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Two cGAS-like receptors induce antiviral immunity in *Drosophila*

Andreas Holleufer^{1*}, Kasper Grønbjerg Winther^{2*}, Hans Henrik Gad^{1*}, Xianlong Ai³,
Yuqiang Chen³, Lihua Li³, Ziming Wei³, Huimin Deng³, Jiyong Liu³, Ninna Ahlmann
Frederiksen¹, Bine Simonsen¹, Line Lykke Andersen⁴, Karin Kleigrew⁵, Louise Dalskov¹,
Andreas Pichlmair^{4,6}, Hua Cai^{3**}, Jean-Luc Imler^{2,3**} and Rune Hartmann^{1**}.

Affiliations:

¹Department of Molecular Biology and Genetics, Aarhus University, Aarhus, Denmark.

²University of Strasbourg, CNRS UPR9022, Strasbourg, France.

³Sino-French Hoffmann Institute, School of Basic Medical Science, Guangzhou Medical University, Guangzhou, China.

⁴Institute of Virology, Technical University of Munich, Munich, Germany.

⁵Bavarian Center for Biomolecular Mass Spectrometry, Technical University of Munich, Freising, Germany

⁶German Center for Infection Research (DZIF), Munich partner site, Munich, Germany

* These authors contributed equally to this work.

**Corresponding authors: chjorbe@hotmail.com (H.C.); jl.imler@ibmc-cnrs.unistra.fr (J.L.I.); rh@mbg.au.dk (R.H.)

In mammals, cGAS produces the cyclic dinucleotide (CDN) 2'3'-cGAMP in response to cytosolic DNA and this triggers an antiviral immune response. cGAS belongs to a large family of cGAS/DncV-like nucleotidyltransferases, present in both prokaryotes¹ and eukaryotes²⁻⁵. In bacteria, these enzymes synthesize a range of cyclic oligonucleotide and have emerged as important regulators of phage infections⁶⁻⁸. Here, we identify two novel cGAS-like receptors (cGLRs) in the insect *Drosophila melanogaster*. We show that cGLR1 and cGLR2 activate Sting and NF-κB dependent antiviral immunity in response to infection with RNA or DNA viruses. cGLR1 is activated by dsRNA to produce the novel CDN 3'2'-cGAMP whereas cGLR2 produces a combination of 2'3'-cGAMP and 3'2' cGAMP in response to a yet unidentified stimulus. Our data establish cGAS as the founding member of a family of receptors sensing different types of nucleic acids and triggering immunity through production of CDNs beyond 2'3'-cGAMP.

Insects represent 65% of all living animal species and host a wide diversity of viruses, yet we know relatively little about how viral infections are recognized within this group of animals. The common perception of antiviral immunity in insects has so far been dominated by the extensive characterization of the RNA interference (RNAi) pathway⁹. However, transcriptional responses to virus infections have also been identified in the fruit fly *Drosophila melanogaster*, several genera of mosquitos and other insects. The Toll and immune deficiency (IMD) pathways, as well as the JAK-STAT pathway, have been proposed to participate in antiviral defenses, although the exact mechanisms are poorly described¹⁰. In addition, we and others recently reported that the signaling adapter Sting participates in antiviral immunity in *D. melanogaster* as well as the silkworm *Bombyx mori*¹¹⁻¹³. However, how virus infections are recognized to induce these pathways in insects remains unknown and so far the only identified sensor for viral nucleic acids, a hallmark of viral infection, is the DEX/DH box helicase Dicer-2, which detects double stranded (ds) RNA and activates the RNAi pathway¹⁴. In mammals, STING binds a variety of cyclic dinucleotides (CDN)^{15,16} but is most strongly activated by the second messenger 2'3'-cGAMP, which is produced by the enzyme cGAS^{2-5,17-20}. We recently showed that injection of 2'3'-cGAMP into the body cavity of adult flies induces potent Sting signaling and triggers a broad antiviral protection, suggesting that pattern recognition receptors (PRRs) with CDN synthase activity sense viral infection in flies²¹.

The *D. melanogaster* gene *CG7194* was described as a homologue of cGAS²², but so far no enzymatic activity has been described and *CG7194* deficient flies appear to have a normal

antiviral immune response²³. We identified four hitherto undescribed *D. melanogaster* gene products related to CG7194 and cGAS, of which two, CG12970 and CG30424, harbored a conserved active site compatible with CDN synthase activity (Fig. 1a). In the case of CG30424, the annotated start methionine is situated in the active site and would be incompatible with the fold of a CDN synthase. However, we identified an upstream in-frame start codon allowing for the translation of a functional CDN synthase (Extended Data Fig. 1). Interestingly, all candidates lack the characteristic Zn-finger motif as well as the secondary binding site found in cGAS, suggesting that their presumed interaction with nucleic acids must differ significantly (Extended Data Fig. 1). Thus, the *D. melanogaster* genome encodes two proteins with putative CDN synthase activity besides CG7194 and based on the data presented below, we named them cGAS-like receptor 1 and 2 (cGLR1 and cGLR2, encoded by *CG12970* and *CG30424*, respectively).

To test if the identified CDN synthases can activate signaling, we used the macrophage-like S2 cell line and a luciferase reporter system based on the promoter of the *Sting* gene since activation of *Sting* induces its own transcription¹¹. Expression of human cGAS in S2 cells led to activation of the *Sting* reporter and this activation was abolished by mutation of the active site (Fig. 1b, Extended Data Fig. 2), agreeing with our previous discovery that transfection of 2'3'-cGAMP in S2 cells triggers *Sting* signaling²¹. Expression of cGLR1 and cGLR2 resulted in significant upregulation of the *Sting* reporter, and mutation of their predicted active site abrogated this activity. Despite exhibiting the highest homology to human cGAS, CG7194 did not yield any detectable activity in this assay. We conclude that we have identified two cGAS-like enzymes that could function as PRRs sensing viral infection in *D. melanogaster*.

To confirm these results *in vivo*, we generated transgenic flies expressing wild-type or catalytically inactive mutants of cGLR1 and cGLR2 using the heat-inducible Gal4-Gal80/UAS system²⁴. We confirmed that both genes were overexpressed (Extended Data Fig. 3a, b) at 29°C and that this did not have a major effect on the viability of the flies (Extended Data Fig. 3e). Expression levels of *Sting* and the *Sting-regulated genes* (*Srg*) 1, 2, and 3²¹ were all significantly upregulated in flies overexpressing wild-type cGLR2 compared to the catalytically inactive mutant (Fig. 1c, d and Extended Data Fig. 3c, d). Similarly, overexpression of cGLR1 led to a significant upregulation of *Sting*, *Srg2*, and *Srg3*, whereas the trend observed for *Srg1* did not reach significance. Finally, overexpression of cGLR1 and cGLR2 led to a significant reduction of vesicular stomatitis virus (VSV) replication compared to the catalytically inactive mutants (Fig. 1e). Overexpression of cGLR2 also reduced

replication of *Drosophila* C virus (DCV), a natural *Drosophila* pathogen, whereas the slight reduction observed with cGLR1 was not significant for this virus (Fig. 1f). However, transgenic flies expressing the wild-type versions of cGLR1 and cGLR2, but not the catalytically inactive mutants, exhibited a striking increase in survival following infection with DCV (Fig. 1g). We conclude that cGLR1 and cGLR2 can initiate a transcriptional response *in vivo*, associated with protection against virus infection.

