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## **cGAS-like receptors control RNA sensing and 3'2'-cGAMP antiviral signaling in *Drosophila***

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1           Cyclic GMP–AMP synthase (cGAS) is a cytosolic DNA sensor that produces the  
2 second messenger 2'3'-cGAMP and controls activation of innate immunity in mammalian  
3 cells<sup>1-5</sup>. Animal genomes typically encode multiple proteins with predicted homology to  
4 cGAS<sup>6-12</sup>, but the function of these uncharacterized enzymes is unknown. Here we show  
5 that cGAS-like receptors (cGLRs) are innate immune sensors capable of recognizing  
6 divergent molecular patterns and catalyzing synthesis of distinct nucleotide second  
7 messenger signals. Crystal structures of human and insect cGLRs reveal a  
8 nucleotidyltransferase signaling core shared with cGAS and a diversified primary ligand-  
9 binding surface modified with significant insertions and deletions. We demonstrate that  
10 cGLR surface remodeling enables altered ligand specificity and use a forward biochemical  
11 screen to identify cGLR1 as a double-stranded RNA sensor in the model organism  
12 *Drosophila melanogaster*. Surprisingly, RNA recognition activates *Drosophila* cGLR1 to  
13 synthesize the novel product cG[3'–5']pA[2'–5']p (3'2'-cGAMP). A crystal structure of  
14 *Drosophila* Stimulator of Interferon Genes (STING) in complex with 3'2'-cGAMP explains  
15 selective isomer recognition and we demonstrate that 3'2'-cGAMP induces an enhanced  
16 antiviral state *in vivo* that protects from viral infection. Similar to radiation of Toll-like  
17 receptors in pathogen immunity, our results establish cGLRs as a diverse family of  
18 metazoan pattern recognition receptors.

19           To define the function of cGAS-like enzymes in animals, we screened predicted cGAS-  
20 homologs for suitability in structural analysis and determined a 2.4 Å crystal structure of the  
21 human protein MB21D2 (hMB21D2; C3orf59) and a 1.6 Å crystal structure of a protein from the  
22 beetle species *Tribolium castaneum* (Genbank XP\_969398.1) (Supplementary Table 1). Despite  
23 primary sequence divergence, the hMB21D2 and *T. castaneum* XP\_969398.1 structures each  
24 reveal close homology to human cGAS with a shared bi-lobed architecture, caged  
25 nucleotidyltransferase core, Gly-[Gly/Ser]-activation loop, and putative catalytic triad (Fig. 1a,  
26 Extended Data Fig. 1). In human cGAS, the primary ligand binding surface is a long groove on

27 the back of the enzyme formed by the  $\alpha$ -helix spine and a Zn-ribbon motif that is essential for  
28 recognition of double-stranded DNA<sup>3,13-17</sup>. A conserved groove is present in both the hMB21D2  
29 and *T. castaneum* XP\_969398.1 structures (Fig. 1a) but is notably distinguished by the absence  
30 of a Zn-ribbon and the insertion of a C-terminal  $\alpha$ -helix in hMB21D2 (Fig. 1b). We hypothesized  
31 that the remodeling of this groove controls the detection of distinct activating ligands. The  
32 hMB21D2 surface is overall neutral with no obvious capacity to coordinate nucleic acid and no  
33 enzyme activity was detected in the presence of potential activating ligands (Extended Data Fig.  
34 1d,e). In contrast to hMB21D2, the surface of *T. castaneum* XP\_969398.1 shares highly  
35 conserved basic residues with human cGAS (Fig. 1a) and we therefore tested this enzyme with  
36 candidate DNA and RNA ligands. Remarkably, we observed that *T. castaneum* XP\_969398.1 is  
37 activated to synthesize a nucleotide product in response to recognition of double-stranded RNA  
38 (dsRNA) (Fig. 1c). Despite exhibiting a clear difference in ligand-specificity, analysis of all related  
39 structures in the Protein Data Bank confirms that *T. castaneum* XP\_969398.1 is a close homolog  
40 of mammalian cGAS and is distinct from previously characterized RNA sensors including  
41 oligoadenylate synthase 1 (Extended Data Fig. 1f)<sup>18,19</sup>. Together, these results establish the  
42 existence of cGAS-like Receptors (cGLRs) in animals and demonstrate that remodeling of a  
43 primary ligand-binding surface enables the recognition of divergent molecular patterns.

44 To identify additional cGLRs that respond to dsRNA, we used the *T. castaneum* cGLR  
45 (*Tc*-cGLR) sequence to search for predicted cGAS homologs in species related to the model  
46 organism *Drosophila melanogaster*. We identified 153 cGLR genes across 42 species in the order  
47 *Diptera*, which cluster into distinct clades designated 1–5 (Fig. 2a; Supplementary Table 2).  
48 *Drosophila* exhibit a remarkable radiation of cGLR genes with individual species encoding  
49 between three and seven predicted enzymes (Extended Data Fig. 2a). In a systematic  
50 biochemical screen, we purified and tested 53 recombinant cGLR proteins and identified active  
51 enzymes from the species *Lucilia cuprina*, *Drosophila eugracilis*, *Drosophila erecta*, and  
52 *Drosophila simulans* (Extended Data Figs. 2b–f, 3a). Similar to *Tc*-cGLR, each active *Diptera*

53 enzyme specifically responds to dsRNA indicating that cGLR-based recognition of RNA is  
54 conserved across diverse insect species (Fig. 2b; Extended Data Fig. 4a).

55 The *D. simulans* enzyme identified in our screen shares 91% sequence identity with the  
56 protein product of the uncharacterized *D. melanogaster* gene *CG12970*. We purified recombinant  
57 *D. melanogaster* *CG12970* and found that it synthesizes a nucleotide product specifically in the  
58 presence of dsRNA and we therefore named this gene *cGAS-like Receptor 1 (Dm-cGLR1)* (Fig.  
59 2c). To understand how dsRNA activates *Drosophila* cGLR1, we analyzed the molecular  
60 determinants for enzymatic activity *in vitro*. We observed that *D. simulans* cGLR1 (*Ds-cGLR1*)  
61 and *Dm-cGLR1* recognize long >30 bp dsRNA with no preference for 5' RNA phosphorylation  
62 (Fig. 2d; Extended Data Fig. 4b,c). Notably, activation of *Dm-cGLR1* and *Ds-cGLR1* requires  
63 dsRNA ligands that exceed the length of 21–23 bp RNA molecules commonly produced during  
64 RNA interference (RNAi) in *Drosophila*, suggesting specific avoidance of self-recognition<sup>20-22</sup>. *Ds-*  
65 *cGLR1* selectively binds dsRNA and forms a higher-order complex that is dependent on dsRNA  
66 length, similar to condensate formation previously observed with hcGAS recognition of dsDNA  
67 (Fig. 2e; Extended Data Fig. 5)<sup>23-25</sup>. Using the *Tc-cGLR* and human cGAS–DNA structures as a  
68 template<sup>17</sup>, we modeled interactions between *Drosophila* cGLR1 and dsRNA (Fig. 2f). Single  
69 glutamate substitutions to the conserved basic residues R41, R259, and K269 within the predicted  
70 *Ds-cGLR1* ligand binding surface significantly diminish dsRNA-stimulated activity (Fig. 2g;  
71 Extended Data Fig. 3). We ectopically expressed *Dm-cGLR1* or *Ds-cGLR1* in human cells and  
72 observed that expression is sufficient to enable dsRNA sensing and drive activation of a STING-  
73 dependent immune response (Fig. 2h; Extended Data Fig. 4e). *Dm-cGLR1* and *Ds-cGLR1*  
74 signaling in cells is strictly dependent on dsRNA stimulation and mutations to the enzyme catalytic  
75 site or conserved ligand-binding surface disrupt signaling and prevent downstream STING  
76 activation (Fig. 2h; Extended Data Fig. 3f). Together, these data demonstrate that insect cGLRs  
77 and human cGAS use a shared mechanism of ligand detection and reveal that *Dm-cGLR1* can  
78 function as a foreign RNA sensor.

79 A role in sensing foreign RNA suggests that the function of *Dm*-cGLR1 is to control  
80 activation of a downstream immune response in *Drosophila*. In human cells, cGAS catalyzes  
81 production of the nucleotide second messenger 2'3'-cGAMP (cG[2'-5']pA[3'-5']p) that contains a  
82 non-canonical 2'-5' phosphodiester linkage required for potent activation of immune signaling<sup>2-5</sup>.  
83 To determine how *Dm*-cGLR1 controls cellular signaling, we purified the nucleotide reaction  
84 product for direct comparison to 2'3'-cGAMP. Surprisingly, *Dm*-cGLR1 synthesizes a nucleotide  
85 product that exhibits a C18 chromatography migration profile distinct from 2'3'-cGAMP and all  
86 previously known naturally occurring cyclic dinucleotide (CDN) signals (Fig. 3a; Extended Data  
87 Fig. 6). Production of this nucleotide signal is conserved in *Diptera* with *Ds*-cGLR1, *Lc*-cGLR, and  
88 *Deu*-cGLR reactions each synthesizing the same major reaction product (Extended Data Fig. 6a).  
89 Using nucleobase-specific labeling and nuclease digestion of the *Dm*-cGLR1 product we  
90 observed a 3'-5'-linkage connected to an adenosine phosphate and a protected 2'-5'-linkage  
91 connected to a guanosine phosphate suggesting the existence of a mixed-linkage cyclic GMP-  
92 AMP species (Fig. 3b). We verified these findings with comparative high performance liquid  
93 chromatography and tandem mass-spectrometry profiling against a chemically synthesized  
94 standard and confirmed that the shared *Diptera* cGLR product is the novel isomer 3'2'-cGAMP  
95 (cG[3'-5']pA[2'-5']p) (Figs. 3a,b; Extended Data Figs. 6a,b).

96 *Dm*-cGLR1 synthesizes 3'2'-cGAMP in a two-step reaction through production of the linear  
97 intermediate pppA[2'-5']pG and uses an opposite nucleobase reaction order compared to human  
98 cGAS (Extended Data Fig. 7a)<sup>2,3,26</sup>. We next used mass-spectrometry to further analyze each  
99 lysate sample from our screen of recombinant dipteran cGLR proteins. 3'2'-cGAMP was detected  
100 as a main reaction product from 15 *Diptera* cGLRs including active enzymes from each sub-group  
101 within Clade 5 of the insect cGLR phylogeny (Fig. 3c, Extended Data Fig. 6c). cGLRs clustered  
102 within Clade 5 collectively represent 41 species suggesting widespread conservation of 3'2'-  
103 cGAMP-signaling in *Diptera*. Interestingly, the beetle enzyme *Tc*-cGLR synthesizes 2'3'-cGAMP,  
104 supporting that 2'3'-cGAMP is an ancestral signaling molecule in metazoans and that 3'2'-

105 cGAMP-signaling is a recent adaptation in flies (Fig. 3d; Extended Data Fig. 6a)<sup>10,27,28</sup>. Insect and  
106 mammalian viruses encode 2'3'-cGAMP-specific nucleases named poxins that allow evasion of  
107 cGAS-STING immune responses<sup>29,30</sup>. Remarkably, 3'2'-cGAMP is protected from poxin cleavage  
108 (Extended Data Fig. 7b–d), indicating that an isomeric switch in phosphodiester linkage specificity  
109 endows *Drosophila* with a signaling pathway resistant to a major form of viral immune evasion.

110 *Drosophila* STING (dSTING) is known to function as a cyclic dinucleotide receptor *in*  
111 *vivo*<sup>31-34</sup>, but an endogenous nucleotide second messenger has not previously been identified.  
112 We therefore developed an *in vitro* thermo-fluor binding assay to analyze the ability of dSTING to  
113 recognize specific CDNs. dSTING preferentially forms a thermo-stable complex with 3'2'-cGAMP  
114 and exhibits no detectable complex formation with 2'3'-cGAMP or other CDNs *in vitro* (Fig. 4a;  
115 Extended Data Fig. 8b,c). Using direct delivery of CDNs to permeabilized cells, we confirmed that  
116 dSTING preferentially responds to 3'2'-cGAMP in the cellular environment (Extended Data Fig.  
117 8d). To define the mechanism of selective 3'2'-cGAMP recognition, we next determined a 2.0 Å  
118 crystal structure of the *D. eugracilis* STING CDN-binding domain in complex with 3'2'-cGAMP  
119 (Fig. 4b). dSTING adopts a highly conserved V-shaped homodimeric architecture with a deep  
120 central pocket that binds 3'2'-cGAMP. The dSTING–3'2'-cGAMP structure reveals a tightly  
121 “closed” conformation with dSTING protomers positioned 36 Å apart, similar to the closed  
122 conformation of human STING bound to 2'3'-cGAMP (Extended Data Fig. 8e)<sup>5,35</sup>. Each  
123 nucleobase of 3'2'-cGAMP is stacked between dSTING Y164 and R234, and E257 specifically  
124 coordinates the 3'2'-cGAMP guanosine N2 position (Fig. 4c,d). In human STING, high-affinity  
125 recognition of 2'3'-cGAMP requires readout of the 2'–5' phosphodiester linkage by R232 in the β-  
126 strand lid<sup>5,35</sup>. In dSTING, the equivalent R229 makes no contact with either phosphodiester bond.  
127 Instead, R229 is repositioned to extend outward from the ligand binding pocket by the deletion of  
128 a single lid residue and the formation of a salt bridge with E267 on the opposing protomer,  
129 explaining the diminished affinity of dSTING for 2'3'-cGAMP (Fig. 4c,e). Additionally, a key  
130 asparagine substitution N159 in dSTING extends across the binding pocket to coordinate the

131 adenosine 3' OH in 3'2'-cGAMP and directly replaces the human STING S162 residue that  
132 contacts the guanosine 3' OH in 2'3'-cGAMP (Fig. 4f). We tested a panel of dSTING mutant  
133 proteins and confirmed that mutations to each coordinating residue disrupt dSTING–3'2'-cGAMP  
134 complex formation (Extended Data Fig. 8i). The unique ligand-binding pocket adaptations  
135 observed in the dSTING–3'2'-cGAMP structure are widely conserved in *Diptera* and together  
136 explain a mechanism for how specific 3'2'-cGAMP-dependent signaling drives STING activation.

137 To determine how *Dm*-cGLR1–3'2'-cGAMP–STING signaling controls immune responses  
138 *in vivo*, we next injected 3'2'-cGAMP into *D. melanogaster* to directly monitor the dSTING  
139 response. 3'2'-cGAMP potently induces the expression of *dSTING* and three other STING-  
140 regulated genes in a dose-dependent manner (Fig 5a; Extended Data Fig. 9). Notably, 3'2'-  
141 cGAMP-dependent signaling through dSTING is significantly more potent than the response  
142 triggered by injection of the bacterial CDN signal 3'3'-c-di-GMP (Fig 5a; Extended Data Fig. 9e–  
143 k). Genetic mutations to *dSTING* and the NF-κB homolog *Relish* ablate 3'2'-cGAMP-induced  
144 responses, demonstrating that signaling is dependent upon each downstream pathway  
145 component (Fig. 5a, Extended Data Fig. 9e–k). We challenged flies with viral infection and  
146 observed that 3'2'-cGAMP markedly suppresses the replication of two unrelated RNA viruses,  
147 *Drosophila C virus (Dicistroviridae)*, a natural *Drosophila* pathogen, and vesicular stomatitis virus  
148 (*Rhabdoviridae*) (Figs. 5b,c; Extended Data Fig. 10a,b). 3'2'-cGAMP activation of antiviral  
149 immunity is strictly dependent on dSTING and results in a response that significantly delays  
150 pathogen-mediated mortality (Figs. 5b,c; Extended Data Fig. 10a,b). Direct comparison of the  
151 protective effects against DCV infection demonstrates that the endogenous signal 3'2'-cGAMP  
152 exhibits greater antiviral potency than 2'3'-cGAMP. 3'2'-cGAMP more robustly suppresses RNA  
153 viral loads and extends animal survival (Fig. 5d; Extended data Fig. 10c,d), revealing that the  
154 dSTING antiviral signaling axis is preferentially activated by 3'2'-cGAMP *in vivo*. Together, these  
155 results demonstrate that 3'2'-cGAMP is an antiviral nucleotide second messenger in *D.*



156 *melanogaster* and establish a novel cGLR–STING–NF- $\kappa$ B axis that protects animals from viral  
157 replication.

