score indicates greater homology, confirming the close relationship between animal cGLR enzymes and mammalian cGAS, and more distant similarity to cGAS/DncV-like nucleotidyltransferases (CD-NTases) in bacterial antiphage defence systems and human oligoadenylate synthase 1 (refs. 9,15,45,47–50). Z-score cut-offs are 13 and 15 for hMB21D2 and Tc-cGLR, respectively.
Extended Data Fig. 2 | Forward biochemical screen of predicted cGLRs in Diptera. **a**, Violin plot showing the number of predicted cGLRs in Diptera genomes. *Drosophila* genomes (n = 31 species) have a median of four predicted cGLRs in contrast to a median of two predicted cGLRs in other dipteran insects (n = 11 species). **b**, Schematic of the in vitro screen of predicted cGLRs in the order Diptera. Fifty-three sequences were selected representing each clade in the phylogeny in Fig. 2a. Following recombinant protein expression in *E. coli*, lysates were split into two samples for parallel TLC analysis of in vitro enzymatic activity and HPLC-MS analysis of lysate nucleotide metabolites. **c, d**, Purified cGLR proteins were incubated overnight at 37 °C with α<sup>32</sup>P-radiolabelled nucleotides, a mixture of Mn<sup>2+</sup> and Mg<sup>2+</sup>, and the 45-bp immunostimulatory DNA ISD45 or the synthetic dsRNA analogue poly I:C as potential nucleic acid ligands, and reactions were visualized by PEI-cellulose TLC. Wild-type (WT) and catalytically inactive mouse cGAS enzymes were used as controls for each sample set. Note that mouse cGAS exhibits dsDNA-independent activity in the presence of Mn<sup>2+</sup> (ref. 51). Predicted Diptera cGLRs are grouped by clade (DC01–05) and numbered within each clade. Ligand-dependent activity was identified for DC02_01, DC05_03, DC05_19 and DC05_21; species listed below. We observed ligand-independent activity for two enzymes in clade 3. Data represent n = 2 independent experiments. **e, f**, SDS–PAGE and Coomassie stain analysis of final cGLR proteins used for biochemical studies, which were purified by NITTA-affinity, ion-exchange chromatography and size-exclusion chromatography.
Extended Data Fig. 3 | See next page for caption.
Extended Data Fig. 3 | Sequence analysis and mutagenesis of insect cGLRs.

a, Alignment of the catalytic domain of hcGAS and active cGLRs identified in *T. castaneum*, *D. eugracilis*, *L. cuprina*, *D. erecta*, *D. simulans* and *D. melanogaster*. The EhD[X50–90]D catalytic triad is highlighted with a red outline and the human Zn-ribbon insertion that is absent in insect cGLRs is denoted with a red dashed outline. Predicted basic ligand-binding residues selected for mutational analysis denoted by black circles. cGLRs from *D. erecta* and *D. simulans* are close homologues of *Dm*-cGLR1 (76% and 91% sequence identity, respectively) and thus are also referred to as ‘cGLR1’. All biochemical experiments with *Ds*-cGLR1 were performed with a construct beginning at M19.

b, In vitro reactions demonstrating that mutation of the catalytic residues ablates nucleotide product synthesis by *Ds*-cGLR1 in response to poly I:C. c, d, In vitro reactions analysing dsRNA recognition through the putative ligand-binding surface by *Ds*-cGLR1 in response to poly I:C. The insets for panels c and d show models of the *Tc*-cGLR–dsRNA complex based on the hcGAS–dsDNA structure (PDB: 6CTA)14, indicating predicted dsRNA-interacting residues in *Ds*-cGLR1 (c) or *Tc*-cGLR (d). Charge swap mutation to these residues variably disrupted poly I:C-stimulated activity by *Ds*-cGLR1 and *Tc*-cGLR, shown by TLC (left) and quantified relative to WT activity (right). Data in b–d are representative of *n* = 3 independent experiments.