To test if signaling by cGLR1 and cGLR2 depends upon Sting, we introduced a frameshift deletion in the *Sting* gene of S2 cells (Extended Data Fig. 4d, e). Activation of the Sting reporter by cGLR1, cGLR2 and cGAS was abolished in the knockout cells but was rescued when co-transfecting with Sting (Extended Data Fig. 4a, b). Thus, Sting is required for cGLR1- and cGLR2-mediated signaling. Sting drives the expression of antiviral genes through Relish, a member of the NF- κ B family of transcription factors^{11,21}. Mutation of the Relish binding site within the *Sting* promoter abolished the response to cGLR1 and cGLR2, connecting these receptors to NF- κ B-dependent transcription (Extended Data Fig. 4c). This is in contrast to the mammalian system, where members of the interferon regulatory factor (IRF) family of transcription factors mediate most of the antiviral effect^{25,26}, whereas NF- κ B is thought to primarily drive expression of pro-inflammatory cytokines.

To show that cGLR1 and cGLR2 are required for initiating an antiviral immune response in flies, we generated cGLR1 or cGLR2 knockout (KO) flies by CRISPR/Cas9 mutagenesis (Extended Data Fig. 5). Next, we generated cGLR1 and cGLR2 double KO flies by recombination (denoted cGLR1/2 KO). All flies were viable and resisted the stress of a buffer injection (Extended Data Fig. 6a). Upon infection with DCV cGLR1 KO but not cGLR2 KO flies exhibited reduced survival whereas the cGLR1/2 KO flies had a significantly lower survival than either of the two single KO flies (Fig. 2a). In agreement with the survival data, we observed a trend for increased DCV replication in cGLR1 KO flies compared to control and cGLR2 KO flies, which became statistically significant in the cGLR1/2 double KO flies (Fig. 2c). Finally, expression of Sting-regulated genes was reduced in both cGLR1 KO and cGLR1/2 KO flies (Fig. 2d and Extended Data Fig 6b, c, d). Next, we infected both cGLR1 and cGLR2 KO flies with Kallithea virus (KV), a large DNA virus and also a natural *Drosophila* pathogen, and observed reduced survival in both KO flies (Fig. 2b and Extended Data Fig. 7a). However, this was not accompanied by a significant increase in viral replication in either KO flies (Fig. 2e). As previously reported²⁷, KV did induce expression of Sting-regulated genes and this induction was lost in cGLR1 KO flies. Intriguingly, the

induction of *Srg3*, but not *Sting*, *Srg1*, and *Srg2*, was affected in cGLR2 KO flies (Fig. 2f and Extended Data Fig 7b, c, d). Similar to what was observed for DCV, cGLR1/2 KO flies exhibited a more severe phenotype upon KV infection than single KO flies (Extended Data Fig. 7e, f, g). We also tested infection by VSV and invertebrate iridescent virus 6 (IIV6), but neither of them showed any overt phenotype for survival and viral load (Extended Data Fig. 8 and 9). However, we observed that one of the marker genes, *Srg3*, was induced by VSV and IIV6 infection in control and cGLR1 KO flies, but not in the cGLR2 KO flies (Extended Data Fig. 8g and 9g). We conclude that both cGLR1 and cGLR2 are required for survival of flies upon infection with two natural viral pathogens of *Drosophila*, DCV and KV.

Next, we expressed cGLR1, cGLR2 or cGAS alone or together with human STING in the human cell line HEK293T, which lacks endogenous expression of STING. Here, cGLR2 induced signaling via STING to a level comparable to that of cGAS (Fig. 3a and Extended Data Fig. 10a). Furthermore, this depended upon the active site of cGLR2, suggesting that cGLR2 produces a CDN capable of activating STING (Extended Data Fig. 10b, c). In contrast, cGLR1 did not activate STING signaling in HEK293T cells, (Fig. 3a and Extended Data Fig. 10a). To identify the CDN synthesized by cGLR2, we isolated the nucleotide-containing fraction from HEK293T cells expressing cGLR2 and then subjected this extract to analysis by mass spectrometry. Interestingly, Slavik *et al.* demonstrate that cGLR1 produces 3'2'-cGAMP²⁸. We therefore established a protocol to detect both 2'3'-cGAMP and 3'2'-cGAMP, which revealed that the two CDNs are synthesized in approximately equivalent amounts by cGLR2 in HEK293T cells (Fig. 3b and Extended Data Fig. 10d, e, f). Both 2'3'-cGAMP and 3'2'-cGAMP induced expression of *Sting* and *Srg3* when introduced into S2 cells. The 2'3'-cGAMP mediated induction was lower than that of 3'2'-cGAMP but the difference was not statistically significant. The two CDNs also activated endogenous STING in the human cell line HT-1080, with 2'3'-cGAMP appearing the most potent activator (Extended Data Fig. 10g, h, i). Therefore, we hypothesize that the absence of activity by cGLR1 in HEK293T cells is caused by the absence of a suitable activator. The cGLRs share similarity with oligoadenylate synthetases, a family of proteins activated by dsRNA, and thus we transfected HEK293T cells first with cGLR1 and then poly(I:C), which led to activation of cGLR1 (Fig. 3c). To confirm that cGLR1 is indeed activated by dsRNA, we expressed the protein in *E. coli*, purified it and then performed an *in vitro* activity assay as described by Kranzusch and colleagues²⁸. This showed that cGLR1 produces 3'2'-cGAMP and minor amounts of 2'3'-c-diAMP upon stimulation with either poly(I:C) or a 100 base pair long

dsRNA produced by T7 transcription, but not with a similar 100 base pair long dsDNA (Fig. 3d and Extended Data Fig. 11a, b). We also attempted to purify cGLR2 but only achieved poor quality protein preparations that did not display any activity.

Since cGLR2 is constitutively active when expressed in both HEK293T cell and S2 cells, we assume that these cells produce a suitable activator. When performing transfections, the transfected DNA can act as a ligand activating cGAS and potentially cGLR2. To circumvent this issue, we generated HEK293T cells stably expressing either cGAS or cGLR2 by retroviral transduction. cGAS only produced 2'3'-cGAMP upon transfection with DNA (Fig. 3e). However, cGLR2 produced both 3'2'-cGAMP and 2'3'-cGAMP, regardless of the presence of cytosolic DNA supplied by transfection. Thus, we conclude that the ligand activating cGLR2 is not dsDNA. As demonstrated above, cGLR1 is inactive in HEK293T cells unless dsRNA is provided by transfection, indicating that dsRNA is not present in our HEK293T cells. Thus, the activity of cGLR2 in HEK293T cells cannot be explained by the presence of either dsRNA or dsDNA. It is possible that cGLR2 is constitutively active and to address this, we mutated residues in the putative nucleic acid-binding domain (K256E/R267E and K256E/R271E). Those residues are conserved in cGAS and oligoadenylate synthetases (OAS) and are known to be critical for DNA- and RNA-induced activation of these enzymes^{3,29}. Mutation of those residues led to significant loss of activity in S2 cells and a complete loss of activity in HEK293T cells (Fig. 3f, Extended Data Fig. 11c, d). In summary, our data suggest that cGLR2 requires allosteric activation by a nucleic acid.