158         Along with cGAS recognition of dsDNA, the discovery of animal cGLR dsRNA sensors  
159 establishes a diverse class of pattern recognition receptors conserved throughout metazoans.  
160 Divergent structural homologs of cGAS in humans and insects demonstrate that cGLRs constitute  
161 a rapidly evolving family of proteins in which remodeling of a shared primary binding surface  
162 enables the detection of diverse ligands. Our characterization of the mechanism of *Drosophila*  
163 cGLR1 activation shows that cGLRs function as direct sensors of pathogen-associated molecular  
164 patterns and synthesize distinct second messengers to control a conserved downstream signaling  
165 axis. *Drosophila* were previously thought to respond to foreign nucleic acid exclusively through  
166 RNAi and direct cleavage of pathogen RNA<sup>21,22</sup>. *Drosophila* cGLR1 reveals a parallel signaling  
167 system for sensing foreign RNA and directing an inducible immune response through STING.  
168 Synthesis of the novel second messenger 3'2'-cGAMP by *Drosophila* cGLR1 and selective  
169 recognition by dSTING provides the first evidence that metazoans use CDNs beyond 2'3'-cGAMP  
170 as endogenous second messengers and highlights the evolutionary plasticity of cGLR signaling.  
171 Our structural analysis also reveals that the human cGLR MB21D2 is competent for nucleotide  
172 second messenger synthesis and has a remodeled ligand-binding groove likely adapted for  
173 detection of an unknown stimulus. Together with the known high frequency of hMB21D2 mutation  
174 in cancer<sup>37,38</sup>, these results support a more extensive role for cGLR signaling in human biology.  
175 The existence of multiple unique cGLRs encoded within a single species (Extended Data Fig. 2a)  
176 suggests a model in which the cGLR signaling scaffold is harnessed to detect a number of distinct  
177 stimuli (Fig. 5e). In support of this conclusion, Hartmann, Imler, Cai and colleagues identify cGLR2  
178 as a second functional cGLR in *Drosophila* and demonstrate *in vivo* that cGLR1 and cGLR2 have  
179 distinct functions in *Drosophila* immunity<sup>36</sup>. Together, our results define cGLRs as receptors in  
180 animal cells capable of detecting diverse pathogen-associated molecular patterns and dictating  
181 response to the foreign environment.

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304 structural experiments were performed by K.M.S., hMB21D2 structural experiments were  
305 performed by B.R.M, and dSTING structural experiments were performed by K.M.S and A.E.R.  
306 hMB21D2, cGLR and dSTING biochemical experiments were performed by K.M.S. and A.E.R.  
307 Phase separation and RNA-binding analysis were performed by W.Z and A.E.R. Cell biology  
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316 **Figure Legends**

317 **Figure 1 | Structural remodeling in animal cGLRs enables divergent pattern recognition.**

318 **a**, Crystal structures and surface electrostatic views of human MB21D2 and a cGLR from the  
319 beetle *T. castaneum* (*Tc*-cGLR). Structural comparison with the human cGAS–DNA complex  
320 (PDB 6CTA) reveals that cGLRs adopt a conserved overall architecture with a  
321 nucleotidyltransferase signaling core and shared primary ligand-binding surface (indicated by  
322 dashed lines). **b**, Zoom-in cutaways highlighting structural insertions and deletions unique to each  
323 cGLR. hMB21D2 and *Tc*-cGLR lack the Zn-ribbon motif present in cGAS (left) and hMB21D2  
324 contains a C-terminal  $\alpha$ -helix extension that contacts the central “spine” helix (right). Specific  
325 alterations in the predicted ligand-binding surfaces suggest individual cGLRs are remodeled for  
326 recognition of different molecular patterns. **c**, Thin-layer chromatography (TLC) analysis and  
327 quantification of *Tc*-cGLR enzymatic reactions in the presence of candidate 40 nt or bp nucleic  
328 acid ligands. In contrast to mouse cGAS recognition of dsDNA, *Tc*-cGLR synthesizes a nucleotide  
329 product specifically in response to recognition of dsRNA confirming altered ligand specificity. Data  
330 are relative to maximum enzymatic activity and represent the mean  $\pm$  SEM for  $n = 3$  independent  
331 experiments.

332

333 **Figure 2 | *Drosophila* cGLR1 senses long double-stranded RNA.**

334 **a**, Summary of a forward biochemical screen of 53 cGLR proteins in the order *Diptera*. cGLR  
335 genes were selected based on predicted structural homology to *Tc*-cGLR and human cGAS, and  
336 conservation of putative nucleotidyltransferase catalytic residues. Screened enzymes are  
337 denoted with a red dot, and active dsRNA-sensors are denoted with a blue circle. The phylogeny  
338 represents 153 proteins clustered into Clades 1–5, with <30% sequence identity between clades.  
339 41/42 *Diptera* species are represented in Clade 5, which contains *D. melanogaster* CG12970  
340 (cGLR1) and CG30424. **b**, Functional *Diptera* cGLRs identified in the biochemical screen are  
341 activated to form a nucleotide product by the dsRNA mimic I:C. **c**, A single mutation to the

342 *Dm*-cGRL1 active site disrupts all enzymatic activity. **d**, *Dm*-cGRL1 *in vitro* activity was monitored  
343 in the presence of a panel of synthetic dsRNAs from 10–40 bp and quantified relative to 40 bp  
344 reactions. **e**, Electrophoretic mobility shift assay (EMSA) shows binding between *Ds*-cGRL1 and  
345 a 40 bp dsRNA. Formation of a higher order protein–nucleic acid complex which does not migrate  
346 through the gel is also observed with human cGAS and a 45 bp dsDNA ligand, in contrast to 2:2  
347 binding between mouse cGAS and dsDNA. **f**, Model of the *Tc*-cGRL–dsRNA complex based on  
348 the hcGAS–dsDNA structure (PDB 6CTA). Predicted *Dm*-cGRL1 (*Ds*-cGRL1) ligand-binding  
349 residues R23 (41), K42 (60), K52 (70), R 241 (259) and K251 (269) are analogous to *Tc*-cGRL  
350 residues R22, K40, R51, R249, and K259 respectively. **g**, Analysis and quantification of *in vitro*  
351 *Ds*-cGRL1 activity demonstrates that charge-swap mutations to ligand-binding residues  
352 significantly impair enzyme activation (see also **Extended Data Fig. 3**) **h**, Analysis of *Dm*-cGRL1  
353 activity in human cells using a mammalian STING-controlled IFN- $\beta$  luciferase reporter. IFN- $\beta$  is  
354 quantified relative to vector control and shown here relative to wild-type activity. *Dm*-cGRL1  
355 signaling to STING is strictly dependent on poly I:C stimulation and mutation of the catalytic site  
356 or predicted ligand-binding residues ablates this activity. Data in **h** are mean  $\pm$  SEM of  $n = 3$   
357 technical replicates and representative of  $n = 3$  independent experiments; all other data are the  
358 mean  $\pm$  SEM of  $n = 3$  independent experiments.

359

### 360 **Figure 3 | Discovery of 3'2'-cGAMP as a metazoan nucleotide second messenger.**

361 **a**, HPLC chromatogram of the *Dm*-cGRL1 reaction (orange) and comparison with synthetic  
362 nucleotide standards. *Dm*-cGRL1 synthesizes 3'2'-cGAMP as a major product that migrates  
363 identical to a synthetic standard (black). The retention time of other standards are indicated with  
364 a dotted line, demonstrating that a minor *Dm*-cGRL1 reaction product is 2'3'-c-di-AMP (see also  
365 **Extended Data Fig. 6a**). **b**, TLC analysis of mouse cGAS and *Dm*-cGRL1 reactions labeled with  
366 either  $\alpha$ -<sup>32</sup>P-ATP or  $\alpha$ -<sup>32</sup>P-GTP and treated as indicated. Schematic on right demonstrates how  
367 pairwise labeling and Nuclease P1 digestion verifies that cGAS and *Dm*-cGRL1 synthesize

368 distinct cGAMP isomers with opposite phosphodiester linkage specificities. High-resolution mass  
369 spectrometry confirms the major *Diptera* cGLR product as 3'2'-cGAMP (see also **Extended Data**  
370 **Fig. 6b**). **c**, Inset of Clade 5 in the *Diptera* cGLR phylogeny from **Fig. 2a** annotated to denote all  
371 enzymes identified to synthesize 3'2'-cGAMP by bacterial lysate analysis. **d**, HPLC analysis and  
372 quantification of product formation by insect cGLRs. 3'2'-cGAMP is the dominant product of each  
373 identified *Diptera* cGLR, and 2'3'-cGAMP is the dominant product of cGAS and *Tc*-cGLR. Data  
374 are mean  $\pm$  SEM for n = 3 independent experiments.

375

#### 376 **Figure 4 | Structural basis for 3'2'-cGAMP recognition by *Drosophila* STING**

377 **a**, Thermal denaturation assay showing selective recognition of 3'2'-cGAMP *Drosophila* STING  
378 (see also **Extended Data Fig. 8b,c**). Data shown are representative of n = 3 independent  
379 experiments. **b**, Crystal structure of the dSTING–3'2'-cGAMP complex reveals a tightly closed  
380 homodimer conformation and an ordered  $\beta$ -strand lid, indicating high-affinity engagement with the  
381 endogenous *Drosophila* second messenger 3'2'-cGAMP. **c**, Phylogenetic alignment of the stem  
382 helix and  $\beta$ -strand lid in human and insect STING proteins colored by amino-acid conservation.  
383 Critical ligand binding residues are denoted with a navy circle and adaptations specific to *Diptera*  
384 are highlighted in red outline. **d**, Highlight of 3'2'-cGAMP in the CDN-binding pocket of dSTING  
385 showing key ligand contacts. **e**, Superposition of the dSTING–3'2'-cGAMP (blue-orange) and  
386 human STING–2'3'-cGAMP (gray-pink) complexes reveals human STING readout of the 2'–5'  
387 phosphodiester bond by the conserved R229 is absent in dSTING. dSTING R229 is stabilized in  
388 a conformation extended outward from the ligand-binding pocket interaction with E267 on the  
389 opposite dimer. **f**, Human STING S162 (gray) contacts the free 3' OH of the guanosine base in  
390 2'3'-cGAMP (pink). dSTING N159 (blue) extends across the ligand binding pocket to contact the  
391 the free 3' OH of the adenosine base in 3'2'-cGAMP (orange).

392

#### 393 **Figure 5 | 3'2'-cGAMP activates STING-dependent antiviral immunity in *Drosophila*.**



394 **a**, Synthetic 3'2'-cGAMP or 3'3'-c-di-GMP was injected into the body cavity of wildtype or mutant  
395 flies and gene expression measured after 24 h. *STING-regulated gene 1 (Srg1)* RNA levels shown  
396 as fold induction compared to buffer control in wildtype. dSTING<sub>Mut</sub> = RXN mutant, as previously  
397 characterized<sup>31,34</sup>. Data represent RNA levels measured relative to the house-keeping gene  
398 *RpL32* and are from 3 independent experiments \*P ≤ 0.05; \*\*P ≤ 0.01; \*\*\*P ≤ 0.001; and n.s., P  
399 > 0.05. P value n.s. unless otherwise noted. **b**, Viral RNA loads 3 days after infection with the  
400 RNA pathogen Drosophila C virus (DCV) demonstrate significantly diminished viral replication in  
401 wildtype flies injected with 3'2'-cGAMP. **c**, Survival analysis of animals injected with 3'2'-cGAMP  
402 or buffer control and infected with DCV. 3'2'-cGAMP injection results in a dSTING-dependent  
403 response that significantly delays mortality. **d**, Survival analysis directly comparing the effects of  
404 cGAMP isomers 7 days after DCV infection. cGAMP injection increases animal survival in a dose-  
405 dependent manner compared to buffer control and injection of 3'2'-cGAMP confers greater  
406 protection than 2'3'-cGAMP at each dose tested (see also **Extended Data Fig. 10 c,d**). **e**,  
407 Proposed model for the cGLR-STING signaling. Diverse animal cGLRs recognize distinct  
408 molecular patterns, respond by synthesizing a nucleotide second messenger, and induce  
409 activation of STING-dependent antiviral immunity.

## 410 **Methods**

### 411 **Bioinformatics and Dipteran cGLR sequence analysis**

412 Building on previous analyses<sup>6-12</sup>, animal cGAS homologs suitable for crystallography were  
413 identified using the amino acid sequences of human cGAS and *D. melanogaster* CG7194 to seed  
414 a position-specific iterative BLAST (PSI-BLAST) search of the NCBI non-redundant protein  
415 database. The PSI-BLAST search was performed with an E value cutoff 0.005 for inclusion into  
416 the next search round, BLOSUM62 scoring matrix, gap costs settings existence 11 and extension  
417 1, and using conditional compositional score matrix adjustment. Candidate homologs identified  
418 from this search included the uncharacterized human protein MB21D2 and the *Tribolium*  
419 *castaneum* sequence XP\_969398.1. Pairwise structural comparison between human MB21D2,  
420 *Tc*-cGLR, and protein structures in the Protein Data Bank was performed using DALI<sup>37</sup> and Z-  
421 scores for homologs less than 90% identical to one another (PDB90) were plotted in GraphPad  
422 Prism. A Z-score of 15 for *Tc*-cGLR and 13 for human MB21D2 was selected as a lower cut-off  
423 to emphasize direct relevant homologs in analysis.

424         Following structure determination of human MB21D2 and *T. castaneum* XP\_969398.1,  
425 predicted cGLRs were further identified in *Diptera* using PSI-BLAST searches seeded with either  
426 *D. melanogaster* CG7194 or the *Tc*-cGLR sequence selecting in each round for proteins matching  
427 known cGLR domain organization and active-site residues. *Diptera* cGLR sequences were  
428 aligned using MAFFT (FFT-NS-i iterative refinement method)<sup>38</sup> and used to construct a  
429 phylogenetic tree in Geneious Prime v2020.12.23 using the neighbor-joining method and Jukes-  
430 Cantor genetic distance model with no outgroup. Further manual analysis and curation of  
431 candidate cGLR sequences was performed based on alignments and predictive structural  
432 homology using HHPred<sup>39</sup> and Phyre2<sup>40</sup>. Sequences were selected for predicted structural  
433 homology to cGAS, including the presence of a conserved nucleotidyltransferase domain with a  
434 G[S/G] activation loop and [E/D]h[E/D] X<sub>50-90</sub> [E/D] catalytic triad. Manual refinement was also  
435 used to exclude duplicate sequences, gene isoforms, and proteins less than 250 or greater than

436 700 residues. NCBI available genomes from 42 species in *Diptera* are represented in the final  
437 tree, including 31 species in the genus *Drosophila*. Clustering of sequences in the final unrooted  
438 tree was used to define clades, with no more than 30% sequence identity shared between  
439 members of different clades. Further manual analysis of the tree was used to determine the  
440 number and distribution of predicted cGLRs by species (see Extended Data Fig. 2a).  
441 PROMALS3D<sup>41</sup> used for structure guided alignment of apo human cGAS (PDB 4KM5)<sup>14</sup>,  
442 hMB21D2, and *Tc*-cGLR in Extended Data Fig. 1a. MAFFT (FFT-NS-i iterative refinement  
443 method)<sup>38</sup> was used to align STING sequences in Extended Data Fig. 8a. Geneious Prime  
444 software was used to generate the sequence alignments in Figure 4 and Extended Data Figs. 1a,  
445 3a and 8a.

446

#### 447 **Protein expression and purification**

448 Recombinant cGLR and dSTING proteins were expressed and purified used methods previously  
449 optimized for human cGAS<sup>17</sup>. Animal cGLR and dSTING sequences were codon-optimized for  
450 expression in *E. coli* and cloned from synthetic constructs (GeneArt or Integrated DNA  
451 Technologies) into a custom pET16 expression vector with an N-terminal 6×His-MBP fusion tag  
452 or an N-terminal 6×His-SUMO2 fusion. The full-length coding sequence was used except for  
453 human cGAS 157–522, mouse cGAS 147–607, human MB21D2 S29–F491, *Ds*-cGLR1 19–393,  
454 and *D. eugracilis* STING 150–340 as specified. The N-terminus of *D. eugracilis* STING 150–340  
455 was fused to the full-length coding sequence of T4 lysozyme connected by a Gly-Ser linker  
456 sequence. Briefly, transformed BL21-CodonPlus(DE3)-RIL *E. coli* (Agilent) were grown in MDG  
457 media overnight prior to inoculation of M9ZB media at an OD<sub>600</sub> of 0.0475. M9ZB cultures were  
458 grown to OD<sub>600</sub> of 2.5 (approximately 5 h at 37°C with shaking at 230 rpm) followed by cooling on  
459 ice for 20 min. Cultures were induced with 500 μM IPTG prior to incubation at 16°C overnight with  
460 shaking at 230 rpm. Cultures were pelleted the following day and either flash frozen in liquid  
461 nitrogen for storage at –80°C or directly lysed for purification. Selenomethionine-substituted

462 proteins for crystallography experiments were purified using a modified growth protocol as  
463 previously described<sup>30</sup>.