e, SDS–PAGE and Coomassie stain analysis of purified WT and mutant proteins, as labelled in the above TLC images. f, IFNβ luciferase assay in which cGLRs are expressed in human cells and CDN synthesis is detected by mammalian STING activation, as in Fig. 2e. IFNβ was quantified relative to the empty vector control. In comparison to hcGAS control, which is activated by expression vector-plasmid DNA, *Dm*-cGLR1 (left) and *Ds*-cGLR1 (right) strictly require poly I:C stimulation to activate a downstream STING response. Mutation to catalytic residues or putative ligand-binding residues ablates cGLR1 signalling. See Fig. 2e: *Dm*-cGLR1 activity quantified relative to WT activity upon poly I:C stimulation. Data are mean ± s.e.m. of *n* = 3 technical replicates and representative of *n* = 3 independent experiments.
Extended Data Fig. 4 | See next page for caption.
Extended Data Fig. 4 | Analysis of RNA recognition by insect cGLRs. 
a–c, In vitro activity assays for each active insect cGLR demonstrating that dsRNA recognition is required for enzyme activation. Reactions were performed with 40-nt or 40-bp synthetic ligands. Weak Deu-cGLR ssRNA-stimulated activity may be explained by transient short duplex formation similar to observations that some ssDNA oligos can stimulate mouse cGAS dsDNA-dependent activity3. 
b, TLC and quantification for enzyme activation in the presence of a panel of 10–40-bp synthetic dsRNA ligands. dsRNA (30 bp) is sufficient to stimulate maximal activity for Tc-cGLRs, Dm-cGLRs and Lc-cGLRs, while Ds-cGLR1 requires 35 bp and Deu-cGLR can be activated by dsRNAs as short as 15 bp. Data are mean ± s.e.m., quantified relative to maximum observed activity. c, Reactions with 146-bp in vitro-transcribed dsRNAs containing either a 5′ triphosphate or 5′ OH termini demonstrate that dsRNA recognition by insect cGLRs does not involve 5′-end discrimination. 
d, Deconvolution of catalytic metal requirements for enzymatic activity by insect cGLRs. Insect cGLRs require Mn^{2+} for maximal catalytic activity, with weak product formation observed in the presence of Mg^{2+}. e, Poly I:C titration demonstrates that dsRNA stimulation of Drosophila cGLR1 activity in cells is dependent on RNA concentration. IFNβ luciferase assay in which cGLRs are expressed in human cells and CDN synthesis is measured by mammalian STING activation, as in Fig. 2e and Extended Data Fig. 3f. IFNβ quantified relative to the empty vector control. Data are mean ± s.e.m. of n = 3 technical replicates. All data in a–e represent n = 3 independent experiments.
Extended Data Fig. 5 | Characterization of Ds-cGLR1–dsRNA condensate formation. a, Electrophoretic mobility shift assay (EMSA) showing binding between Ds-cGLR1 or the C-terminal NTase domain of hcGAS (hcGAS-NTase) and a 40-bp dsRNA or 45-bp dsDNA. Ds-cGLR1 preferentially binds to dsRNA and more weakly interacts with dsDNA, consistent with observed binding between hcGAS and dsRNA. b, EMSA comparison of Ds-cGLR1–dsRNA binding and mammalian cGAS–dsDNA binding. Similar to hcGAS, Ds-cGLR1 forms a higher-order protein–nucleic acid complex that does not migrate through the gel, in contrast to the 2:2 binding observed between mouse cGAS and dsDNA. Data in a and b are representative of \( n = 3 \) independent experiments. c, Analysis of the effect of AF488 labelling on Ds-cGLR1 enzymatic activity. Similar to previous observations with hcGAS, AF488 labelling negatively impacts enzymatic activity but has minimal effect at the ratio of 90% unlabelled and 10% labelled protein used for all imaging experiments. Data are mean ± s.e.m. of \( n = 3 \) independent experiments. d, e, Analysis of hcGAS (d) and Ds-cGLR1 (e) phase separation with AF488-labelled protein. Mammalian cGAS contains a highly disordered N-terminal extension of approximately 150 residues, but this unstructured extension is absent in insect cGLR sequences. In the presence of dsDNA, full-length hcGAS forms highly dynamic liquid droplets18,43,52, whereas the minimal hcGAS NTase domain forms rigid protein–DNA condensates similar to those formed by Ds-cGLR1–RNA complexes. hcGAS exhibits a preference for condensate formation in the presence of dsDNA (d), whereas Ds-cGLR1 exhibits a preference for dsRNA (e), as observed in panel a. Scale bars, 10 μm. Analysis of Ds-cGLR1 dsRNA length specificity for condensate formation demonstrates clear length dependency (e) and supports that long dsRNA and condensate formation are required for maximal Ds-cGLR1 activation.
Extended Data Fig. 6 | Synthesis of 3′2′-cGAMP by Diptera cGLRs. a, HPLC analysis of the nucleotide products of Tc-cGLR, Dm-cGLR1, Dv-cGLR1, Lc-cGLR and Deu-cGLR reactions compared with relevant synthetic controls. Integration of major and minor product peaks in n = 3 independent experiments was used to calculate relative product ratios shown in Fig. 3c. b, The Drosophila cGLR major reaction product was purified from Deu-cGLR reactions and compared with synthetic 3′2′-cGAMP with tandem mass spectrometry analysis. Parent mass extracted ion trace (left) and tandem mass spectra comparison (right) validate the chemical identity of the Drosophila cGLR product as 3′2′-cGAMP. c, Identification of widespread 3′2′-cGAMP synthesis by Diptera cGLRs. The heat map shows the relative concentrations of cGAMP isomers detected by HPLC-MS in bacterial lysates expressing Diptera cGLRs (as described in Extended Data Fig. 2b) (left). In all cases, 3′2′-cGAMP was present as the dominant product with trace amounts of 3′3′-cGAMP and 2′3′-cGAMP detected in some samples as minor species. Right, inset of clade 5 in the Diptera cGLR phylogeny from Fig. 2a annotated to show all enzymes identified to synthesize 3′2′-cGAMP.
Extended Data Fig. 7 | Mechanism of 3′2′-cGAMP bond formation and resistance to degradation by viral poxin enzymes. a, Analysis of Dm-cGLR1 reactions with pairwise combinations of α-32P-labelled nucleotides and non-hydrolyzable nucleotides reveals reaction intermediates and identifies the order of bond formation during 3′2′-cGAMP synthesis. Left: TLC analysis demonstrates that Dm-cGLR1 forms a linear intermediate in the presence of GTP and non-hydrolyzable ATP (Apcpp), indicating that the 2′–5′ phosphodiester bond is synthesized first. Exposed γ-phosphates removed by phosphatase treatment before analysis are indicated in parentheses. Note that while a linear intermediate cannot be formed in the presence of non-hydrolyzable GTP (Gpcpp), Dm-cGLR1 will synthesize the off-product 2′3′-c-di-AMP. Mouse cGAS, which synthesizes 2′3′-cGAMP through the linear intermediate pppG[2′–5′]pA, is shown here for comparison. Right: schematic of the reaction mechanism for each enzyme. b, Poxins are 2′3′-cGAMP-specific viral nucleases that disrupt cGAS–STING signalling. HPLC analysis of synthetic 2′3′-cGAMP or 3′2′-cGAMP treated with poxin from the insect baculovirus Autographa californica nucleopolyhedrovirus (AcNPV) is shown. In 1 min, AcNPV poxin cleaves 2′3′-cGAMP into a mixture of intermediate and full-cleavage product; and after 1 h, turnover is complete. No cleavage of 3′2′-cGAMP is observed by AcNPV poxin under these reaction conditions. c, Using TLC as a more sensitive assay, we observed minimal cleavage of 3′2′-cGAMP following overnight incubation with AcNPV poxin. Data in a–c are representative of n = 3 independent experiments. d, Schematic highlighting how an isomeric switch in phosphodiester linkage specificity makes 3′2′-cGAMP remarkably resistant to poxin-mediated cleavage.
Extended Data Fig. 8 | See next page for caption.
Extended Data Fig. 8 | Structural and biochemical analysis of dSTING.