To conclude, we have identified two novel PRRs, cGLR1 and cGLR2, which orchestrate a Sting and NF- κ B dependent antiviral immune response in *D. melanogaster*. Several primordial functions of Sting have been suggested, including sensing of bacteria through recognition of bacterially derived CDNs²³ or autophagy^{12,30}. Our data suggest that a cGLR-Sting-NF- κ B axis was present already during early metazoan evolution. They further reveal that the production of CDNs by metazoan enzymes is not limited to 2'3'-cGAMP. Different CDNs could lead to diverse signaling outcomes, interact with alternative receptors³¹ or have different stability towards degradation by both host and viral encoded degrading enzymes, e.g. poxins³²⁻³⁴. Future studies are needed to determine the biological role of different cGAMP isomers.

The cGLRs are the first reported class of antiviral PRRs responsible for inducing a transcriptional response in insects. One striking difference between the cGLRs and cGAS is the specificity in activation. Whereas cGAS contains a Zn-finger motif inserted early in

vertebrate evolution, which confers specificity for DNA, the cGLRs have a nucleic acid-binding groove more akin to OAS. We show that cGLR1 is activated by dsRNA and this is consistent with our *in vivo* data, which reveal a strong susceptibility of cGLR1 mutant flies to DCV, a (+)ssRNA virus known to produce large amounts of long dsRNAs. Interestingly, our data suggest that cGLR2 is activated by a yet unidentified ligand. Both receptors may recognize different characteristics of the virally derived RNA, in a manner conceptually similar to the receptors RIG-I and MDA5 in mammals. In addition, we note that the expression of cGLR2 appears to be tightly regulated, with several splice isoforms differentially expressed (flybase.org/reports/FBgn0050424) and poor protein stability in S2 but not HEK293T cells, suggesting alternative modes of regulation of this receptor.

We speculate that the cGLR class of PRRs is not only limited to cGAS, cGLR1, and cGLR2 but might have expanded even further in metazoans. In particular, we cannot rule out that the previously identified cGAS candidate CG7194 is a third cGLR in *D. melanogaster* and that the missing activity is due to a lack of either the correct activator or a *bona fide* readout. Finally, the human genome harbors at least one uncharacterized gene, which shows similarity to cGAS and has a sequence compatible with a functional active site, making it a potential cGLR³⁵.

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Competing interests. The authors declare that they have no competing interests.

Methods

Alignment of cGAS-like proteins

The five cGAS-like candidate proteins from *D. melanogaster*, CG12970 (UniProt: A1ZA55), CG30424 (UniProt: A8DYP7), CG4746 (UniProt: Q9U3W6), CG4766 (UniProt: Q9Y106), and CG7194 (UniProt: Q9VSH0) were aligned with porcine OAS1 (UniProt: Q29599), human OAS1 (UniProt: P00973-1), human cGAS (UniProt: Q8N884-1), porcine cGAS (UniProt: I3LM39), murine cGAS (UniProt: Q8BSY1) using CLC Main Workbench 7.7.2 (QIAGEN). Structurally homologous secondary structure elements of the mammalian nucleotidyltransferases were used as alignment fix points.

Cell lines

HEK293T and HT-1080 cells were obtained from ATCC (CRL3216) and the German Collection of Microorganisms and Cell Cultures (ACC 315), respectively, whereas HEK293T/MAVS KO cells were a kind gift from Veit Hornung (Gene Center Munich). All mammalian cells were cultured in Dulbecco's modified Eagle medium (DMEM) (Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS) (Biowest), 100 U ml⁻¹ penicillin (Sigma-Aldrich) and 100 µg ml⁻¹ streptomycin (Sigma-Aldrich) at 37° C and 5% CO₂. Schneider 2 (S2) cells were obtained from the German Collection of Microorganisms and Cell Cultures (ACC 130) and cultured in Schneider's Drosophila Medium (Biowest) supplemented with 10% FBS, 100 U ml⁻¹ penicillin (Sigma-Aldrich) and 100 µg ml⁻¹ streptomycin (Sigma-Aldrich) at 27 °C.

Fly lines

Fly stocks were raised on standard cornmeal agar medium at 25°C. All fly lines used in this study were free of Wolbachia. The drivers used were [*actin5C*-Gal4/CyO; *tubulin*-

Gal80^{ts}/TM6,Tb]. Transgenic lines for expression of GFP and β -galactosidase (P{UAS-GFP.nls}8 line (BDSC #4776) and P{UAS-LacZ.Exel}2 (BDSC #8529)) were obtained from the Bloomington Drosophila Stock Center. For the UAS transgenic lines of wild-type and AFA mutant versions of cGLR1 and cGLR2, the corresponding versions of the cDNAs (RD isoform in the case of cGLR2) were cloned into pUAST-attB vectors. The resulting plasmids were injected into embryos of [*y^l M{vas-int.Dm}ZH-2A w*; M{3xP3-RFP-attP}ZH-86Fb*] flies (BDSC #24749). Individual males from the injected embryos were then crossed with flies containing the third chromosome balancers *TM6B,Tb/TM3,Sb* to establish balanced stocks. *cGLR1* and *cGLR2* knockout flies were generated by CRISPR-Cas mediated mutagenesis. Briefly, sgRNA encoding pUAST-attB plasmids were injected into embryos of *y^l M{vas-int.Dm}ZH-2A w*; M{3xP3-RFP.attP}ZH-86Fb* (BDSC#24749) flies. The resulting transgenic flies were then crossed with *y^l w¹¹¹⁸; attP2{nos-Cas9}/TM6C, Sb Tb [y+]* flies obtained from the National Institute of Genetics. Individual males from the F1 were then crossed with *yw; Bc,Gla/CyO* flies to establish stocks from the *CyO* progeny. This progeny was then scored for mutations by Sanger sequencing (Guangzhou IGE Biotechnology). *cGLR1* (2R: 52C8) and *cGLR2* (2R: 60E11) knockout flies were isogenized to the DrosDel *w¹¹¹⁸* isogenic background to reduce the genetic background effects. For each line, the non-mutated chromosomes were replaced using balancer chromosomes (*w¹¹¹⁸; If/CyO; TM3/TM6* and *w¹¹¹⁸; If/CyO*) whereas the mutations were recombined to the respective DrosDel *w¹¹¹⁸* isogenic chromosome for seven generations. We confirmed that the isogenized lines retained the mutation of interest by DNA sequencing. *cGLR1* and *cGLR2* double knockout flies were generated by crossing the two single knockout flies. Females from the progeny were crossed with *w¹¹¹⁸; If/CyO* to establish the stocks from the *CyO* progeny. Presence of the double mutation upon recombination on the right arm of the second chromosome in this progeny was established by DNA sequencing. All the crossing schemes and detailed injection protocols are available upon request.