464 For large-scale protein purification, proteins were expressed with a 6×His-SUMO2 (*Tc*-  
465 cGLR, *Ds*-cGLR1, *Deu*-cGLR, *Lc*-cGLR, dSTINGs) or 6×His-MBP (*Dm*-cGLR1, *Der*-cGLR1)  
466 fusion tag and grown as ~4–8× 1 L cultures in M9ZB media. Pellets were lysed by sonication in  
467 lysis buffer (20 mM HEPES pH 7.5, 400 mM NaCl, 30 mM imidazole, 10% glycerol and 1 mM  
468 DTT) and clarified by centrifugation at ~47,850 × g for 30 min at 4°C and subsequent filtration  
469 through glass wool. Recombinant protein was purified by gravity-flow over NiNTA resin (Qiagen).  
470 Resin was washed with lysis buffer supplemented to 1 M NaCl and then eluted with 20 mL of lysis  
471 buffer supplemented to 300 mM imidazole. SUMO2-fusion proteins were cleaved by  
472 supplementing elution fractions with ~250 µg of human SENP2 protease (D364–L589 with M497A  
473 mutation) during overnight dialysis at 4°C against dialysis buffer (20 mM HEPES pH 7.5, 250 mM  
474 KCl, 1 mM DTT). MBP-tagged fusion proteins were buffer exchanged into lysis buffer with 4%  
475 glycerol and no imidazole to optimize conditions for overnight cleavage by recombinant TEV  
476 protease at ~10°C. cGLR proteins were next purified by ion exchange chromatography using  
477 HiTrap Heparin HP columns (GE Healthcare) and eluted across a 150–1000 mM NaCl gradient.  
478 Target protein fractions were pooled and further purified by size-exclusion chromatography using  
479 a 16/600 Superdex 75 column or 16/600 Superdex 200 column and storage buffer (20 mM  
480 HEPES-pH 7.5, 250 mM KCl, 1 mM TCEP). Final proteins were concentrated to ~20–30 mg mL<sup>-1</sup>  
481 and flash-frozen with liquid nitrogen and stored at -80°C for crystallography or supplemented with  
482 10% glycerol prior to freezing for biochemistry experiments. *Tc*-cGLR and *Ds*-cGLR1 mutant  
483 proteins were purified from 1 L M9ZB cultures using NiNTA affinity chromatography and overnight  
484 dialysis directly into storage buffer (20 mM HEPES-pH 7.5, 250 mM KCl, 10% glycerol, 1 mM  
485 TCEP) without SUMO2 tag cleavage.

486 For small-scale protein purification used in the *Diptera* cGLR screen, recombinant proteins  
487 were expressed with a 6×His-MBP fusion tag with the exception of human cGAS, mouse cGAS,

488 *Tc*-cGLR, *Deu*-cGLR, *Lc*-cGLR, and *Ds*-cGLR1 which were expressed with a 6×His-SUMO2  
489 fusion tag. Small-scale cultures were grown in 20 mL of M9ZB media, lysed with sonication, and  
490 recombinant protein was purified as previously described<sup>11</sup>. Briefly, protein was purified directly  
491 from lysates by centrifuge flow-through over NiNTA resin (Qiagen) in 2 mL Mini Spin columns  
492 (Epoch Life Sciences). Following elution with elution buffer (20 mM HEPES pH 7.5, 400 mM NaCl,  
493 300 mM imidazole, 10% glycerol and 1 mM DTT) proteins were buffer exchanged into storage  
494 buffer (20 mM HEPES-pH 7.5, 250 mM KCl, 10% glycerol, 1 mM TCEP). Fresh protein  
495 preparations were immediately used for *in vitro* nucleotide synthesis reactions.

496

#### 497 **Protein crystallization and structure determination**

498 Crystals of native and selenomethionine-substituted human MB21D2 S29–F491, *Tc*-cGLR, and  
499 T4 lysozyme-dSTING L150–I340, were grown at 18°C using hanging-drop vapor diffusion.  
500 Optimized crystals were grown in EasyXtal 15-well trays (NeXtal Biotechnologies) with 350 µL of  
501 reservoir solution and 2 µL drops set with a ratio of 1 µL protein solution and 1 µL of reservoir  
502 solution. Human MB21D2 crystals were grown using a reservoir solution (1.2 M ammonium  
503 sulfate, 5 mM MgCl<sub>2</sub>, 100 mM MES pH 6.2) previously identified by Pei Wang and Raven Huang  
504 (University of Illinois at Urbana-Champaign)<sup>42</sup> for 1 day prior to cryoprotection with reservoir  
505 solution supplemented with 30% glycerol and freezing in liquid nitrogen. *Tc*-cGLR crystals were  
506 grown using the reservoir solution (0.3 M potassium thiocyanate, 10–16% PEG-3350) for 5–16  
507 days prior to cryoprotection with reservoir solution supplemented with 15% ethylene glycol and  
508 freezing in liquid nitrogen. Apo T4 lysozyme-dSTING crystals were grown using the reservoir  
509 solution (0.2 M sodium citrate, 0.1 M Tris-HCl, 22% PEG-3350) 7 days prior to cryoprotection with  
510 reservoir solution supplemented with 15% ethylene and freezing in liquid nitrogen. T4 lysozyme-  
511 dSTING–3'2'-cGAMP crystals were grown using the reservoir solution (0.1–0.2 M sodium acetate  
512 pH 4.8, 0.2 M ammonium formate, 20–22% PEG-3350) supplemented with 250 µM 3'2'-cGAMP  
513 (Biolog) for 10 days prior to cryoprotection with reservoir solution supplemented to 35% PEG-

514 3350 and freezing in liquid nitrogen. Growth of single MB21D2 and *Tc*-cGLR crystals was further  
515 optimized with streak seeding. X-ray diffraction data were collected at the Advanced Photon  
516 Source beamlines 24-ID-C and 24-ID-E and at the Advanced Light Source beamlines 5.0.1 and  
517 8.2.2. Data were processed with XDS and Aimless<sup>43</sup> using the SSRL autoxds script (A. Gonzales).  
518 Experimental phase information for all proteins was determined using data collected from  
519 selenomethionine-substituted crystals. Anomalous sites were identified, and an initial map  
520 generated with AutoSol within PHENIX<sup>44</sup>. Structural modeling was completed in Coot<sup>45</sup> and  
521 refined with PHENIX. Refinement statistics are described in Supplementary Table 1, and all  
522 structure figures were generated with PyMOL 2.3.0.

523

#### 524 **Nucleotide product synthesis analysis**

525 cGLR nucleotide synthesis activity was analyzed by thin-layer chromatography as previously  
526 described<sup>11</sup>. For the *Diptera* cGLR screen, recombinant protein preparations were incubated in  
527 10  $\mu$ L reactions containing 0.5  $\mu$ L  $\alpha$ -<sup>32</sup>P labeled NTPs (~0.4  $\mu$ Ci each of ATP, CTP, GTP, and  
528 UTP), 200  $\mu$ M unlabeled NTPs, 10 mM MgCl<sub>2</sub>, and 1 mM MnCl<sub>2</sub> in a final reaction buffer of 50  
529 mM Tris-HCl pH 7.5, ~50 mM KCl, 1 mM TCEP. Reactions were additionally supplemented with  
530 ~1  $\mu$ g poly I:C or 5  $\mu$ M ISD45 dsDNA as indicated. Reactions were incubated at 37°C overnight  
531 and subsequently treated with 1  $\mu$ L Quick CIP phosphatase (New England Biolabs) for 20 min at  
532 37°C to remove unreacted phosphate signal. Each reaction was diluted 1:10 in 100 mM sodium  
533 acetate pH 5.2 and 0.5  $\mu$ L was spotted on a 20-cm  $\times$  20-cm PEI-cellulose thin-layer  
534 chromatography plate. Plates were run with 1.5 M KH<sub>2</sub>PO<sub>4</sub> solvent until ~2.5 cm from top of the  
535 plate, dried at room-temperature, and exposed to a phosphor-screen prior to signal detection with  
536 a Typhoon Trio Variable Mode Imager System (GE Healthcare). For all other nucleotide synthesis  
537 reactions visualized by thin-layer chromatography, enzymes were tested at 5  $\mu$ M with 5  $\mu$ M  
538 nucleic acid ligands and either 1 mM MnCl<sub>2</sub> or 10 mM MgCl<sub>2</sub> for insect cGLRs or cGAS  
539 respectively. hMB21D2 activity was tested with 1 mM MnCl<sub>2</sub> and 10 mM MgCl<sub>2</sub> using the following

540 synthetic innate immune agonists: lipopeptide Pam3CSK4 (Invivogen), *S. aureus* lipoteichoic acid  
541 (LTA-SA) (Invivogen), *S. cerevisiae* cell wall preparation (Zymosan) (Invivogen), *B. subtilis*  
542 peptidoglycan (PGN-BS) (Invivogen), synthetic lipid A mimic (CRX-527) (Invivogen), *B. subtilis*  
543 flagellin (FLA-BS) (Invivogen), imidazoquinoline (Imiquimod) (Invivogen), CpG oligonucleotide  
544 (ODN 2006) (Invivogen), *S. aureus* 23S rRNA oligonucleotide (ORN Sa19) (Invivogen). Besides  
545 *Diptera* screen reactions, samples were not diluted in sodium acetate prior to spotting on PEI-  
546 cellulose TLC plates. TLC images were adjusted for contrast using FIJI<sup>46</sup> and quantified using  
547 ImageQuant (8.2.0). Nucleotide product formation was measured according to the ratio of product  
548 to total signal for each reaction. For Figs. 1c, 2d, 2g and Extended Data Figs. 3e, 4b, 5d relative  
549 activity was calculated as percent conversion for each reaction relative to maximal conversion  
550 observed by wildtype enzyme or in the presence of 40 bp dsRNA for insect cGLRs and 45 bp  
551 dsRNA for cGAS.

552

### 553 **Electrophoretic mobility shift assay (EMSA)**

554 Analysis of *in vitro* protein–nucleic acid complex formation was conducted as previously  
555 described<sup>17</sup>. Briefly, 1  $\mu$ M 40-bp dsRNA or 45-bp dsDNA was incubated with *Ds*-cGLR1 or hcGAS  
556 NTase-domain (D157–522) respectively at a gradient of protein concentrations as indicated in  
557 each figure. Complex formation was performed with the final reaction buffer 20 mM HEPES-NaOH  
558 pH 7.8, 75 mM KCl, 1 mM DTT. 20  $\mu$ L reactions were incubated at 4°C for 20 min before  
559 separation on a 2% agarose gel using 0.5  $\times$  TB buffer (45 mM Tris, 45 mM boric acid) as a running  
560 buffer. The agarose gel was post-stained in 0.5  $\times$  TB buffer supplemented with 10  $\mu$ g mL<sup>-1</sup>  
561 ethidium bromide with gentle shaking at 25°C for 45 min. Complex formation was visualized using  
562 a ChemiDoc MP Imaging System (Bio-Rad).

563

### 564 ***In vitro* phase separation assays**

565 *In vitro* phase separation was performed as previously described with minor modifications<sup>23,25</sup>.  
566 Briefly, *Ds*-cGLR1 was labeled with AlexaFluor-488 (AF488) carboxylic acid (succinimidyl ester)  
567 (Thermo Fisher Scientific) according to manufacturer's manuals using a molar ratio of 1:10 at 4°C  
568 for 4 h. Excess free dye was removed by dialysis against buffer (20 mM HEPES-KOH pH 7.5,  
569 250 mM KCl, 1 mM DTT) at 4°C overnight and AF488-labeled *Ds*-cGLR1 was then further purified  
570 on a PD-10 desalting column (GE Healthcare) eluted with storage buffer (20 mM HEPES-KOH  
571 pH 7.5, 250 mM KCl, 1 mM TCEP). Final AF488-labeled *Ds*-cGLR1 was concentrated to ~5 mg  
572 mL<sup>-1</sup>, flash-frozen in liquid nitrogen, and stored as aliquots at -80°C. hcGAS and hcGAS NTase-  
573 domain (D157–F522) proteins were prepared as previously described<sup>25</sup>.

574 To induce phase separation, *Ds*-cGLR1 (10 μM, containing 1 μM AF488-labeled *Ds*-  
575 cGLR1) was mixed with various lengths of RNA (10 μM each) in buffer (20 mM Tris-HCl pH 7.5,  
576 1 mg mL<sup>-1</sup> BSA, 1 mM TCEP) in the presence of various salt concentrations at 25°C in a total  
577 reaction volume of 20 μL. The details of proteins, nucleic acids, and salt concentrations are  
578 indicated in figures. *Ds*-cGLR1–RNA reactions were placed in 384-well non-binding microplates  
579 (Greiner Bio-One) and incubated at 25°C for 30 min prior to imaging to allow condensates to  
580 settle. Fluorescence microscopy images were acquired at 25°C using a Leica TCS SP5 X (Leica  
581 Microsystems) mounted on an inverted microscope (DMI6000; Leica Microsystems) with an oil  
582 immersion 63×/numerical aperture 1.4 objective lens (HCX PL APO; Leica Microsystems).  
583 AF488-labeled *Ds*-cGLR1, hcGAS and hcGAS NTase-domain proteins were detected with  
584 excitation at 488 nm (emission at 500–530 nm). Microscopy images were processed with FIJI<sup>46</sup>,  
585 and contrast adjusted with a uniform threshold setup for each enzyme.

586

### 587 **Cellular STING signaling assays**

588 Human HEK293T cells were maintained in complete media (DMEM supplemented with penicillin,  
589 streptomycin, and 10% fetal bovine serum) at 37°C. For all assays 4.5 × 10<sup>4</sup> cells were plated in  
590 96-well plates. STING and cGLR activity assays were performed using Dual-Luciferase Reporter



591 Assay System (Promega) as previously described<sup>14</sup>, with modifications. Lipofectamine-2000 was  
592 used to transfect IFN $\beta$ -firefly luciferase and TK-Renilla luciferase reporters and 5 ng of pcDNA4-  
593 mouse STING or 15 ng pcDNA4-dSTING. For cGLR signaling assays 150 ng of *Drosophila*  
594 cGLR1, 30 ng human cGAS balanced with empty vector, or 150 ng empty vector were additionally  
595 transfected. Native cGLR and STING coding sequences were expressed from a pcDNA4 vector.  
596 24–30 h after transfection luciferase was measured using a GloMax microplate reader (Promega)  
597 and relative IFN $\beta$  expression calculated by normalizing firefly to Renilla readings. For poly I:C  
598 stimulation of cGLR activity, cells were transfected with 100 ng poly I:C (6.125–200 ng for titration  
599 experiment) 5 h after plasmid transfection. For dSTING signaling assays a final concentration of  
600 500 pM to 50  $\mu$ M 2'3'-cGAMP or 3'2'-cGAMP was delivered to cells using a digitonin  
601 permeabilization buffer<sup>47</sup> 10 h prior to luciferase measurement.

602

### 603 **Nucleotide purification and HPLC analysis**

604 Enzymatic synthesis of cGLR nucleotide products for HPLC analysis was performed using 100  
605  $\mu$ L reactions containing 10  $\mu$ M cGLR enzyme, 200  $\mu$ M ATP, 200  $\mu$ M GTP, 10  $\mu$ g poly I:C, 1 mM  
606 MnCl<sub>2</sub> and 50 mM Tris-HCl pH 7.5. Protein storage buffer (20 mM HEPES pH 7.5, 250 mM KCl,  
607 1 mM TCEP) was used as necessary to adjust KCl concentration to ~100 mM. Reactions were  
608 incubated at 37°C for 1 h and then nucleotide product was recovered by filtering reactions through  
609 a 30-kDa cutoff concentrator (Amicon) to remove protein. Nucleotide products were separated on  
610 an Agilent 1200 Infinity Series LC system using a C18 column (Zorbax Bonus-RP 4.6  $\times$  150 mm,  
611 3.5  $\mu$ m) at 40°C. Products were eluted at a flow rate of 1 mL min<sup>-1</sup> with a buffer of 50 mM NaH<sub>2</sub>PO<sub>4</sub>  
612 pH 6.8 supplemented with 3% acetonitrile.