a. Alignment of the C-terminal CDN-binding domains of human STING, mouse STING, *D. eugracilis* STING and *D. melanogaster* STING. Architecture of the core CDN-binding domain is conserved across metazoans; the disordered C-terminal tail, which controls IRF3–IFNβ signalling, is specific to vertebrates. Ligand-interacting residues selected for mutational analysis are denoted with a black circle; Diptera-specific adaptations are highlighted with a red outline. All structural and biochemical experiments were performed with a *D. eugracilis* STING construct terminating at I340.

b. In vitro thermal denaturation assay analysing dSTING interactions with a panel of CDNs. Only 3′2′-cGAMP forms a thermostable complex with dSTING in vitro (see also Fig. 3d). 2′3′-cGAMP is known to be capable of stimulating dSTING-dependent signalling in vivo, supporting that dSTING can engage with 2′3′-cGAMP with lower affinity. This observation is consistent with the weaker recognition of bacteria-derived 3′3′-cGAMP and 3′3′-c-di-GMP by human STING.

c. In vitro thermal denaturation assay demonstrating concentration-dependent thermal shift induced by 3′2′-cGAMP. Dose titration of 2′3′-cGAMP and 3′2′-cGAMP in human cells demonstrating selective response by dSTING to 3′2′-cGAMP. The *D. eugracilis* CDN-binding domain (CBD) was adapted for downstream signalling in human cells by addition of N-terminal human transmembrane (hTM) domains and the human C-terminal tail (hCTT).