Plasmids

cDNAs encoding human cGAS (amino acids 155-522), cGLR1, cGLR2 or CG7194 with a C-terminal V5- or triple FLAG-tags were cloned into the pAc5.1 vector for transient expression in S2 cells, the pcDNA3.1 vector for transient expression in HEK293T cells or the pCCL-PGK vector for production of lentiviral particles. For expression of cGLR1 in *E. coli*, the cDNA was cloned into the pET-9d vector in between an N-terminal TEV protease cleavage

site and maltose-binding protein (MBP) and a C-terminal polyhistidine-tag. The following active site mutants were generated by site-directed mutagenesis: cGAS AFA (E225A D227A), cGLR1 AFA (E71A D73A), cGLR2 AFA (E79A D81A), and CG7194 AFA (E70A D72A). cDNAs encoding human or *D. melanogaster* STING/Sting without or with a C-terminal V5-tag were cloned into the pcDNA3.1 and pAc5.1 vector, respectively. Plasmids expressing firefly luciferase under transcriptional control of the proximal 200 base pairs of the *D. melanogaster* *Sting* promoter or a mutated version of the promoter containing two point mutations in the Relish binding site have previously been described and so has the plasmid constitutively expressing *Renilla* luciferase in S2 cells¹¹. Plasmids expressing firefly luciferase under transcriptional control of the proximal 200 base pairs of the human *IFNBI* promoter or constitutively expressing *Renilla* luciferase in HEK293T cells have also been previously described³⁶.

Transfection of S2 cells

To test induction of *D. melanogaster* Sting by cGLRs, 12-well tissue culture plates were seeded with 6×10^5 S2 cells per well. After 24 h, each well was transfected with 485 ng pGL3 plasmid expressing firefly luciferase under transcriptional control of the *Sting* promoter, 15 ng pAc5.1 plasmid constitutively expressing *Renilla* luciferase, 400 ng pAc5.1 plasmid expressing cGAS, cGLR1, cGLR2 or CG7194 or their corresponding mutants (except Extended Data Fig. 10b where 10 ng was used) and finally empty Ac5.1 plasmid to reach a total amount of 1 μ g plasmid per well. To test if the activity of cGLRs is Sting-dependent, 12-well tissue culture plates were seeded with 6×10^5 S2 or Sting knockout S2 cells per well. After 24 h, each well was transfected with 485 ng pGL3 plasmid expressing firefly luciferase under transcriptional control of the *Sting* promoter, 15 ng pAc5.1 plasmid constitutively expressing *Renilla* luciferase, 30 ng pAc5.1 plasmid expressing Sting, 100 ng pAc5.1 plasmid expressing cGAS, cGLR1, cGLR2 or CG7194 and finally Ac5.1 plasmid to reach a total amount of 1 μ g plasmid per well. All transfections of S2 cells were performed using jetOPTIMUS (Polyplus Transfection) according to the manufacturer's instructions.

Transfections of HEK293T cells

To test induction of human STING by cGLRs, 12-well tissue culture plates were seeded with 3×10^5 HEK293T cells per well. After 24 h, each well was transfected with 970 ng pGL3 plasmid expressing firefly luciferase under transcriptional control of the *IFNBI* promoter, 30 ng pRL plasmid constitutively expressing *Renilla* luciferase, 100 ng pcDNA3.1 plasmid

expressing STING, 400 ng pcDNA3.1 plasmid expressing cGAS, cGLR1, cGLR2 or CG7194 and finally empty pcDNA3.1 plasmid to reach a total amount of 2 µg plasmid per well. For each well, the DNA was dissolved in 100 µl DMEM and likewise 6 µg polyethylenimine (PEI)(Polysciences) was dissolved in 100 µl DMEM. The DNA and PEI was then mixed and incubated for 20 min before adding it dropwise to the cells.

To test activation of cGLR1 by poly(I:C), 48-well tissue culture plates were seeded with 9 x 10⁴ HEK293T/MAVS KO cells per well. After 24 h, each well was transfected with 242.5 ng pGL3 plasmid expressing firefly luciferase under transcriptional control of the *IFNBI* promoter, 7.5 ng pRL plasmid constitutively expressing *Renilla* luciferase, 10 ng pcDNA3.1 plasmid expressing STING, 240 ng pcDNA3.1 plasmid expressing cGLR1 and finally empty pcDNA3.1 plasmid to reach a total amount of 500 ng plasmid per well. After another 3 h, cells were transfected 150 ng VacciGrade™ Poly(I:C)(InvivoGen) per well. Both kind of transfection were done using Lipofectamine 2000 (Fisher Scientific) following to the manufacturer's instructions.

To test production of CDNs by cGLR2 via mass spectrometry, HEK293T cells were grown in T175 tissue culture flasks and transfected at a confluence of 60-70%. For each flask 87.5 µg pcDNA3.1 plasmid expressing EGFP, cGAS or cGLR2 was dissolved in 4.4 ml DMEM and 262.5 µg PEI were dissolved in 4.4 ml DMEM. The DNA and PEI was then mixed and incubated for 20 min before added to the cells.

Transduction of HEK293T cells

Lentiviral particles were made as previously described³⁷. For transduction of HEK293T cells, the cells were grown in T75 tissue culture flasks and transduced at a confluence of 60-70% with filtered supernatants. The cells were then passaged for 1 week before being subjected to further experiments. Transfection of transduced cells was performed using pcDNA3.1 plasmid expressing EGFP as described above.

Measuring luciferase activity of transfected cells

Cells were lysed in 200 µl Passive Lysis Buffer (Promega) per well and afterwards the lysates were centrifuged at 14,000g for 5 min to remove cell debris. Firefly and *Renilla* luciferase activity was measured on 10 µl lysate using the Dual-Luciferase® Reporter Assay System (Promega).

Immunoblotting

Cell lysates were subjected to SDS-PAGE and proteins were transferred to a polyvinylidene fluoride membrane. Blotted proteins were detected with Anti-V5 Antibody (Invitrogen)(diluted 1:5,000), Anti-Actin, clone 4 (EMD Millipore)(diluted 1:5,000), Anti-Sting (BioGenes)(1:500), Monoclonal ANTI-FLAG® M2 (Sigma-Aldrich)(diluted 1:2,000) or STING (D2P2F) Rabbit mAb (Cell Signaling Technology)(diluted 1:1,000) primary antibody and a Peroxidase-conjugated AffiniPure F(ab')₂ Fragment Donkey Anti-Mouse IgG (JacksonImmunoResearch)(diluted 1:15,000) or Anti-Rabbit IgG, HRP-linked Antibody (Cell Signalling Technology)(diluted 1:3,000) secondary antibody. Blots were developed with SuperSignal™ West Dura Extended Duration Substrate. The Anti-Sting antibody was raised in rabbit against the peptide HMQNKTKTIDEISN. The antibody was affinity purified from serum using the peptide and verified by immunoblotting of recombinant Sting made in *Escherichia coli*.