613 To purify the *Deu*-cGLR product for mass-spectrometry analysis, nucleotide synthesis  
614 reaction conditions were scaled as previously described for bacterial cGAS/DncV-like  
615 Nucleotidyltransferase reactions<sup>11,48</sup>. Briefly, a 10 mL reaction containing 528 nM *Deu*-cGLR  
616 enzyme, 125  $\mu$ M ATP, 125  $\mu$ M GTP, ~250  $\mu$ g poly I:C, 1 mM MnCl<sub>2</sub>, 50 mM Tris-HCl 7.5, and ~25

617 mM KCl was incubated with gentle rotation for 36 h at 37°C follow by Quick CIP (NEB) treatment  
618 for 6 h. The reaction was monitored using a 20 µL aliquot supplemented with  $\alpha$ -<sup>32</sup>P labeled NTPs  
619 and to visualize product formation by thin-layer chromatography. Following incubation, the large-  
620 scale reaction was filtered through a 10-kDa concentrator (Amicon) and purified by anion  
621 exchange chromatography using a 1 mL Q-sepharose column washed with water and eluting with  
622 a 0–2 M ammonium acetate gradient. Fractions corresponding to main product 3'2'-cGAMP were  
623 differentiated from fractions corresponding to 2'3'-c-di-AMP by HPLC analysis. Product fractions  
624 were further purified by size-exclusion chromatography using a Superdex 30 Increase 10/300 GL  
625 with dH<sub>2</sub>O as a running buffer. Peak fractions were eluted in 1 mL volumes, pooled, and  
626 evaporated for storage prior to mass-spectrometry analysis.

627

#### 628 **Nucleotide mass spectrometry analysis and 3'2'-cGAMP identification**

629 Purified nucleotide product samples were evaporated at 40°C under a gentle nitrogen stream.  
630 The residual pellet was resuspended in 200 µL HPLC grade water (J.T. Baker), and 40 µL was  
631 then mixed with 40 µL of water containing 50 ng mL<sup>-1</sup> tenofovir as internal standard and  
632 transferred to measuring vials.

633 Experiments for 3'2'-cGAMP identification were performed on an ACQUITY UPLC I-  
634 Class/Vion IMS-QTOF high-resolution LC-MS system (Waters Corporation). Reverse phase  
635 chromatographic separation was carried out at 30°C on a C18 column (Nucleodur Pyramid C18  
636 50 x 3 mm; 3 µm Macherey Nagel, Düren, Germany) connected to a C18 security guard  
637 (Phenomenex, Aschaffenburg, Germany) and a 2 µm column saver. Separation was achieved  
638 using a binary gradient of water containing 10 mM ammonium acetate and 0.1% acetic acid  
639 (solvent A) and methanol (solvent B). The analytes were eluted at a flow rate of 0.6 mL min<sup>-1</sup>.  
640 The eluting program was as follows: 0 to 4 min: 0% B, 4 to 7.3 min: 0 to 10% B. This composition  
641 of 10% B was held for 1 min, then the organic content was increased to 30% within 2.7 min. The  
642 column was then re-equilibrated to 0% B for 2 min. Total analysis run time was 13 min. High

643 resolution MS-Data were collected on a Vion IMS-QTOF mass spectrometer equipped with an  
644 electrospray ionization source, operating in positive ionization mode. The capillary voltage was  
645 set at 2.5 kV and the cone voltage at 40 V. The source temperature and desolvation gas  
646 temperature was 150°C and 600°C, respectively. Analyte fragmentation was achieved using  
647 argon as the collision gas. Collision energy of 10 V was used to obtain a low collision energy  
648 spectrum. For high collision energy spectrum, the collision energy was ramped from 15 to 30 V.  
649 Data acquisition was controlled by the UNIFI 1.9.4.0 software (Waters). For 3'2'-cGAMP  
650 identification the retention times, drift times and fragment spectra of a synthetic 3'2'-cGAMP  
651 standard (Biolog) were collected as a reference and compared to those of the suspected 3'2'-  
652 cGAMP in the samples.

653

#### 654 **3'2'-cGAMP quantification**

655 For quantification of 3'2'-cGAMP, chromatographic conditions were transferred to a API4000  
656 mass spectrometer (Sciex) coupled to a Shimadzu HPLC-system (Shimadzu, Duisburg,  
657 Germany). The analytes were ionized by means of electro spray ionization in positive mode  
658 applying an ion spray voltage of 3000 V. Further ESI parameters were: curtain gas (CUR): 30 psi,  
659 collision gas (CAD): 9, source temperature: 650°C, gas 1: 60 psi and gas 2: 45 psi, respectively.  
660 Detection was performed in SRM mode, selecting first for the double-protonated parent ion of  
661 3'2'-cGAMP and 3'3'-cGAMP (used in calibrator series). This resulted in the following mass  
662 transitions: 3'2'-cGAMP and 3'3'-cGAMP:  $m/z$  338.2  $\rightarrow$  152 (quantifier),  $m/z$  338.2  $\rightarrow$  136  
663 (identifier). Tenefovir served as the internal standard ( $m/z$  288  $\rightarrow$  176).

664 For 3'2'-cGAMP semi-quantitative quantification from lysate samples in the *Diptera* cGLR  
665 screen, calibration curves were created by plotting peak area ratios of 3'3'-cGAMP as an internal  
666 standard versus the nominal concentration of the calibrators. The calibration curve was calculated  
667 using quadratic regression and 1/x weighing.

668

669 **Synthetic cyclic dinucleotide standards**

670 Synthetic nucleotide standards used for HPLC analysis and mass-spectrometry analysis were  
671 purchased from Biolog Life Science Institute: 3'3'-cGAMP (cat no. C 117), 2'3'-cGAMP (cat no. C  
672 161), 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  
673 182).

674

675 **Nuclease P1 and poxin cleavage analysis**

676 Nuclease P1 cleavage analysis was performed using *Dm*-cGLR1 reactions labeled with either  $\alpha$ -  
677  $^{32}\text{P}$ -ATP or  $\alpha$ - $^{32}\text{P}$ -GTP as previously described<sup>11,26</sup>. Briefly, radiolabeled nucleotide products were  
678 incubated with Nuclease P1 (80 mU, Sigma N8630) in 1× P1 buffer (30 mM NaOAc pH 5.3, 5 mM  
679 ZnSO<sub>4</sub>, 50 mM NaCl) for 30 min in the presence of Quick CIP (NEB).

680 Poxin cleavage reactions were carried out using purified insect viral AcNPV enzyme as  
681 previously described<sup>29,30</sup>. For HPLC analysis of poxin cleavage, 100  $\mu\text{L}$  reactions were performed  
682 using 100  $\mu\text{M}$  synthetic 2'3'-cGAMP or 3'2'-cGAMP, 50 nM AcNPV poxin, 50 mM HEPES pH 7.5,  
683 10 mM KCl, and 1 mM TCEP. Reactions were incubated at 37°C and at each specified time  
684 reactions were terminated by heat-inactivation at 95°C for 2 min prior to HPLC analysis as  
685 described above. For thin-layer chromatography analysis of poxin cleavage, reactions were  
686 performed using  $\alpha$ - $^{32}\text{P}$ -GTP-labeled 2'3'-cGAMP synthesized by mcGAS or 3'2'-cGAMP  
687 synthesized by *Deu*-cGLR in 5  $\mu\text{L}$  reactions containing 2.5  $\mu\text{M}$  nucleotide product and 1  $\mu\text{M}$   
688 AcNPV poxin, 50 mM HEPES pH 7.5, 10 mM KCl, and 1 mM TCEP. Reactions were incubated  
689 at 37°C and at each specified time reactions were terminated by heat-inactivation at 80°C for 5  
690 min prior to PEI-cellulose thin-layer chromatography analysis as described above.

691

692 **STING CDN thermal shift assay**

693 A final concentration of 15  $\mu\text{M}$  dSTING was mixed with 3× SYPRO orange dye and 100  $\mu\text{M}$   
694 synthetic CDN (Biolog) (or as described in figure) in 20 mM HEPES-KOH pH 7.5 and 100 mM

695 KCl. Samples were heated from 20–95°C in a BioRad CFX thermocycler with HEX channel  
696 fluorescence measurements every 0.5°C. The derivative of each curve over time was calculated  
697 using GraphPad Prism and graphed as a percent maximum change in fluorescence or used to  
698 calculate the melting temperature.

699

#### 700 ***D. melanogaster* cyclic dinucleotide injection and signaling analysis**

701 Fly stocks were raised on standard cornmeal agar medium at 25°C. All fly lines used in this study  
702 were *Wolbachia* free.  $w^{1118}$ ,  $dSTING^{Control}$ , and  $dSTING^{Rxn}$  stocks have been described  
703 previously<sup>31,34</sup>. *Relish<sup>E20</sup>* flies isogenized to the DrosDel  $w^{1118}$  isogenic background were a kind  
704 gift from Luis Teixeira (Instituto Gulbenkian de Ciência)<sup>49</sup>. Cyclic dinucleotides including 3'2'-  
705 cGAMP (Biolog), 2'3'-cGAMP (Invivogen) and 3'3'-c-di-GMP (Invivogen) were dissolved in 10 mM  
706 Tris-HCl pH 7.5 and diluted to the indicated concentrations. 3–5 days old adult flies were injected  
707 with 69 nL of cyclic dinucleotide solution or 10 mM Tris-HCl pH 7.5 (negative control) by  
708 intrathoracic injection using a Nanoject II apparatus (Drummond Scientific). Flies were collected  
709 24 h later in pools of 6 individuals (3 males and 3 females) and homogenized for RNA extraction  
710 and RT-qPCR analysis, as described<sup>34</sup>.

711

#### 712 ***D. melanogaster* viral challenge assays**

713 For 3'2'-cGAMP and virus co-injection, flies were injected with 69 nL of virus (DCV: 5 PFU, VSV:  
714 2000 PFU) in 10 mM Tris-HCl pH 7.5 or in a 0.9 mg mL<sup>-1</sup> 3'2'-cGAMP solution. For titration  
715 experiments comparing cGAMP isomers, 69 nL of DCV (5 pfu) in serial diluted concentrations of  
716 2'3'-cGAMP or 3'2'-cGAMP were injected in the body cavity of the flies. Survival was monitored  
717 daily and flies were collected in pools of 6 individuals (3 males and 3 females) at the indicated  
718 time points to monitor the viral RNA load by RT-qPCR.

719

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805 **Data Availability Statement:** Coordinates and structure factors of human MB21D2, *T.*  
806 *castaneum* cGLR, *Drosophila* STING, and the *Drosophila* STING–3'2'-cGAMP complex have  
807 been deposited in PDB under the accession codes 7LT1, 7LT2, 7MWY, and 7MWZ. All other data  
808 are available in the manuscript or the supplementary materials.

809

## 810 **Extended Data Figure Legends**

### 811 **Extended Data Figure 1 | Sequence and structural analysis of hMB21D2 and *Tc*-cGLR.**

812 **a**, Structure guided sequence alignment of the catalytic domain of human cGAS (PDB 4KM5),  
813 human MB21D2, and *Tc*-cGLR. Strict secondary structure conservation further supports  
814 conserved structural homology despite primary sequence divergence. The [D/E]hD[X<sub>50-90</sub>]D  
815 catalytic triad is highlighted with red outline and the human Zn-ribbon insertion that is absent in  
816 other cGLRs is denoted with magenta line. hMB21D2 contains an additional 61 residues which  
817 are not resolved in the crystal structure and are absent from the alignment. **b,c**, Zoom-in cutaways  
818 of the human MB21D2 and *Tc*-cGLR crystal structures highlighting positioning of conserved  
819 catalytic residues in the nucleotidyltransferase active site. In human cGAS the analogous residues  
820 coordinate two Mg<sup>2+</sup> metal ions to control synthesis of 2'3'-cGAMP (inset, middle; PDB 6CTA).

821 The hMB21D2 structure is in an inactive state distinguished by misaligned catalytic residues and  
822 occlusion by an extended Gly-Gly-activation loop, indicating that catalytic activation is likely  
823 controlled by a conformational rearrangement. **d, e**, TLC analysis of *in vitro* tests for potential  
824 activating ligands of hMB21D2. No nucleotide products were identified upon stimulation with 40  
825 nt or bp nucleic acid ligands (**d**) or ligands known to activate mammalian Toll-like receptors (**e**) **f**,  
826 Z-score structural similarity plot showing homology between human MB21D2 and *Tc*-cGLR with  
827 representative structures in the Protein Data Bank (PDB90). Increasing Z-score indicates greater  
828 homology confirming the close relationship between animal cGLR enzymes and more distantly  
829 related similarity with cGAS/DncV-like Nucleotidyltransferases (CD-NTases) in bacterial anti-  
830 phage defense systems<sup>11,50</sup>. Z-scores cutoffs are 13 and 15 for hMB21D2 and *Tc*-cGLR  
831 respectively.

832

### 833 **Extended Data Figure 2 | Forward biochemical screen of predicted cGLRs in Diptera.**

834 **a**, Violin plot showing the number of predicted cGLRs in *Diptera* genomes. *Drosophila* genomes  
835 (n = 31 species) have a median of four predicted cGLRs in contrast to a median of two predicted  
836 cGLRs in other Dipteran insects (n = 11 species). **b**, Schematic of *in vitro* screen of predicted  
837 cGLRs in the order *Diptera*. 53 sequences were selected representing each clade in the  
838 phylogeny in **Fig. 2a**. Following recombinant protein expression in *E. coli*, lysates were split into  
839 two samples for parallel TLC analysis of *in vitro* enzymatic activity and HPLC-MS analysis of  
840 lysate nucleotide metabolites. **c, d**, Purified cGLR proteins were incubated overnight at 37°C with  
841  $\alpha^{32}\text{P}$ -radiolabeled nucleotides, a mixture of  $\text{Mn}^{2+}$  and  $\text{Mg}^{2+}$ , and the 45 bp immunostimulatory DNA  
842 ISD45 or the synthetic dsRNA analog poly (I:C) as potential nucleic acid ligands, and reactions  
843 were visualized by PEI-cellulose TLC. Wild-type and catalytically inactive mouse cGAS enzymes  
844 were used as controls for each sample set. Note that mouse cGAS exhibits dsDNA-independent  
845 activity in the presence of  $\text{Mn}^{2+}$ <sup>51</sup>. Predicted *Diptera* cGLRs grouped by clade (DC01–05) and  
846 numbered within each clade. Ligand-dependent activity was identified for DC02\_01, 05\_03,



847 05\_19, and 05\_21; species listed below. We observed ligand-independent activity for two  
848 enzymes in Clade 3. Data represent n = 2 independent experiments. **e**, SDS-PAGE and  
849 Coomassie stain analysis of NiNTA purified cGLR protein fractions used for the biochemical  
850 screen. **f**, SDS-PAGE and Coomassie stain analysis of final NiNTA, ion-exchange, and size-  
851 exclusion purified cGLR proteins used for biochemical studies.

852

### 853 **Extended Data Figure 3 | Sequence analysis and mutagenesis of insect cGLRs.**

854 **a**, Alignment of the catalytic domain of human cGAS and active cGLRs identified in *T. castaneum*,  
855 *D. eugracilis*, *L. cuprina*, *D. erecta*, *D. simulans*, and *D. melanogaster*. The EhD[X<sub>50-90</sub>]D catalytic  
856 triad is highlighted with red outline and the human Zn-ribbon insertion that is absent in insect  
857 cGLRs is denoted with a dashed red outline. cGLRs from *D. erecta* and *D. simulans* are close  
858 homologs of *Dm*-cGLR1 (76% and 91% sequence identity, respectively) and thus are also  
859 referred to as “cGLR1”. All biochemical experiments with *Ds*-cGLR1 were performed with a  
860 construct beginning at M19. **b–c**, *In vitro* nucleotide synthesis reactions demonstrating effect of  
861 mutations to catalytic residues (**b**) or putative ligand binding groove (**c**) on insect cGLR enzymatic  
862 activity. Catalytic active-site mutations ablate nucleotide product synthesis and ligand-binding  
863 groove mutations that disrupt predicted RNA contacting residues significantly impair product  
864 synthesis. **d**, SDS-PAGE and Coomassie stain analysis of purified wild-type and mutant proteins,  
865 as labeled in above TLC images. **e**, *Above*, structure of *Tc*-cGLR1 modeled with dsRNA as shown  
866 in **Fig. 2f**, indicating putative ligand binding residues in *Tc*-cGLR selected analysis. *Below*,  
867 quantification of *in vitro* activity of *Tc*-cGLR in **c**, as displayed in **Fig. 2g** for *Ds*-cGLR1. Data are  
868 mean ± SEM, quantified relative to wild-type activity and represent n = 3 independent  
869 experiments. **f**, IFN-β luciferase assay in which cGLRs are expressed in human cells and CDN-  
870 synthesis is detected by mammalian STING activation. IFN-β quantified relative to empty vector  
871 control. In comparison to human cGAS control which is activated by expression vector-plasmid  
872 DNA, *Dm*-cGLR1 (left) and *Ds*-cGLR1 (right) require poly I:C stimulation to activate a downstream

873 STING response. Mutation to catalytic residues or putative ligand binding residues (as indicated  
874 in **Fig. 2f**) ablates cGLR activity. See also **Fig. 2h**. Data are mean  $\pm$  SEM of n = 3 technical  
875 replicates and representative of n = 3 independent experiments. \*P  $\leq$  0.05; \*\*P  $\leq$  0.01; \*\*\*P  $\leq$   
876 0.001; and n.s., P > 0.05. P value n.s. unless otherwise noted.