d. Comparison of the human STING–2′3′-cGAMP and dSTING–3′2′-cGAMP crystal structures reveals a conserved closed homodimer architecture in which apical ‘wings’ are spread 32–36 Å, demonstrating high-affinity engagement with an endogenous ligand.

e. Enlarged cutaways of 3′2′-cGAMP in the dSTING crystal structure. Above: the simulated annealing Fo−Fc omit map (contoured at 3 σ). Below: a top-down view highlighting key dSTING–3′2′-cGAMP contacts.

f. Full crystal structure used to determine the structure of *D. eugracilis* STING in complex with 3′2′-cGAMP. T4 lysozyme is fused to the N terminus of the *D. eugracilis* STING CBD.

h. Thermal denaturation assay as in Fig. 3d demonstrating that N-terminal fusion of T4 lysozyme does not impair dSTING recognition of 3′2′-cGAMP.

i. Mutational analysis of key ligand-interacting residues in dSTING; the thermal denaturation assay was used to analyse 3′2′-cGAMP recognition. Mutations that conserve functional contacts with 3′2′-cGAMP (Y164F) maintain ligand recognition; mutations that ablate contacts abrogate ligand binding. N159S exhibits diminished ability to recognize 3′2′-cGAMP. Data in panels b and i are mean ± s.e.m. of the average Tm calculated from n = 2 technical replicates in n = 3 independent experiments. Data in panels c and d are mean ± s.e.m. of n = 3 technical replicates and representative of n = 3 independent experiments. j. SDS–PAGE and Coomassie stain analysis of purified WT and mutant proteins.
Extended Data Fig. 9 | 3′2′-cGAMP induces the expression of dSTING-regulated genes. a–d, Injection of 3′2′-cGAMP into *D. melanogaster* has a dose-dependent effect on the expression of Sting-regulated genes (*srgs*). 2′3′-cGAMP was used as positive control as previously characterized. Synthetic nucleotide was injected into the body cavity of WT (*w1118*) flies and gene expression was measured after 24 h. RNA levels were measured relative to the control gene *RpL32*, and nucleotide concentrations are displayed in μg μl⁻¹. Note that for *srg2* measurement after injection of 9E⁻⁷ μg μl⁻¹ 3′2′-cGAMP, there was one outlier replicate with a value of 0.5977 (data not shown, included in mean analysis). e–k, As in Fig. 4a, RNA expression analysis of Sting-regulated genes (*srgs*) 24 h after injection with synthetic 3′2′-cGAMP or 3′3′-c-di-GMP. RNA levels are shown as fold induction compared with buffer control in WT flies. dSTINGMut = RXN mutant; RelishMut = RelishE20 mutant, as previously characterized. All data in a–k represent the mean ± s.e.m. of n = 3 independent experiments and each point represents a pool of 6 flies. P value ns (>).05) unless otherwise noted: ****P < 0.0001 (e); ***P = 0.0002 (g); ****P < 0.0001, ***P < 0.0002 (g); ****P < 0.0001 (h); ****P < 0.0001, ***P < 0.0002 (j); ****P < 0.0001, ***P = 0.0009 (k).
Extended Data Fig. 10 | 3′2′-cGAMP functions as a potent antiviral ligand.