Generation of Sting knockout cells by CRISPR-Cas9

Three CRISPR RNAs targeting the first translated exon in the *D. melanogaster* *Sting* gene were cloned into the pAc-sgRNA-Casp vector. After transfection, S2 cells were grown under selection with 5 µg ml⁻¹ puromycin for two weeks. Individual clones were isolated by limiting dilution and then screened for a non-functional Sting pathway. Knockout of Sting was verified by immunoblotting and Sanger sequencing.

Mass spectrometry

Transfected HEK293T cells were washed with PBS, trypsinized and resuspended in DMEM before being harvested by centrifugation at 800g for 5 min. The medium was carefully removed and the cells resuspended in PBS before being collected by centrifugation at 800g for 5 min. After another final washing step, the cells were lysed with 500 µL 60% MeOH and vortexed for 15 seconds. For the spike in samples, 20 nmol of either 2'3'-cGAMP (Invivogen) or 3'2'-cGAMP (BioLog) were added to lysates from GFP transfected cells. The lysates were frozen in liquid nitrogen and stored at -80°C for further analysis. 20 nmol ATP-γ-S (Jena Bioscience NU-506-5) were added to all lysates and nucleotides were purified from the lysates as previously described³⁸ and then analyzed by means of LC-MS/MS with a 5500 QTrap (AB Sciex, Darmstadt, Germany) and a ExionLC AD UPLC (AB Sciex). For chromatographic separation, a Xbridge Amid 3.5 µm, 150 x 2.1 mm column (Waters) was used with acetonitrile/water with 5 mM ammonium acetate (50/50, v/v, pH 9.5) as solvent A and acetonitrile/water with 5 mM ammonium acetate (95/5, v/v, pH 9.5) as solvent B. A

linear gradient from 97% B to 50% B in 16 min was used. Afterward the column was flushed and equilibrated to starting conditions. The separation of 2'3'-cGAMP, 3'2'-cGAMP and ATP- γ -S was performed using a 400 μ L min⁻¹ flowrate at 40°C column oven temperature. Ions were analyzed by mass spectrometry in the negative ionization mode. The spray voltage was set to -4,500 V at a source temperature of 450°C using nitrogen as collision gas. The parameters for the collision-activated dissociation (CAD) were: medium, curtain gas: 35 psi, ion source gas 1: 55 psi, ion source gas 2: 65 psi, entrance potential (EP) -10 V and the dwell time 150 msec. The MRM (multiple reaction monitoring) transition for each compound were as follow: cGAMP: 672.987 $\rightarrow$ 79.1 (quantifier), declustering potential (DP) -205 V, collision energy (CE) -160 V, Cell Exit Potential (CXP) -5 V; 672.987 $\rightarrow$ 150.0 (qualifier), DP -205V, CE -52 V, CXP -7 V; ATP- γ -S: 521.70 $\rightarrow$ 78.8 (quantifier), DP -115 V, CE -128 V, CXP -11 V; 521.70 $\rightarrow$ 426.0 (qualifier), DP -115 V, CE -32 V, CXP -17 V. Analyst 1.7. was used to acquire the data and MultiQuant 3.0.3 was used to analyze the data (both AB Sciex).

Expression and purification of recombinant protein

E. coli BL21 (DE3) cells transformed with a pET-9d plasmid encoding cGLR1 were grown at 37 °C in lysogenic broth medium containing 30 μ g/ml kanamycin until the culture reached an OD₆₀₀ of 0.4–0.5. Protein expression was then induced with 1 mM isopropyl-b-d-thiogalactopyranoside and the cells were incubated for another 12 h at 16 °C. Afterwards, the cells were harvested by centrifugation, resuspended in Lysis Buffer (50 mM NaH₂PO₄, 500 mM NaCl, 10% glycerol, 5 mM β -mercaptoethanol, 1 mM PMSF, 200 μ g ml⁻¹ lysozyme, pH 7.4) and lysed by sonication. Following lysis, cell debris was removed by centrifugation at 18,500 ref for 60 min after which the supernatant was incubated with HisPur Ni-NTA Resin (ThermoFisher Scientific) at 4 °C for 1 h while continuously shaking. The resin was then transferred to a gravity flow column and washed with 20 column volumes (CV) of Wash Buffer (50 mM NaH₂PO₄, 200 mM NaCl, 2 M urea, 10% glycerol, 5 mM β -mercaptoethanol, pH 7.4) before eluting the protein with 5 CV of Elution Buffer (25 mM HEPES, 500 mM NaCl, 500 mM imidazole, 10% glycerol, 5 mM β -mercaptoethanol, pH 7.4).). The eluate was diluted 2.5 times with Dilution Buffer (25 mM HEPES, 200 mM KCl, 5% glycerol, 5 mM dithiothreitol (DTT), pH 7.4) and then applied to a MBPTrap 1 ml column (Cytiva). Bound protein was eluted with 5 CV Elution Buffer (20 mM HEPES, 200 mM KCl, 10% glycerol, 10 mM maltose, pH 7.4) and TEV protease (one twentieth of the total protein concentration) was added before dialysis overnight at 4°C against Dialysis Buffer (25 mM

Tris, 200 mM KCl, 5% glycerol, 5 mM DTT, pH 7.4). Finally, the protein was aliquoted and flash frozen in liquid nitrogen before being stored at -80°C .

***In vitro* activity assay**

cGRL1 activity was measured in a 50 μl reaction volume containing 100 $\mu\text{g/ml}$ recombinant cGRL1, 240 μM ATP, 240 μM GTP, 1 mM MnCl_2 , 1 mM MgCl_2 , 120 mM KCl and 2.5 μg poly(I:C) (GE Healthcare), dsRNA (100 bp) or dsDNA (100 bp). The dsRNA was made as previously described³⁹ whereas the dsDNA was made by purchasing complementary sense and antisense ssDNAs (Sigma-Aldrich) with sequences corresponding to the dsRNA and then annealing them. The reactions were incubated at 27°C for 4 h and then terminated at 95°C for 15 min. To digest non-cyclic nucleotides, the reactions were afterwards incubated with 10 U alkaline phosphatase (Roche) at 37°C overnight and again terminated at 95°C for 15 min. The terminated reactions were applied to anion exchange chromatography as previously described⁴⁰ and 3',2'-cGAMP was quantified by integrating the corresponding peaks on the chromatogram using the software Unicorn 5.10 (GE Healthcare) with default settings.

Treatment with CDNs

12-well tissue culture plates were seeded with 3×10^5 HT-1080 or 3×10^6 S2 cells per well. After 20 h, the medium was removed and 800 μl digitonin buffer (100 mM KCl, 85 mM sucrose, 50 mM HEPES, 3 mM MgCl_2 , 1 mM ATP, 0.1 mM GTP, 0.1 mM DTT, 0.2 % bovine serum albumin, pH 7.0) with or without 5 μM 2',3'-cGAMP or 3',2'-cGAMP (Biolog Life Science Institute) was added to the wells. The cells were incubated for 10 min before the digitonin buffer was removed and replaced with normal medium.