877

#### 878 **Extended Data Figure 4 | Analysis of RNA-recognition by insect cGLRs.**

879 **a–c**, *In vitro* activity assays for each active insect cGLR demonstrating dsRNA recognition is  
880 required for enzyme activation. Reactions were performed with synthetic 40 nt or bp ligands.  
881 Weak *Deu*-cGLR ssRNA-stimulated activity may be explained by transient short duplex formation  
882 similar to observations that some ssDNA oligos can stimulate mouse cGAS dsDNA-dependent  
883 activity<sup>3</sup>. **b**, TLC and quantification for enzyme activation in the presence of a panel of 10–40 bp  
884 synthetic dsRNA ligands. 30 bp dsRNA is sufficient to stimulate maximal activity for *Tc*-, *Dm*-, and  
885 *Lc*- cGLRs, while *Ds*-cGLR1 requires 35 bp and *Deu*-cGLR can be activated by dsRNAs as short  
886 as 15 bp. **c**, Reactions with 146 bp *in vitro* transcribed dsRNAs either retaining a 5' triphosphate  
887 or 5' OH termini demonstrate that dsRNA-recognition by insect cGLRs does not involve 5' end  
888 discrimination. Data are mean  $\pm$  SEM, quantified relative to maximum observed activity and  
889 represent n = 3 independent experiments. **d**, Deconvolution of catalytic metal requirements for  
890 enzymatic activity by insect cGLRs. Insect cGLRs require Mn<sup>2+</sup> for maximal catalytic activity with  
891 weak product formation observed in the presence of Mg<sup>2+</sup>. Data represent n = 3 independent  
892 experiments. **e**, Poly I:C titration demonstrates dsRNA-stimulation of *Drosophila* cGLR1 activity  
893 in cells is dependent on RNA concentration. IFN- $\beta$  luciferase assay in which cGLRs are expressed  
894 in human cells and CDN-synthesis is measured by mammalian STING activation, as in **Fig. 2h**  
895 and **Extended Data Fig. 3f**. IFN- $\beta$  quantified relative to empty vector control. Data are mean  $\pm$   
896 SEM of n = 3 technical replicates and representative of n = 3 independent experiments.

897

#### 898 **Extended Data Figure 5 | Characterization of *Ds*-cGLR1–dsRNA condensate formation.**

899 **a**, Electrophoretic mobility shift assay (EMSA) showing binding between *Ds*-cGLR1 or the C-  
900 terminal nucleotidyltransferase domain of human cGAS (hcGAS-NTase) and a 40 bp dsRNA or  
901 45 bp dsDNA. *Ds*-cGLR1 preferentially binds dsRNA and more weakly interacts with dsDNA,  
902 consistent with observed binding between human cGAS and dsRNA<sup>13</sup>. **b–c**, Analysis of *Ds*-  
903 cGLR1 and human cGAS (hcGAS) phase separation with AF488-labeled protein. Mammalian  
904 cGAS contains a highly disordered N-terminal extension of ~150 residues, but this unstructured  
905 extension is absent in insect cGLR sequences. In the presence of dsDNA, full-length hcGAS  
906 forms highly dynamic liquid droplets, whereas the minimal hcGAS NTase-domain forms rigid  
907 protein–DNA condensates similar to those formed by *Ds*-cGLR1–RNA complexes. Human cGAS  
908 exhibits a preference for condensate formation in the presence of dsDNA while *Ds*-cGLR1  
909 exhibits a preference for dsRNA (scale bar = 10  $\mu$ m) (see also **Fig. 2e**). **c**, Analysis of *Ds*-cGLR1  
910 dsRNA length-specificity for condensate formation demonstrates clear length-dependency and  
911 supports that long dsRNA and condensate formation are required for maximal *Ds*-cGLR1  
912 activation. **d**, Analysis of the impact of AF488-labeling on *Ds*-cGLR1 enzymatic activity. Similar  
913 to previous observations with hcGAS<sup>25</sup>, AF488-labeling negatively impacts enzymatic activity but  
914 has minimal effect at the ratio of 90% unlabeled and 10% labeled protein used for all imaging  
915 experiments. Data represent n = 3 independent experiments, and are quantified in **c** as the mean  
916  $\pm$  SEM.

917

### 918 **Extended Data Figure 6 | Synthesis of 3'2'-cGAMP by *Diptera* cGLRs.**

919 **a**, HPLC analysis of the nucleotide products of *Tc*-cGLR, *Dm*-cGLR1, *Ds*-cGLR1, *Lc*-cGLR, and  
920 *Deu*-cGLR reactions compared to relevant synthetic controls. Integration of major and minor  
921 product peaks in n = 3 independent experiments were used to calculate relative product ratios  
922 shown in **Fig. 3d**. **b**, The *Drosophila* cGLR major reaction product was purified from *Deu*-cGLR  
923 reactions and compared to synthetic 3'2'-cGAMP with tandem mass-spectrometry analysis.  
924 Parent mass extracted ion trace (left) and tandem mass spectra comparison (right) validate the

925 chemical identity of the *Drosophila* cGLR product as 3'2'-cGAMP. **c**, Heat map showing the  
926 relative concentrations of cGAMP isomers detected by HPLC-MS in bacterial lysates expressing  
927 *Diptera* cGLRs (as described in **Extended Data Fig. 2b**). In all cases 3'2'-cGAMP was present  
928 as the dominant product with trace amounts of 3'3'- and 2'3'-cGAMP detected in some samples  
929 as minor species.

930

931 **Extended Data Figure 7 | Mechanism of 3'2'-cGAMP bond formation and resistance to**  
932 **degradation by viral poxin enzymes.**

933 **a**, Analysis of *Dm*-cGLR1 reactions with pairwise combinations of  $\alpha$ -<sup>32</sup>P-labeled nucleotides and  
934 non-hydrolyzable nucleotides reveals reaction intermediates and identifies the order of bond  
935 formation during 3'2'-cGAMP synthesis. *Left*, TLC analysis demonstrates *Dm*-cGLR1 forms a  
936 linear intermediate in the presence of GTP and non-hydrolyzable ATP (Apcpp) indicating the 2'–  
937 5' phosphodiester bond is synthesized first. Exposed  $\gamma$ -phosphates removed by phosphatase  
938 treatment prior to analysis indicated by parentheses. Note that while a linear intermediate cannot  
939 be formed in the presence of non-hydrolyzable GTP (Gpcpp), *Dm*-cGLR1 will synthesize the off-  
940 product 2'3'-c-di-AMP. Mouse cGAS, which synthesizes 2'3'-cGAMP through the linear  
941 intermediate pppG[2'–5']pA, is shown here for comparison<sup>26</sup>. *Right*, Schematic of the reaction  
942 mechanism for each enzyme. Data are representative of n = 3 independent experiments. **b**,  
943 Poxins are 2'3'-cGAMP-specific viral nucleases that disrupt cGAS-STING signaling. HPLC  
944 analysis of synthetic 2'3'-cGAMP or 3'2'-cGAMP treated with poxin from the insect baculovirus  
945 *Autographa californica* nucleopolyhedrovirus (AcNPV)<sup>29,30</sup>. In 1 min, AcNPV poxin cleaves 2'3'-  
946 cGAMP into a mixture of intermediate and full cleavage product; and after 1 h turnover is  
947 complete. No cleavage of 3'2'-cGAMP is observed by AcNPV poxin under these reaction  
948 conditions. **c**, Using TLC as a more sensitive assay, we observed minimal cleavage of 3'2'-  
949 cGAMP following overnight incubation with AcNPV poxin. **d**, Schematic highlighting how an  
950 isomeric switch in phosphodiester linkage specificity makes 3'2'-cGAMP remarkably resistant to

951 poxin-mediated cleavage.

952

### 953 **Extended Data Figure 8 | Structural and biochemical analysis of *Drosophila* STING**

954 **a**, Alignment of the C-terminal cyclic dinucleotide (CDN)-binding domains of human STING,  
955 mouse STING, *D. eugracilis* STING and *D. melanogaster* STING. Architecture of the core CDN-  
956 binding domain is conserved across metazoans; the disordered C-terminal tail which controls  
957 IRF3-IFN $\beta$  signaling is specific to vertebrates<sup>10,28</sup>. Ligand-interacting residues selected for  
958 mutational analysis annotated with a navy circle; *Diptera*-specific adaptations highlighted with red  
959 outline. All structural and biochemical experiments were performed with a *D. eugracilis* STING  
960 construct ending at I340. **b**, *In vitro* thermal denaturation assay analyzing dSTING interactions  
961 with a panel of CDNs. Only 3'2'-cGAMP forms a thermo-stable complex with dSTING (see also  
962 **Fig. 4a**). Data are mean  $\pm$  SEM of the average  $T_m$  calculated from technical duplicates in  $n = 3$   
963 independent experiments. **c**, *In vitro* thermal denaturation assay demonstrating concentration-  
964 dependent thermal shift induced by 3'2'-cGAMP. **d**, Dose titration of 2'3'-cGAMP and 3'2'-cGAMP  
965 in human cells demonstrating selective response by dSTING to 3'2'-cGAMP. *D. eugracilis* CDN  
966 binding domain (CBD) was adapted for downstream signaling in human cells by addition of N-  
967 terminal human transmembrane (hTM) domains and human C-terminal tail (hCTT). **e**,  
968 Comparison of the human STING–2'3'-cGAMP and dSTING–3'2'-cGAMP crystal structures  
969 reveals a conserved closed homodimer architecture in which apical “wings” are spread 32–36 Å,  
970 demonstrating high-affinity engagement with an endogenous ligand. **f**, Enlarged cutaway of 3'2'-  
971 cGAMP in the dSTING crystal structure, shown as simulated annealing  $F_o - F_c$  omit map. **g**, Full  
972 crystal structure used to determine structure of *D. eugracilis* STING in complex with 3'2'-cGAMP.  
973 T4-lysozyme is fused to the N-terminus of the *D. eugracilis* STING CDN binding domain. **h**,  
974 Thermal denaturation assay as in **Fig. 4a** demonstrating that N-terminal fusion of T4 lysozyme  
975 does not impair dSTING recognition of 3'2'-cGAMP. **i**, Mutational analysis of key ligand-interacting  
976 residues in dSTING; thermal denaturation assay used to analyze 3'2'-cGAMP recognition.

977 Mutations which conserve functional contacts with 3'2'-cGAMP (Y164F) maintain ligand  
978 recognition; mutations which ablate contacts abrogate ligand binding. N159S exhibits diminished  
979 ability to recognize 3'2'-cGAMP. Data in **b** and **i** are mean  $\pm$  SEM of the average  $T_m$  calculated  
980 from n=2 technical replicates in n = 3 independent experiments. Data in **c** are representative of n  
981 = 3 independent experiments. Data in **d** are mean  $\pm$  SEM of n = 3 technical replicates and  
982 representative of n = 3 independent experiments. **j**, SDS-PAGE and Coomassie stain analysis of  
983 purified wild-type and mutant proteins.

984

#### 985 **Extended Data Figure 9 | 3'2'-cGAMP induces the expression of dSTING-regulated genes.**

986 **a–d**, Injection of 3'2'-cGAMP into *D. melanogaster* has a dose-dependent effect on the expression  
987 of dSTING-regulated genes. 2'3'-cGAMP was used as positive control as previously  
988 characterized<sup>31,34</sup>. Synthetic nucleotide was injected into the body cavity of wildtype (*w*<sup>1118</sup>) flies  
989 and gene expression was measured after 24 h. RNA levels were measured relative to the house-  
990 keeping gene *RpL32*, and nucleotide concentrations are displayed in  $\mu\text{g } \mu\text{L}^{-1}$ . Note that for *srg2*  
991 measurement after injection of  $9\text{E-}7 \mu\text{g } \mu\text{L}^{-1}$  3'2'-cGAMP there was one outlier replicate with a  
992 value of 0.5977 (data not shown, included in mean analysis). **e–k**, As in **Fig. 5a**, RNA expression  
993 analysis of STING-regulated genes 24 h after injection with synthetic 3'2'-cGAMP or 3'3'-c-di-  
994 GMP. RNA levels are shown as fold induction compared to buffer control in wildtype, *dSTING*, or  
995 *Relish* mutant flies respectively. *dSTING*<sub>Mut</sub> = RXN mutant; *Relish*<sub>Mut</sub> = *Relish*<sup>E20</sup> mutant, as  
996 previously characterized<sup>31,34</sup>. All data represent the mean  $\pm$  SEM of n = 3 independent  
997 experiments, and each point represents a pool of 6 flies \*P  $\leq$  0.05; \*\*P  $\leq$  0.01; \*\*\*P  $\leq$  0.001; and  
998 n.s., P > 0.05. P value n.s. unless otherwise noted.

999

#### 1000 **Extended Data Figure 10 | 3'2'-cGAMP functions as a potent antiviral ligand**

1001 **a**, Analysis of Drosophila C virus (DCV) viral RNA load in flies injected with 3'2'-cGAMP or buffer  
1002 control. *dSTING* wildtype and mutant flies were injected with 3'2'-cGAMP or buffer control and

1003 then infected with DCV. Viral RNA levels were measured at each time as indicated relative to the  
1004 house-keeping gene *RpL32*. DCV is a picornavirus-like (+)ssRNA virus in the family  
1005 *Dicistroviridae*. **b**, Analysis of vesicular stomatitis virus (VSV) viral RNA load in flies injected with  
1006 3'2'-cGAMP or buffer control. *dSTING* wildtype and mutant flies were injected with 3'2'-cGAMP or  
1007 buffer control as in **a** and then infected with VSV. Viral RNA levels were measured 4 days post  
1008 infection relative to the house-keeping gene *RpL32*. VSV is a (-)ssRNA virus in the *Rhabdoviridae*  
1009 family. **c**, (As in **a**) Analysis of Drosophila C virus (DCV) viral RNA load in flies injected with 3'2'-  
1010 cGAMP, 2'3'-cGAMP, or buffer control. Viral RNA levels were measured one, two, or three days  
1011 post-infection (dpi) relative to house-keeping gene *RpL32*. **d**, Survival curves after DCV infection  
1012 showing effect of injection with dose titration of 3'2'-cGAMP or 2'3'-cGAMP compared to buffer  
1013 control. Both cGAMP isomers significantly delay mortality in a dose-dependent manner; 3'2'-  
1014 cGAMP provides greater protection in comparison to 2'3'-cGAMP. All data represent the mean  $\pm$   
1015 SEM of  $n = 3$  independent experiments, and each point represents a pool of 6 flies (**a**, **b**) or 10  
1016 flies (**c**, **d**). \* $P \leq 0.05$ ; \*\* $P \leq 0.01$ ; \*\*\* $P \leq 0.001$ ; and n.s.,  $P > 0.05$ . P value n.s. unless otherwise  
1017 noted.