a, Analysis of the effect of 3′2′-cGAMP on Drosophila C virus (DCV) viral RNA load in flies. dSTING WT and mutant flies were co-injected with DCV and 3′2′-cGAMP or buffer control. Viral RNA levels were measured at each time as indicated relative to the control gene RpL32. DCV is a picornavirus-like (+) ssRNA virus in the family Dicistroviridae. **P = 0.0051, *P = 0.0388.
b, Analysis of the effect of 3′2′-cGAMP on vesicular stomatitis virus (VSV) viral RNA load in flies. dSTING WT and mutant flies were co-injected with VSV and 3′2′-cGAMP or buffer control as in a. Viral RNA levels were measured 4 days post-infection (dpi) relative to the control gene RpL32. VSV is a (+)ssRNA virus in the Rhabdoviridae family. *P = 0.0185.
c, Analysis of DCV viral RNA load in flies injected with increasing doses of 3′2′-cGAMP, 2′3′-cGAMP or buffer control (as in a). Viral RNA levels were measured 2 dpi relative to the control gene RpL32. For 2′3′-cGAMP injection: 9E−1 P = 0.0192. For 3′2′-cGAMP injection: 9E−3 P = 0.0212, 9E−2 P = 0.0075, 9E−1 P = 0.0070.
d, Survival curves after DCV infection showing the effect of injection with dose titration of 3′2′-cGAMP or 2′3′-cGAMP compared with buffer control. Both cGAMP isomers significantly delay mortality in a dose-dependent manner; 3′2′-cGAMP provides greater protection in comparison to 2′3′-cGAMP. For 2′3′-cGAMP injection: 9E−3 P = 0.0047, 9E−2 P = 0.0031, 9E−1 P = 0.0002. For 3′2′-cGAMP injection: 9E−4 P = 0.0344, 9E−3 P = 0.005, 9E−2 P < 0.001, 9E−1 P < 0.0001. All data in a–d represent the mean ± s.e.m. of n = 3 independent experiments and each point represents a pool of 6 flies (a, b) or 10 flies (c, d). P value is ns unless otherwise noted; ns P > 0.05.
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☐ ☐ For null hypothesis testing, the test statistic (e.g. F, t, r) with confidence intervals, effect sizes, degrees of freedom and P value noted

Give P values as exact values whenever suitable.

☐ ☐ For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings

☐ ☐ For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes

☐ ☒ Estimates of effect sizes (e.g. Cohen's d, Pearson's r), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection

All radioactivity-based imaging was collected using Typhoon scanner control 2.0.0.6
Chromatography traces collected using GE Unicorn 7.1
Protein, DNA, and RNA gel images collected using BioRad ImageLab 2.4.0.3
Protein homologs identified using NCBI PSI-BLAST (web-based: https://blast.ncbi.nlm.nih.gov/Blast.cgi)

Data analysis

Phenix 1.19, Coot 0.8.9, PyMOL 2.3, GraphPad Prism 9.0.1, Geneious Prime v2020.12.23, Image Quant 8.2.0

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- Accession codes, unique identifiers, or web links for publicly available datasets
- A description of any restrictions on data availability
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Atomic coordinates and structure factors of human MB21D2, T. castaneum cGLR, Drosophila STING, and the Drosophila STING–3’2’-cGAMP complex have been deposited in PDB under the accession codes 7LT1, 7LT2, 7MWY, and 7MWZ. All other data are available in the manuscript or the supplementary materials.
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For a reference copy of the document with all sections, see nature.com/documents/nr-reporting-summary-flat.pdf

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

| Sample size | Sample size for all Drosophila experiments was determined using previously published protocols (Cai et al., 2020, PMID:33262294). |
|-------------|------------------------------------------------------------------------------------------------------------------|
| Data exclusions | No data were excluded from analyses |
| Replication | All experiments were performed with independent replicates as described in the figure legends. |
| Randomization | X-ray crystal structures were refined with a randomly selected R-free reflection set based on automatic selection in Phenix 1.19. Flies were randomly selected for injection with any of the tested CDNs or buffer control. No other randomization was required for the cell biological, biochemical, and structural analyses in this study. |
| Blinding | Blinding was not performed for data analysis or group allocation for Drosophila experiments. Flies were randomly selected for each experimental group and data were collected by unbiased, quantitative means. |

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We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

| Materials & experimental systems | Methods |
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| n/a | n/a |
| ☒ Antibodies | ☒ Involved in the study |
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| ☒ Animals and other organisms | ☒ MRI-based neuroimaging |
| ☒ Human research participants | |
| ☒ Clinical data | |
| ☒ Dual use research of concern | |

Eukaryotic cell lines

Policy information about cell lines

- HEK 293T (catalog ATCC CRL-3216) cells were purchased directly from ATCC.
- HEK 293T cells were validated by ATCC.
- Cell lines were not tested for mycoplasma contamination.
- No misidentified lines were used.

Animals and other organisms

Policy information about studies involving animals; ARRIVE guidelines recommended for reporting animal research

- All Drosophila melanogaster fly lines are described in methods, were handled according to standards practices in the field, and are Wolbachia free. Equal numbers of male and female flies were selected for each experimental group. Flies were 3-5 days old at the commencement of each experiment.
- No wild animals were used in this study.
| Field-collected samples | No field-collected samples were used in this study. |
|-------------------------|--------------------------------------------------|
| Ethics oversight        | No ethics oversight was required for this study.  |

Note that full information on the approval of the study protocol must also be provided in the manuscript.