Ectopic expression of cGLRs in transgenic flies

cGLRs were expressed in transgenic flies using the heat-controlled Gal4/Gal80 system. Briefly, the UAS-Gal4>cGLR transgenic flies were crossed with the driver line [*actin5C-Gal4/CyO*; *tubulin-Gal80^{ts}/TM6,Tb*] at 25°C . Males from F1 were collected and shifted to 29°C for 5 days to block the repressive function of Gal80 to Gal4 and drive the expression of the transgenes.

Viral infections

Viral stocks were prepared in 10 mM Tris-HCl, pH 7.5. Infections were performed with adult flies by intrathoracic injection (Nanoject II apparatus, Drummond Scientific) with 4.6 nL of

DCV (5 (survival) or 500 (gene induction and viral load) PFU/fly), KV (10,000 ID⁵⁰/fly), VSV (5,000 PFU/fly) or IIV6 (5,000 PFU/fly) viral suspension.

RNA extraction from cells and flies and quantification of gene expression by qPCR

RNA was extracted, cDNA was synthesized, and gene expression was quantified by qPCR as previously described^{21,37}. Gene expression was analysed using the CFX Maestro Software (Bio-Rad).

Statistical analyses

All statistical analyses were done using GraphPad Prism 9.0.1 (GraphPad Software). Comparisons between groups were analysed using two-way ANOVA and Holm Holm-Šidák post hoc test, except for comparison of viral RNA load and survival of flies. For comparison of viral load, data were log transformed and then analyzed using one-way ANOVA and Dunnett T3 post hoc test whereas survival of flies was compared using log-rank test. Sample sizes (n) for each experiment are stated in the figure legends.

Data Availability Statement

The authors declare that the data supporting the findings of this study are available within the paper. The sequences and structures used in this study are CG12970 (UniProt: A1ZA55), CG30424 (UniProt: A8DYP7), CG4746 (UniProt: Q9U3W6), CG4766 (UniProt: Q9Y106), CG7194 (UniProt: Q9VSH0), porcine OAS1 (UniProt: Q29599), human OAS1 (UniProt: P00973-1), human cGAS (UniProt: Q8N884-1), porcine cGAS (UniProt: I3LM39), murine cGAS (UniProt: Q8BSY1), and murine cGAS in complex with DNA and a cGAMP intermediate analog (PDB: 4K98).

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Fig. 1 | cGRL1 and cGRL2 activate the Sting pathway and protect against viral infection. **a**, Truncated alignment of identified cGAS-like candidates, mammalian cGAS and the structurally related OAS1. Essential active site residues (the GS/GG duplet and the metal ion coordinating acidic residues) are indicated above the alignment. **b**, *Sting* reporter activity in S2 cells transfected with cGAS-like candidates or their corresponding active site mutants (AFA). **c**, **d**, Expression of *Sting* (**c**) and *SrgI* (**d**) was monitored by RT-qPCR in transgenic flies ectopically expressing wild-type or mutant cGLRs. In **b**, **c**, **d**, data are from three independent experiments (blue, red, and green, each performed in triplicate) and shown with mean ($n = 9$). **e**, **f**, VSV (**e**) or DCV (**f**) viral RNA load measured at 4 or 3 days, respectively, post infection in transgenic flies ectopically expressing wild-type or mutant cGLRs. Data are from three independent experiments, each performed in triplicates, and shown with mean ($n = 9$). **g**, Flies expressing the indicated transgenes were injected with DCV and survival was monitored daily. Data are from 3 independent experiments, each with 30 flies ($n = 90$). In **b**, **c**, and **d**, data were analyzed using two-way ANOVA and Holm-Šidák post hoc test and compared to mock (**a**) or relevant AFA mutants (**b**, **c**, **d**). In **e**, and **f**, Log transformed data were analyzed using one-way ANOVA and Dunnett T3 post hoc test and compared to relevant AFA mutants. In **g**, log-rank test was used to compare cGRL1 vs. cGRL1 AFA ($P < 0.0001$) and cGRL2 vs. cGRL2 AFA ($P < 0.0001$). ns not significant, ** $P < 0.01$, **** $P < 0.0001$.

Fig. 2 | Loss of cGLR1 or cGLR2 leads to an impaired antiviral immune response *in vivo*. **a, b,** w^{1118} , cGLR1 KO, cGLR2 KO or cGLR1/2 KO flies were injected with DCV and survival was monitored daily. Data are from four independent experiments, each with three independent groups of around 10 flies. **b,** w^{1118} , cGLR1 KO or cGLR2 KO flies were injected with KV and survival was monitored daily. Data are from three independent experiments, each with three independent groups of around 10 flies. **c, d,** w^{1118} , cGLR1 KO, cGLR2 KO or cGLR1/2 KO flies were injected with DCV or Tris and viral load (**c**) as well as expression of *Srg1* (**d**) were monitored by RT-qPCR at 2 and 3 days post-injection (dpi). **e, f,** w^{1118} , cGLR1 KO or cGLR2 KO flies were injected with KV or Tris and viral load (**e**) as well as expression of *Srg1* (**f**) were monitored by RT-qPCR at 5 and 10 days post-infection (dpi). For panels c-f, expression was normalized to the housekeeping gene *RpL32* and data are from three independent experiments, each performed in triplicates ($n = 9$). In **a** and **b**, log-rank test was used to compare the survival curves pairwise followed by a Holm-Šidák multiple comparison correction. In **c** and **e**, Log transformed data were analyzed using one-way ANOVA and Dunnett T3 post hoc test and compared to w^{1118} flies. In **d** and **f**, data were analyzed using two-way ANOVA and Holm-Šidák post hoc test and compared to w^{1118} flies. ns not significant, ** $P < 0.01$. *** $P < 0.001$. **** $P < 0.0001$.

Fig. 3 | cGRL1 is activated by dsRNA whereas cGRL2 is activated by an unidentified molecule. **a**, *IFNB1* reporter activity in HEK293T cells transfected with cGAS-like candidates and human STING. Data are from three independent experiments (blue, red, and green, each performed in triplicates) and are shown with mean (n = 9). **b**, Quantification by mass spectrometry of 2'3'-cGAMP or 3'2'-cGAMP production by HEK293T cells transfected with GFP, cGAS or cGRL2. Data are from four independent experiments and shown as mean $\pm$ s.e.m. (n = 4). **c**, *IFNB1* reporter activity in HEK293T/MAVS KO cells transfected first with cGRL1 and human STING and afterwards with poly(I:C). Data are from two independent experiments (blue and red, each performed in triplicates) and are shown with mean (n = 9). **d**, Quantification by anion exchange chromatography analysis of 3'2'-cGAMP production by recombinant cGRL1 in the presence of different nucleic acids. Data are from three independent experiments and shown as mean $\pm$ s.e.m. (n = 3). **e**, Quantification by mass spectrometry of 2'3'-cGAMP or 3'2'-cGAMP production by HEK293T cells transduced with GFP, cGAS, cGRL1 or cGRL2 and subsequently transfected with DNA. Data are from two independent experiments (n = 2). **f**, *IFNB1* reporter activity in HEK293T cells transfected with wild-type cGRL2 or the indicated mutants and human STING. Data are from three independent experiments (blue, red, and green, each performed in triplicates) and are shown with mean (n = 9). In **a**, **c** and **f**, data were analyzed using two-way ANOVA and Holm-Šidák post hoc test and compared to mock (**a**) or as indicated (**c**, **f**). nd not detected; ns not significant, **** $P < 0.0001$.