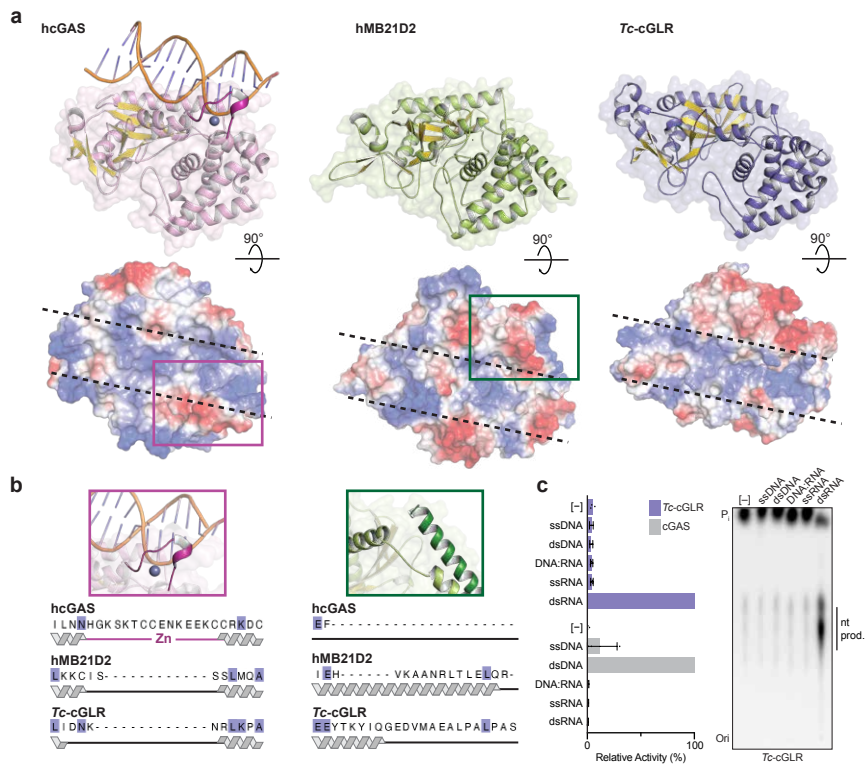
1018

1019 **Supplementary Table 1 | Summary of X-ray crystallography data collection, phasing and**  
1020 **refinement statistics.**

1021

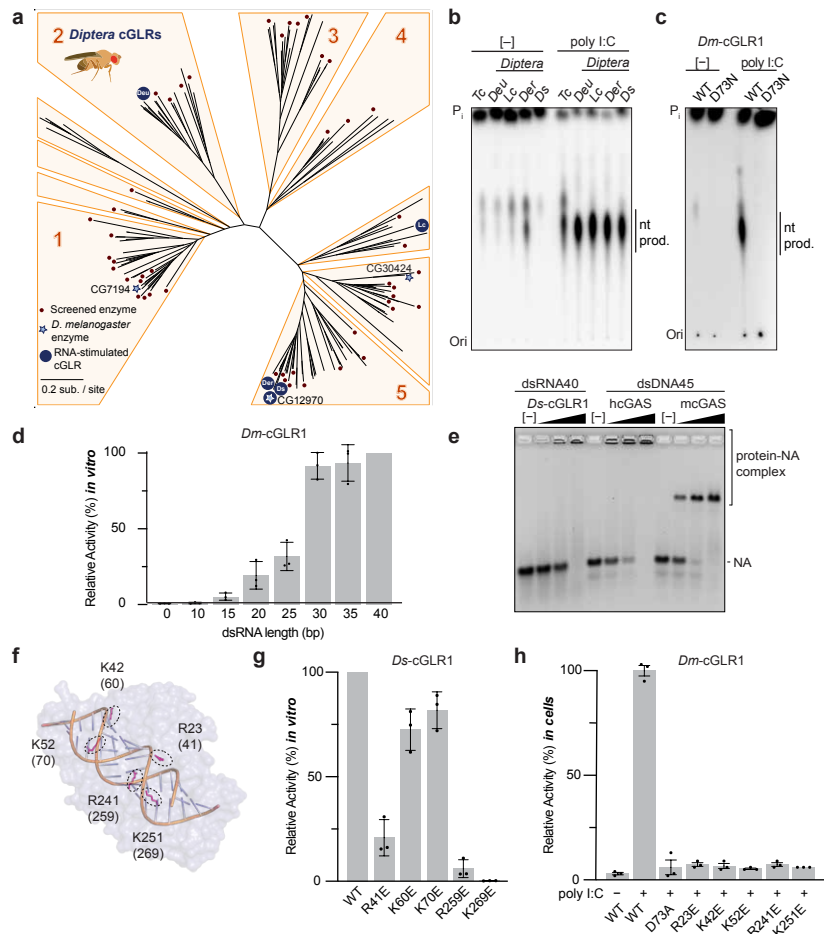
1022 **Supplementary Table 2 | cGLR sequence information.**

**Fig. 1 | Structural remodeling in animal cGLRs enables divergent pattern recognition**

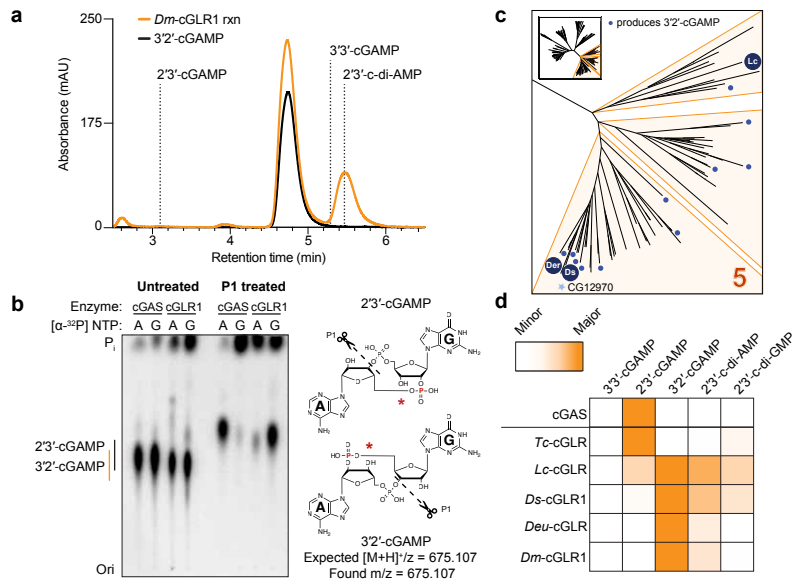




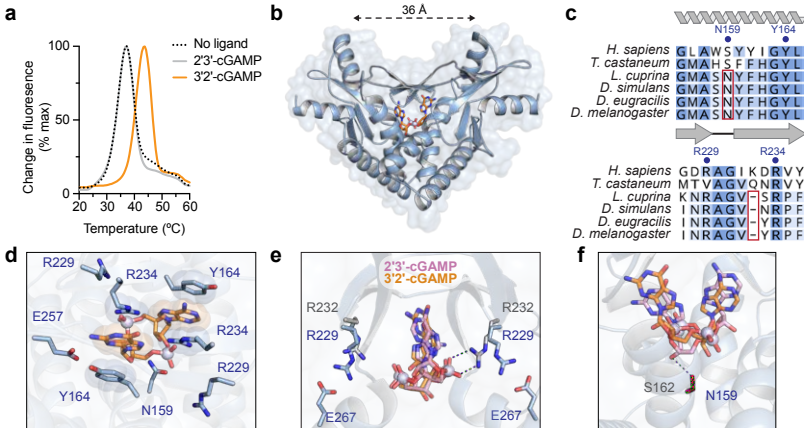
**Fig. 2 | *Drosophila* cGLR1 senses long double-stranded RNA**



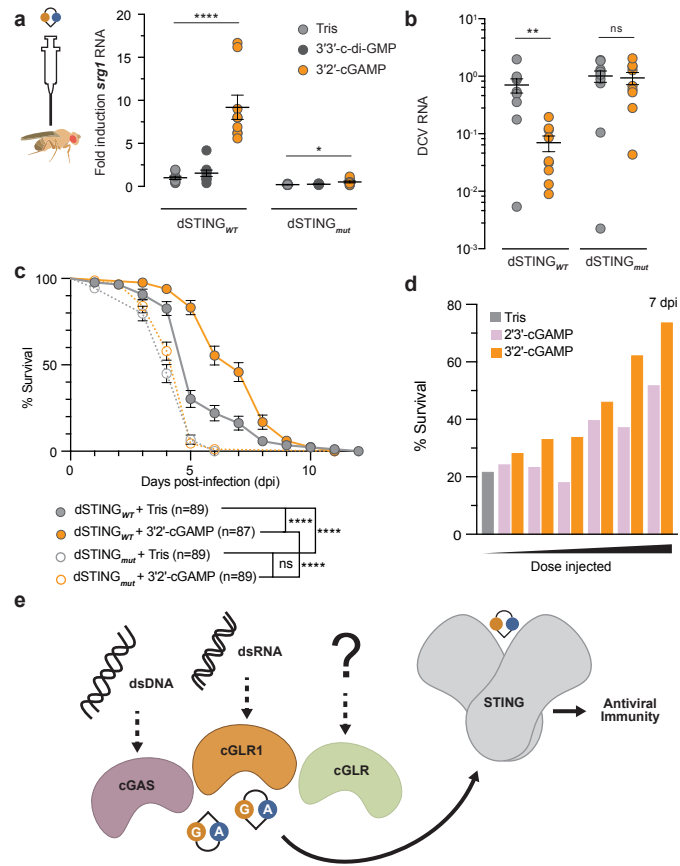
**Fig. 3 | Discovery of 3'2'-cGAMP as a metazoan nucleotide second messenger**



**Fig. 4 | Structural basis for 3'2'-cGAMP recognition by *Drosophila* STING**

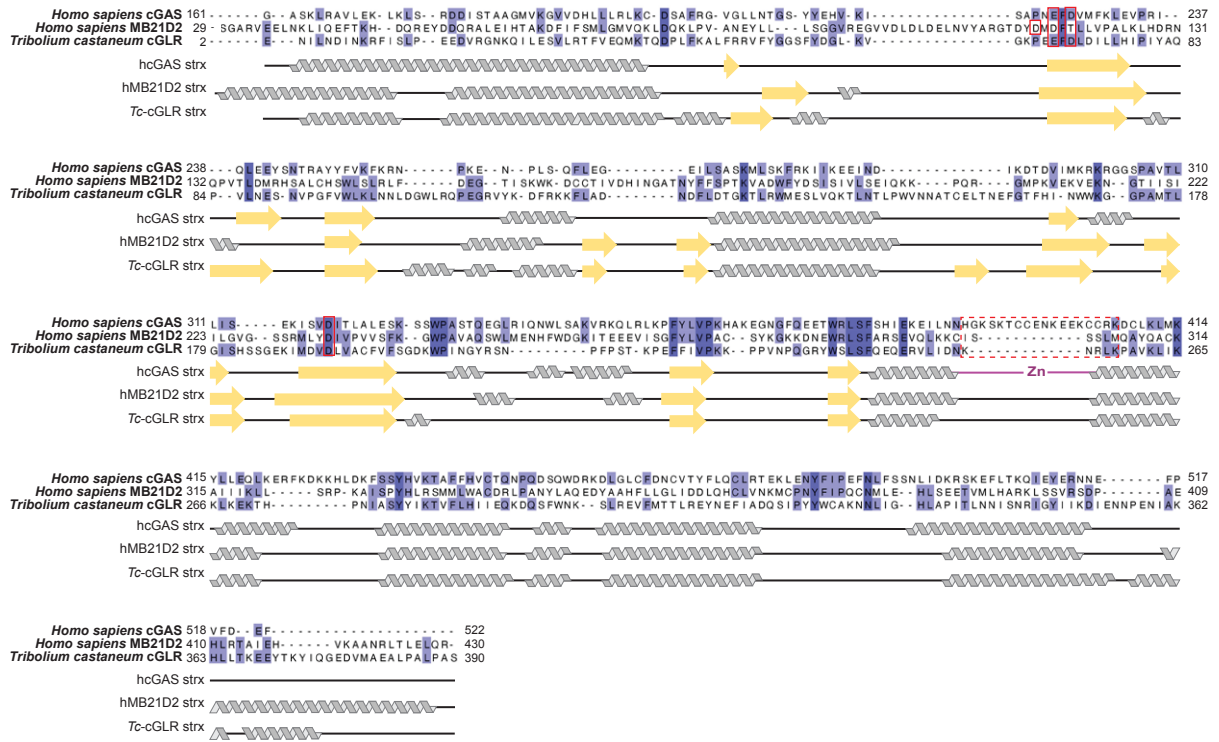


**Fig. 5 | 3'2'-cGAMP activates STING-dependent antiviral immunity in *Drosophila***

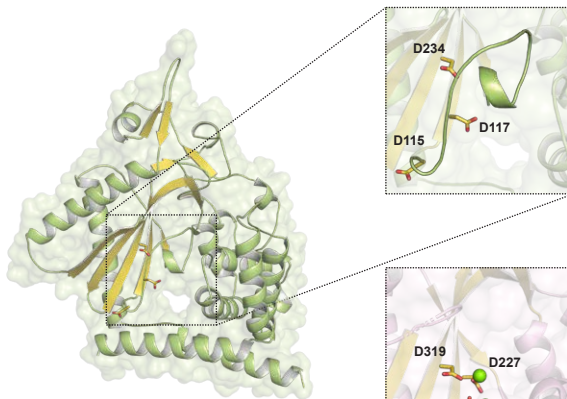


**Extended Data Fig. 1 | Sequence and structural analysis of hMB21D2 and *Tc*-cGLR**

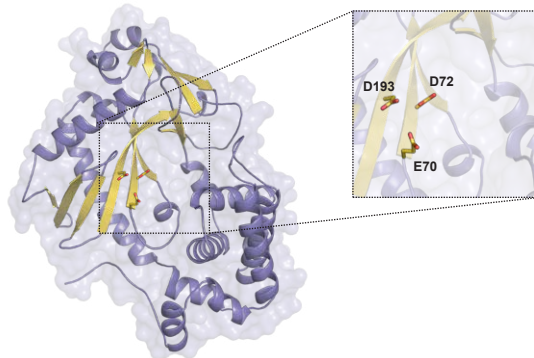
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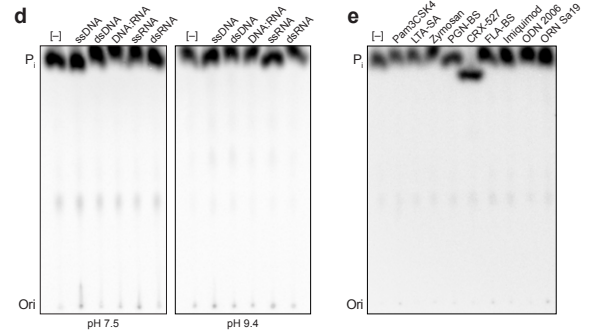
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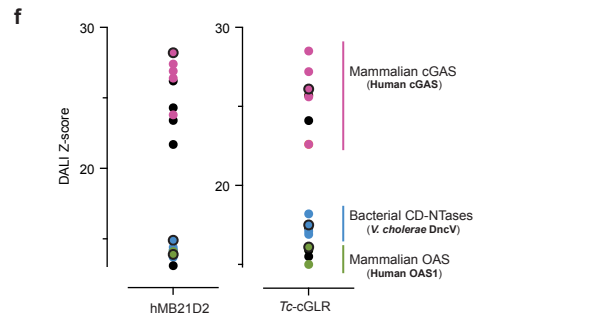
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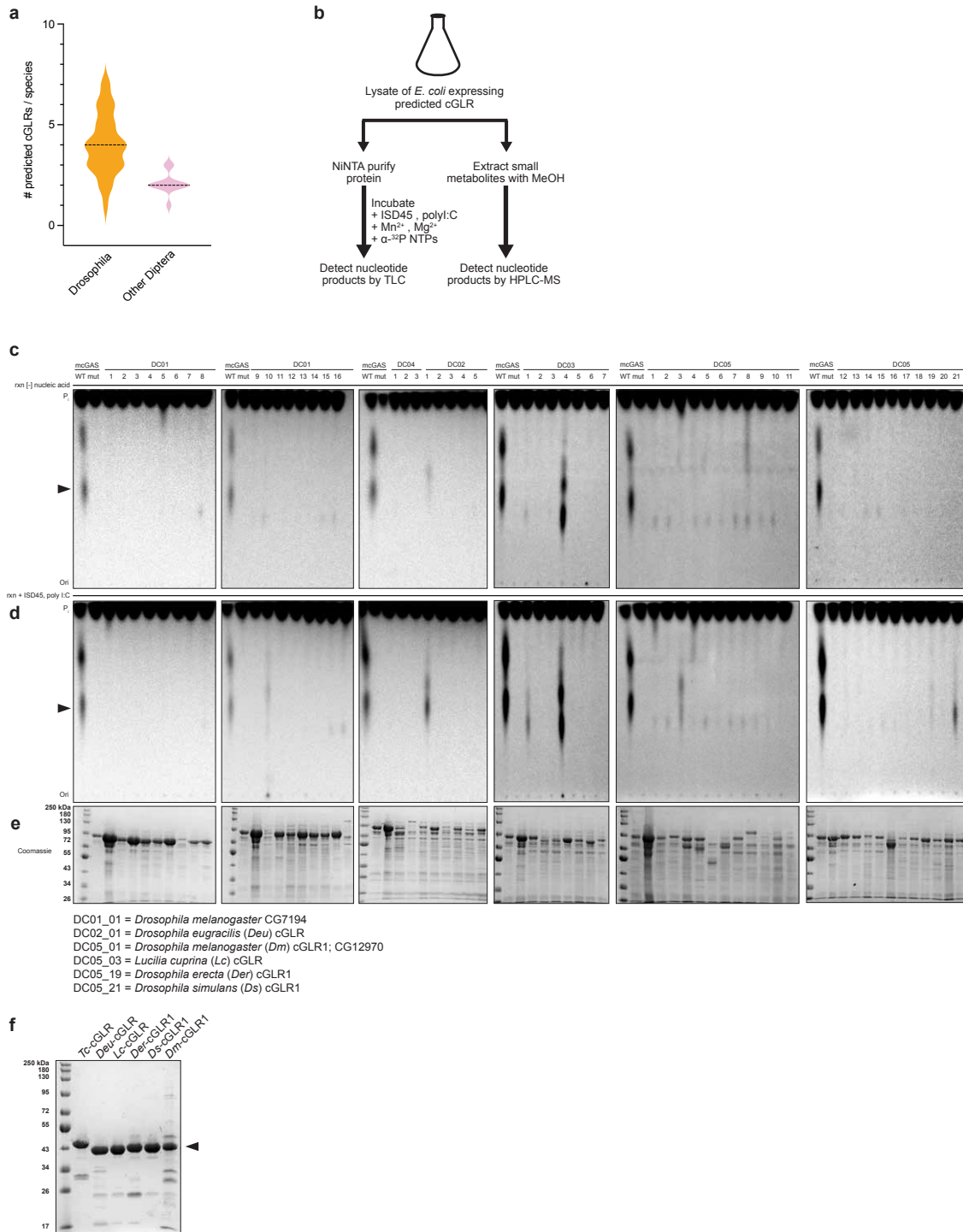
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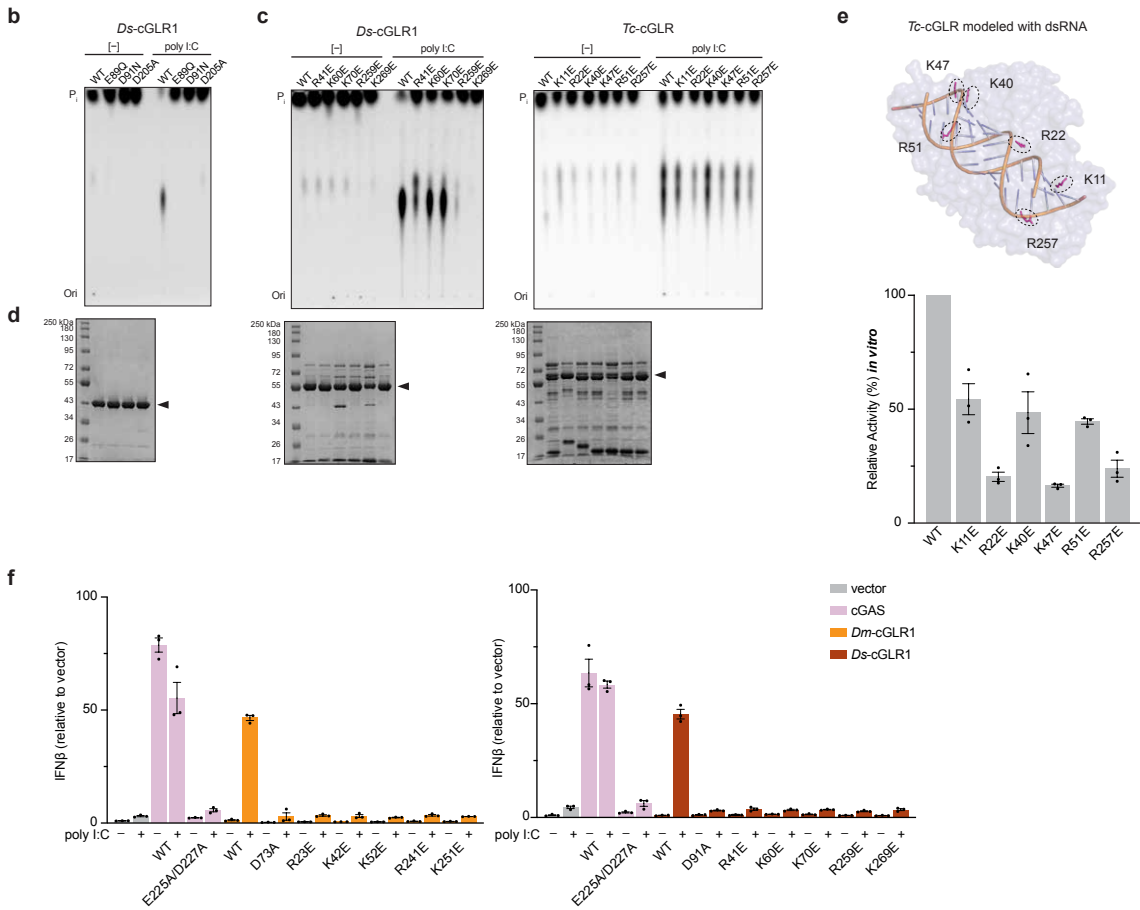
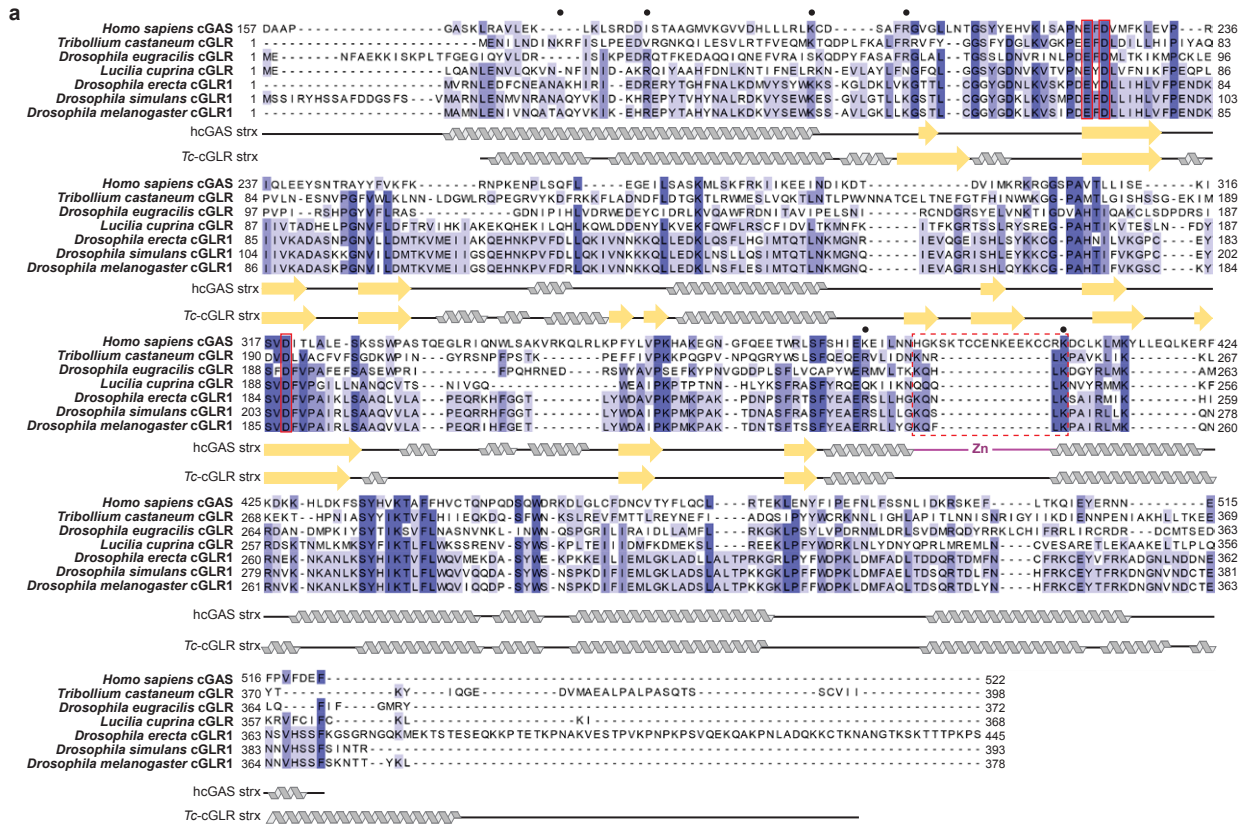
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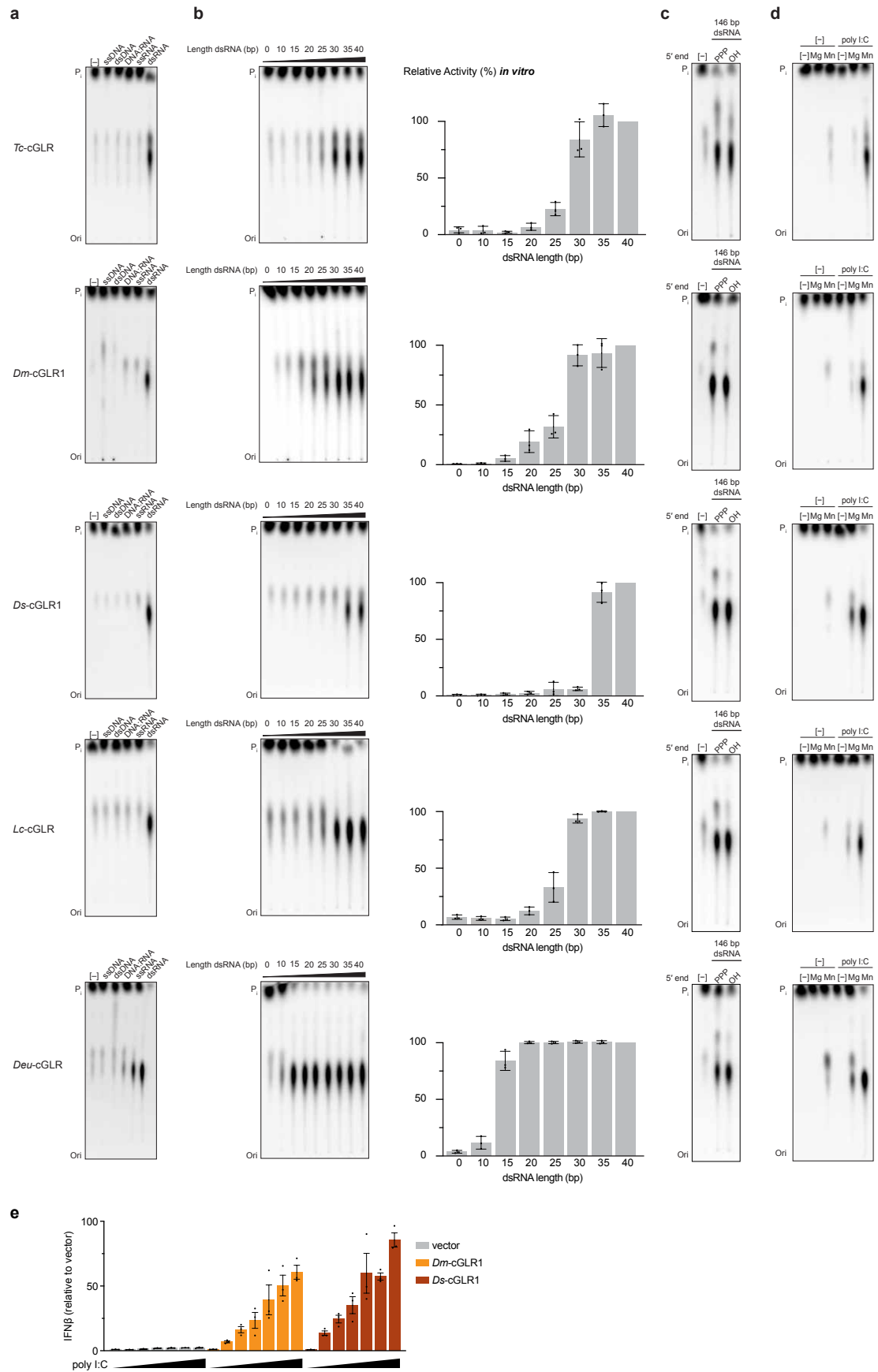
Extended Data Fig. 2 | Forward biochemical screen of predicted cGLRs in *Diptera*