Extended Data Fig. 1 | Alignment of mammalian cGAS/OAS1 proteins and cGAS-like proteins from *D. melanogaster*

a

<i>S. scrofa</i> OAS1	1	-----MEIRHTPARLDKFTEDHLLPNTCF-----RTQVKEATDIYCRFLKERCFQGTADPVRVSKVVKGGSSGKGTTLGRSDADLVV	
<i>H. sapiens</i> OAS1	1	-----MMDLRNTPAKSLDKFTEDYLLPDTCF-----RMOINHADTICGFLKERCFRGSSYPVCVSKVVKGGSSGKGTTLGRSDADLVV	
<i>H. sapiens</i> cGAS	147	PVSAPILVRRDAAPGASKLRAVLEKLLSRDD-----ISTAAGMVKGVVDHLLLR-LKC-DSAFRGVGLLNTGSYVEHVKISAPNEFDVMF	
<i>S. scrofa</i> cGAS	129	-----RMEAPPGAWLQTVLEKVLRSRHE-----ISEAAEYVNVVVEHLLRR-LQGGSEFEKGVALLRTGSYVEHVKISAPNEFDVMF	
<i>M. musculus</i> cGAS	149	-----KLKKVLDKLRLLKRRD-----ISEAAEYVNVVVEHLLRR-MQKRESEFGKVEQLNTGSYVEHVKISAPNEFDVMF	
CG7194	1	-----MSTANHFQRILEKLSISDNERYVYTKAEETQNYVDELLKRV-DKTFRQVFDGLST--GGSVLDKRVKINLDEEDLHM	
CG4766	1	MLVPPDMMAAQTRMYYQNNRCECTERVOARMYKTATA-----ISICKYVQDILKEVE-----LQEPFRTSS--CNGRFECVGVISNFEETII	
CG4766	1	MLVPSDMIAAQSKMYVQNNKYCADRVQRKAQTHQD-----DEVCRVYVQDILKEVE-----VQEPFRTSS--LNDYNGRFECVGVISNFEETII	
CG30424 (cGLR2)	1	-----MKESRNLENFVEKKLYECKKKYVDIPKDEGQRYQKHYEALISKYFETLRK-----ENPELDITIAEFSLE--SVGDKLKIAPNEFDLVF	
CG12970 (cGLR1)	1	-----MAMNLENIVN-----QATAQYVKIK-KEHREPYTAHYNAIKDKVYSEW-K-----SSAVLGKLLKGSTLCCGVYGDKLKVISIPNEFDLII	
<i>S. scrofa</i> OAS1	80	EL-----T-----TKLTSF--EDQLRRRGFEITQEIIRQLE-----ACQREQMF-----KVT	
<i>H. sapiens</i> OAS1	81	EL-----T-----TKLTSF--EDQLRRRGFEITQEIIRQLE-----ACQREQMF-----KVT	
<i>H. sapiens</i> cGAS	231	KLEVPRIQ-----LEEYCNTRAYVYFVKFRNPKNPLSQELEGILSASKMISKFRKIHK-----EEINDIE-----D-TDVI	
<i>S. scrofa</i> cGAS	206	KLEVPRIQ-----LEEYCNTRAYVYFVKFRNPKNPLSQELEGILSASKMISKFRKIHK-----EEIKNIE-----GVT	
<i>M. musculus</i> cGAS	217	KLEVPRIE-----LQEYYETGAFYLVKFKRIPRGNPLSHFLEGEVLSATKMLSKFRKIHK-----EEVKEIID-----DVS	
CG7194	76	KLKFPFDI-----RPTRIDQGFIYLEADLTVI--NP--QRTHRIVLQ-DWLRNARFKVFR--S--NQTIIATTSNRV-YKLT	
CG4766	85	YLN--QMGVFNFDVDDGTLPGCAVLKLSD-----GRKRSMSLVVEFITASGYLSRRKIRARFQTLVAOACDSSVYRDMVKMI	
CG4766	85	YLN--QMGVLFNFVDDGTLPGCAVLKLSD-----GRKRSMSLVVEFITASGYLSRRKIRARFQTLVAOACDSSVYRDMVKMI	
CG30424 (cGLR2)	85	YLN--QMGVLFNFVDDGTLPGCAVLKLSD-----GRKRSMSLVVEFITASGYLSRRKIRARFQTLVAOACDSSVYRDMVKMI	
CG12970 (cGLR1)	77	MLVFPENDKIIVKADASKPGNVLDMTKVMEIGSQEHNKPVFDR--KQIVNNK-----KQLEEDKLSFLESINTQTLNMGQNI-E-VA	
<i>S. scrofa</i> OAS1	118	FEVQSPRRN--PRALSFLVSSPQLQGEVEFDVLPADF-ALGQM-----TPGYKPNPEIYVOLI-----ECKS	
<i>H. sapiens</i> OAS1	119	FEVQAPRWGN--PRALSFLVSSPQLQGEVEFDVLPADF-ALGQL-----TPGYKPNPEIYVOLI-----ECTD	
<i>H. sapiens</i> cGAS	298	MKRK--RGCS--PAVTLTLLISE--KESVDITALE--SKSSWPASTQEG--LRIGNWLSAKVRKQLRKPFVLPVKKHA-----KEGN	
<i>S. scrofa</i> cGAS	291	VERK--RRVE--PAVTLTLLISKP--KEESVDITALE--SKSSWPASTQEG--LRIGNWLSAKVRKQLRKPFVLPVKKHA-----KEGS	
<i>M. musculus</i> cGAS	284	VEKE--KPGC--PAVTLTLLINP--KEESVDITALE--SKSSWPASTQEG--LRIGNWLSAKVRKQLRKPFVLPVKKHA-----KDCN	
CG7194	142	YTLF--GYGC--A-HTILAVCGS--RSLSHDVLPAPFESGSQWPEDI--CPVP--ADSVNN--PFAIPQOK-----KK-S	
CG4766	159	GDTSEVK-----LRIRERFVVDITPAFKCS--GIWPRSAHWPLPGLPMPHPNVAEVKTEG--DGLLESVIMQNKNNAA	
CG4766	159	ADTTEVK-----LRIRERFVVDITPAFKCA--GIWPRSAHWPLPGLPMPHPNVAEVKTEG--DGLLESVIMQNKNNAA	
CG30424 (cGLR2)	172	GVSHLKYRTCCGHAHTIFVYGDY--EYSVDYVPAICLAAEQVLPPTQL-ECF-----KRANTSWEAIPKPL-----KPLT	
CG12970 (cGLR1)	160	GRISHLQYRTCCGHAHTIFVYKGC--KYSVDYVPAICLAAEQVLPPTQL-ECF-----KRANTSWEAIPKPL-----KPLT	
<i>S. scrofa</i> OAS1	179	RGKEGEFSTCFTELQDFLRNRPTKL-----KSLIRLVKHVYQTCRKTHGN--KLPP--QYALELLTVYAWENGSRKTOF---	
<i>H. sapiens</i> OAS1	180	LQKEGEFSTCFTELQDFLRNRPTKL-----KSLIRLVKHVYQTCRKTHGN--KLPP--QYALELLTVYAWENGSRKTHF---	
<i>H. sapiens</i> cGAS	369	GFQETWRLSFSHIEKELNNHGSKTCCENKEEKCKRKDCLLMKYLLLEQLKERFKDKKHLDFKFSYHVKTAFHFVCTNNPQDSQWDRK	
<i>S. scrofa</i> cGAS	344	GFQETWRLSFSHIEKELNNHGSKTCCENKEEKCKRKDCLLMKYLLLEQLKERFKDKKHLDFKFSYHVKTAFHFVCTNNPQDSQWDRK	
<i>M. musculus</i> cGAS	357	SFQETWRLSFSHIEKELNNHGSKTCCENKEEKCKRKDCLLMKYLLLEQLKERFKDKKHLDFKFSYHVKTAFHFVCTNNPQDSQWDRK	
CG7194	205	AKPRTTFEMVSCAPHWERNLLQG--GKDNL-----GCRNRLSLTKTLRDRHLDLPGN--PVSYSYHLKLLLYEKEKHPREMEWEN	
CG4766	233	AMEGDWVLSFTDAENRLLQG--GKDNL-----GCRNRLSLTKTLRDRHLDLPGN--PVSYSYHLKLLLYEKEKHPREMEWEN	
CG4766	230	AMEGDWVLSFTDAENRLLQG--GKDNL-----GCRNRLSLTKTLRDRHLDLPGN--PVSYSYHLKLLLYEKEKHPREMEWEN	
CG30424 (cGLR2)	241	ETSMISFRSSFYAVEKILLQDVHENC-----RNAIKREKK--FRDVKTNLGNCK--SYVTKTLFVKKIIEPE--ESYV--LN	
CG12970 (cGLR1)	226	TDNSTSETSSFYAEERLLYQ-KQFL-----CPAIRLMKQ--NRNVK--NKANLK-----SYHTKTLFVKKIIEPE--PSYV--LN	
<i>S. scrofa</i> OAS1	250	STAQGFRTVLELVKXOKLCTFEWAYDYDTNPPVYGRCLMLOQLKPRPVILDPAADPTGNVGGCOT-----HSWQRTAQEARVWLGYP	
<i>H. sapiens</i> OAS1	250	NTAQGFRTVLELVKXOKLCTFEWAYDYDTNPPVYGRCLMLOQLKPRPVILDPAADPTGNVGGCOT-----HSWQRTAQEARVWLGYP	
<i>H. sapiens</i> cGAS	459	DGLCFDNCVYTFLO--CLRTEKLENYLPEFNLFS--NLI-----DK-----PSKEFLSKQIYI-----	
<i>S. scrofa</i> cGAS	434	NLECCFDNCVYTFLO--CLRTEKLENYLPEFNLFS--NLI-----DK-----PSKEFLSKQIYI-----	
<i>M. musculus</i> cGAS	444	NLSCCFDKLLAFFLE--CLRTEKLDHYFIPKFNLSQ--ELI-----DR-----KSKEFLSKQIYI-----	
CG7194	271	DGLTLLVEMWSHVVD--HURARRLEFLAEDHNVNRMNQNEIKC-----LE-----NASTLRLKCTA	
CG4766	304	CIADRINGIFLOLIS--CLQYRRCPHYELPALDMFGKGSPPALEQA-----AKQVWRLLTRILLTNANA	
CG4766	301	CIADRINGIFLOLIS--CLQYRRCPHYELPALDMFGKGSPPALEQA-----AKQVWRLLTRILLTNANA	
CG30424 (cGLR2)	293	PKDITFTEMGLKADSLALTPKKCKLPFFWDPKLDMHAQLTDSQRTDLY-----NHFRKCEYTFKDNQNVN--DC	
CG12970 (cGLR1)	293	PKDITFTEMGLKADSLALTPKKCKLPFFWDPKLDMHAQLTDSQRTDLY-----NHFRKCEYTFKDNQNVN--DC	
<i>S. scrofa</i> OAS1	332	CKNLDGSLVGAWTML-----QK-----	
<i>H. sapiens</i> OAS1	332	FKNWDGSPVSSWILLAESNSADDETDPRRYQKYGYIGTHEYPHFSHRPTLQAASTPQAEEDWTCTI--F	
<i>H. sapiens</i> cGAS	511	ERNNEF-----PVFD-E-----	
<i>S. scrofa</i> cGAS	486	ERNNEF-----PVFD-E-----	
<i>M. musculus</i> cGAS	486	ERNNEF-----PVFD-E-----	
CG7194	329	UTCGGSYHHVAQLFNIP-----	
CG4766	365	FEK-----	
CG4766	362	FEK-----	
CG30424 (cGLR2)	395	SSRRKRNVIKGLK-----TTSICKLRNARTNGWTAGLWTRPGHAYRGPSSETVSTWDTVKDAWSEGIVE	
CG12970 (cGLR1)	362	T--ENNVSFSF-----KNTTYKL-----	