Extended Data Fig. 3 | Sequence analysis and mutagenesis of insect cGLRs

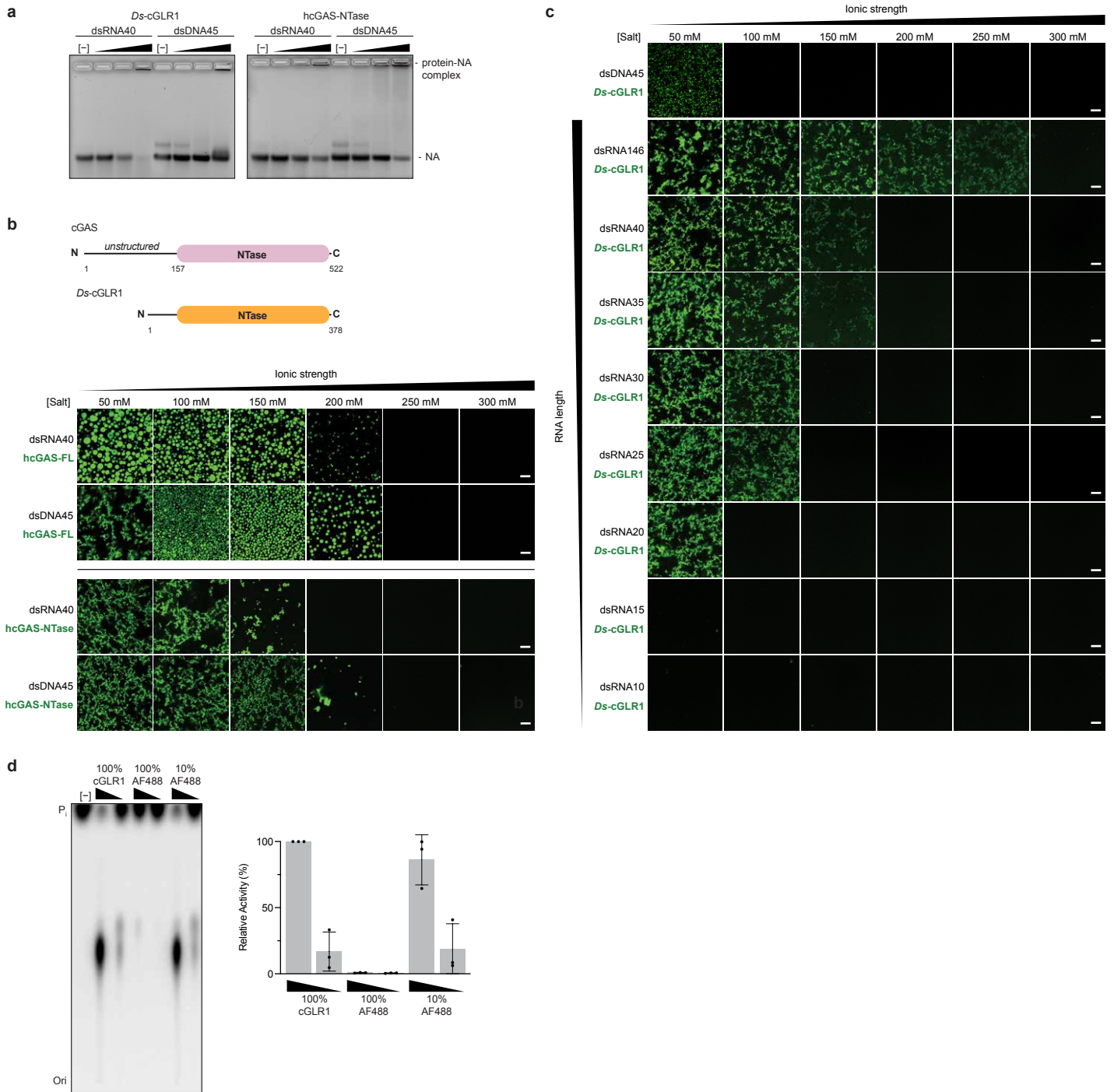


Extended Data Fig. 4 | Analysis of RNA-recognition by insect cGLRs

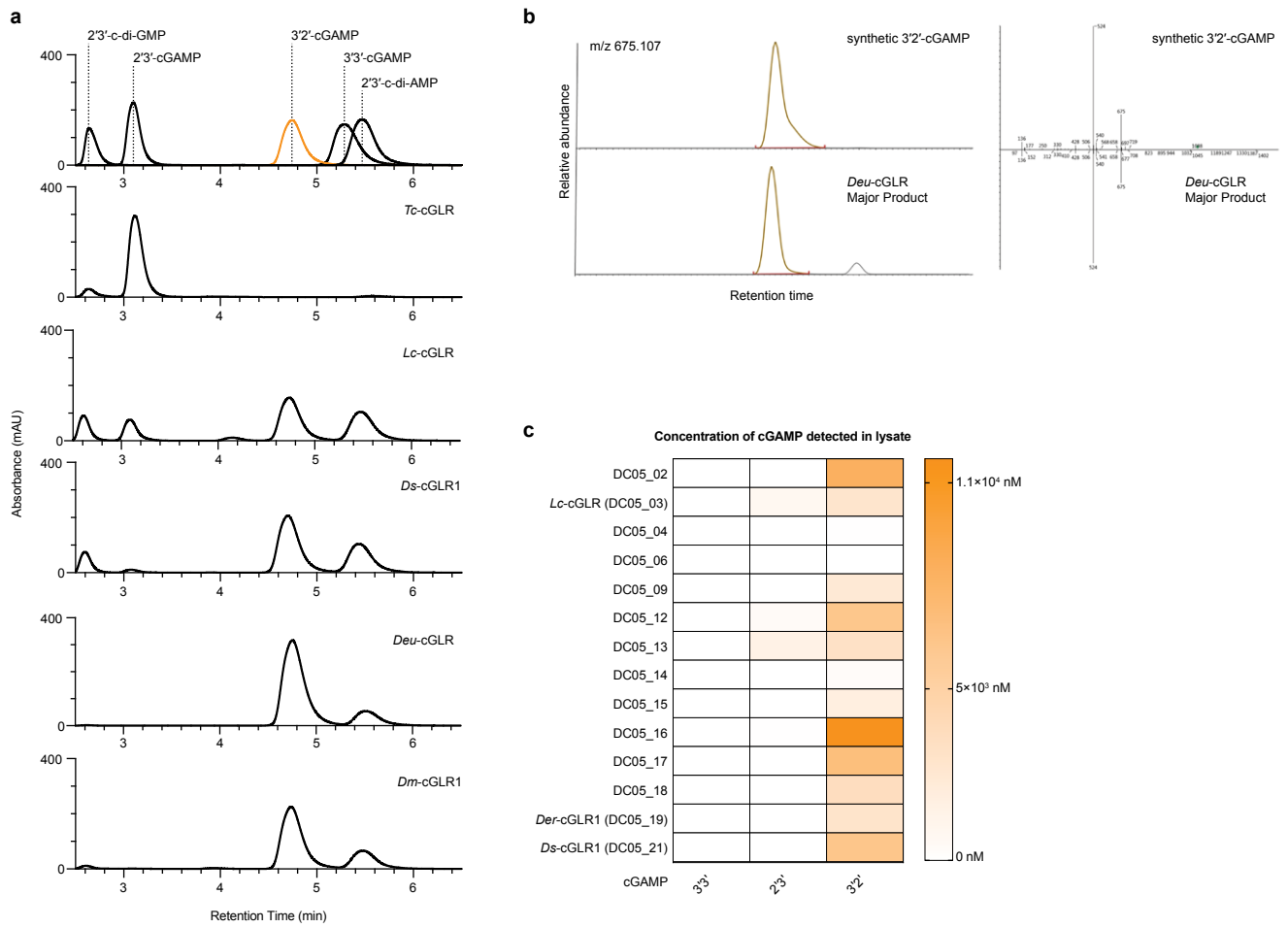




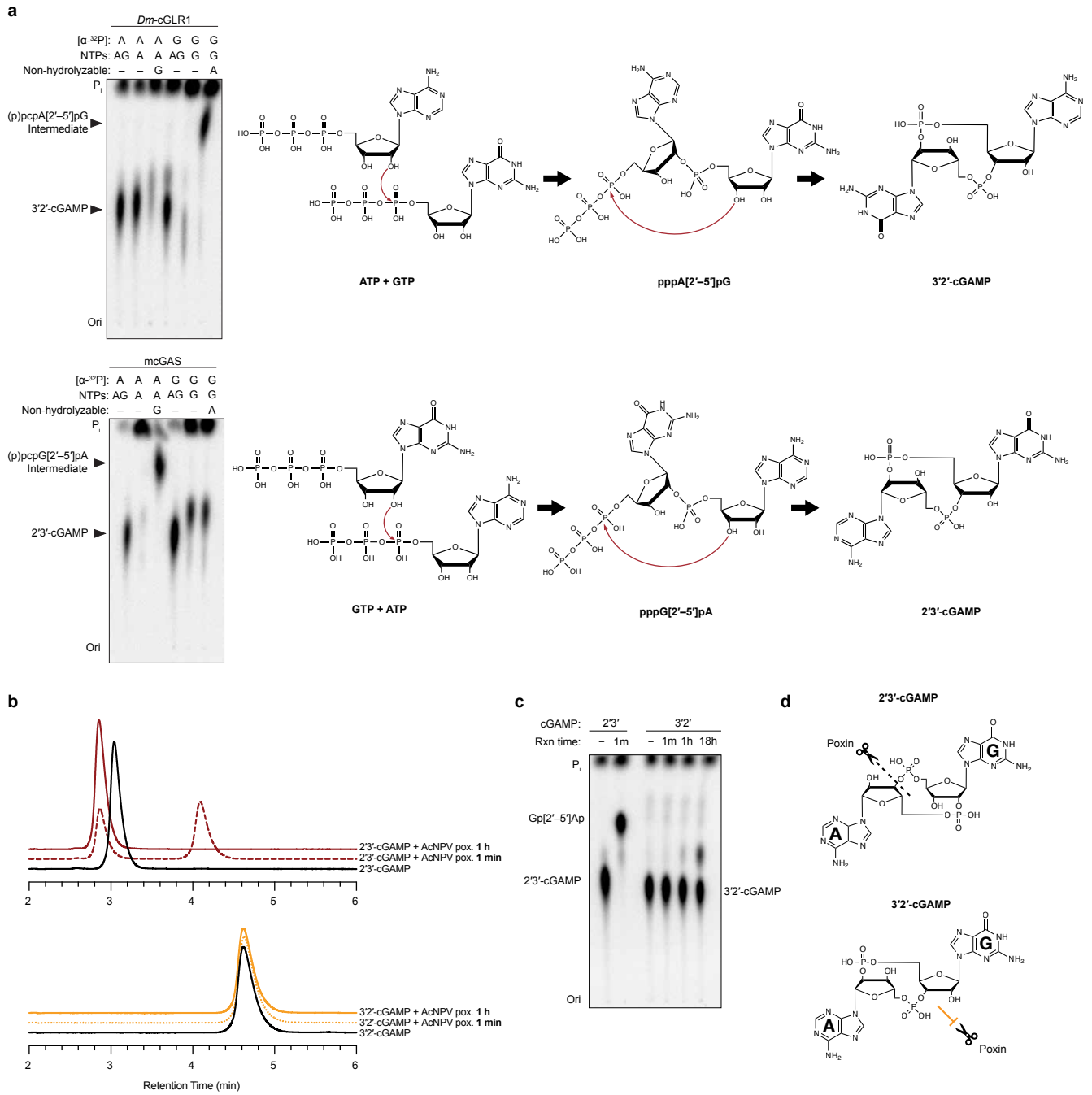
Extended Data Fig. 5 | Characterization of *Ds*-cGRL1–dsRNA condensate formation



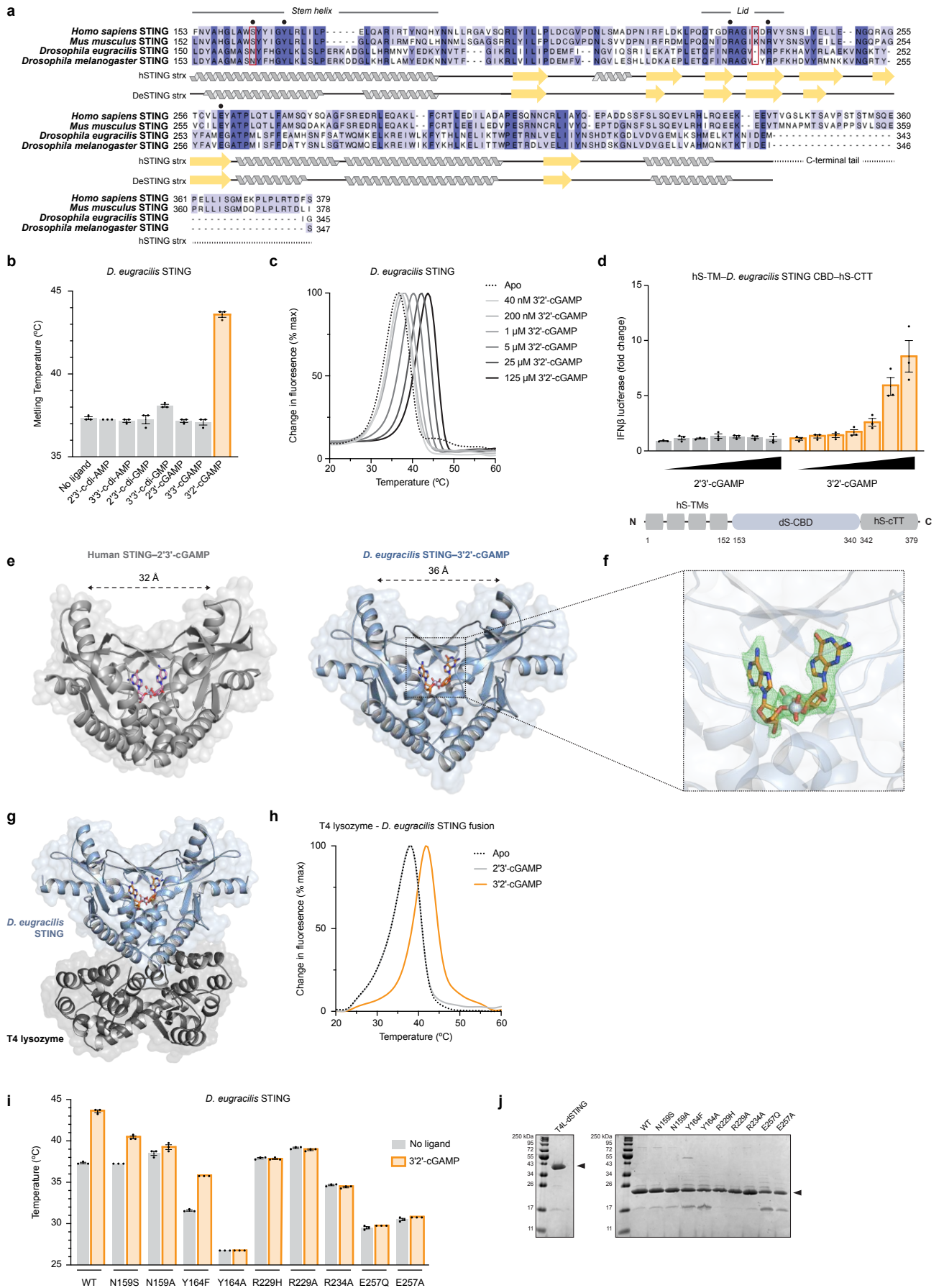
Extended Data Fig. 6 | Synthesis of 3'2'-cGAMP by *Diptera* cGLRs



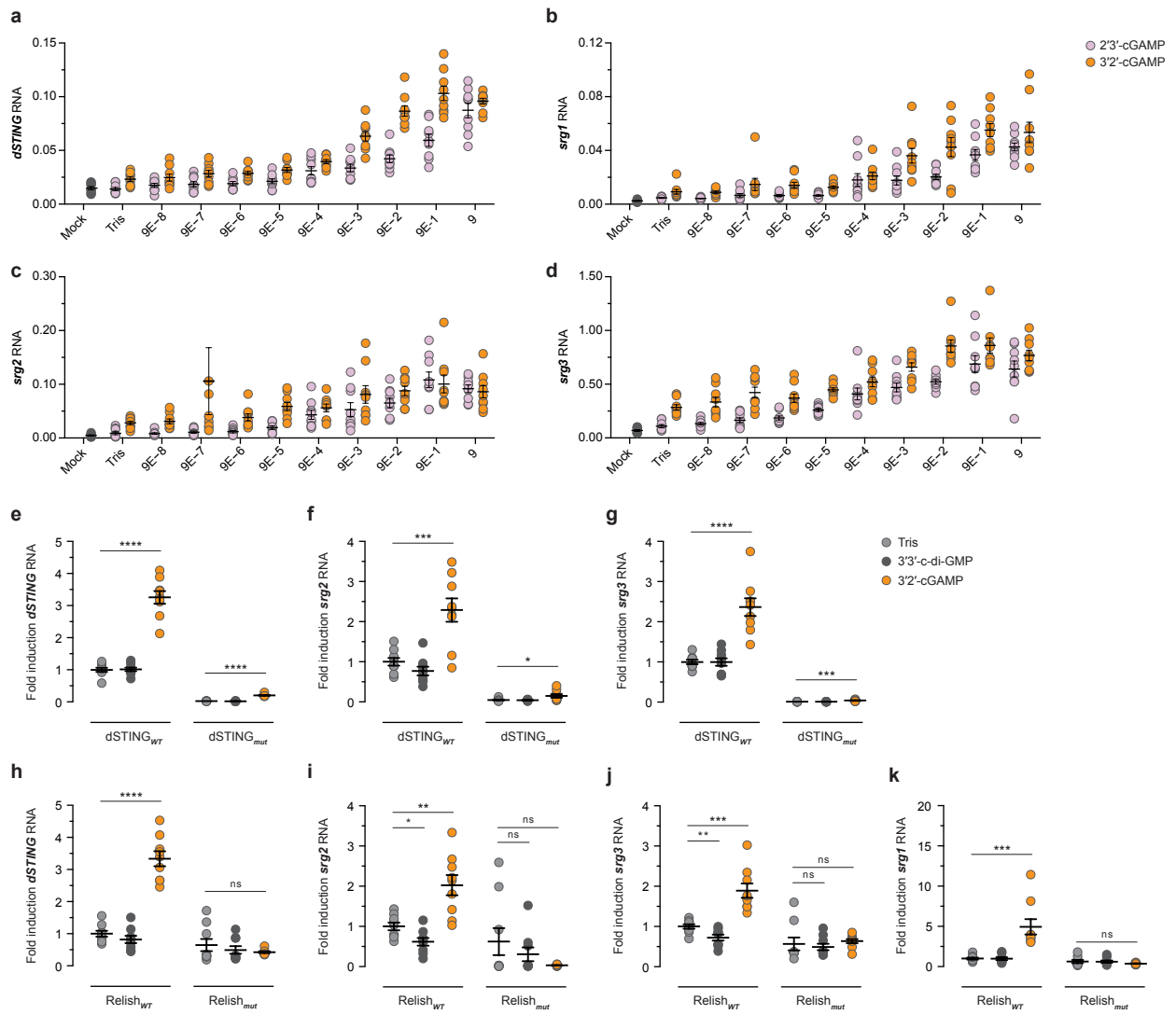
Extended Data Fig. 7 | Mechanism of 3'2'-cGAMP bond formation and resistance to degradation by viral poxin enzymes



Extended Data Fig. 8 | Structural and biochemical analysis of *Drosophila* STING



Extended Data Fig. 9 | 3'2'-cGAMP induces the expression of dSTING-regulated genes



Extended Data Fig. 10 | 3'2'-cGAMP functions as a potent antiviral ligand