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c

Extended Data Fig. 1 | Alignment of mammalian cGAS/OAS1 proteins and cGAS-like proteins from *D. melanogaster*. a, Extended view of the alignment shown in Fig. 1a with the positions of the GS/GG duplet, the metal ion coordinating acidic residues, and the Zn-finger indicated above the sequences with stars, arrows and bar, respectively. The annotated start methionine in the NCBI reference sequence for isoform D of CG30424 (cGLR2), which would delete the entire spine helix as well as part of the active site, is highlighted in yellow.

Deletion of the spine helix is incompatible with a folded enzyme. Furthermore, the GS-containing loop, which coordinates the γ -phosphate of the donor nucleotide, is a universally conserved feature of nucleotidyltransferases. **b, c**, Structure (PDB: 4K98) of murine cGAS in complex with DNA and a cGAMP intermediate analog (represented in sticks) and two magnesium ions (yellow spheres). The sidechains of the acidic active site residues and the serine in the GS motif are also represented in sticks and their oxygen atoms are colored red. Grey coloring represents the proportion of the protein upstream of the annotated start methionine site in CG30424 (cGLR2). **b**, Full view of the structure. **c**, Enhanced view of the active site.

Extended Data Fig. 2 | Transient expression of cGLR1 and cGLR induces the Sting pathway in S2 cells

Extended Data Fig. 2 | Transient expression of candidate cGAS-like receptors in S2 cells. **a**, Due to issues with the ANTI-FLAG® M2 antibody giving rise to non-specific bands when performing immunoblots on S2 cell lysates, we replaced the FLAG-tag we initially used with a V5-tag and reproduced the experiment from Fig. 1b. Cells were transfected with cGAS, cGLR1, cGLR2 and CG7194 or mutants thereof as well as plasmids encoding firefly or *Renilla* luciferase under transcriptional control of the *Sting* or *Actin5C* promoter, respectively. At 24 h post transfection, luciferase activity was measured. Data are from one experiment performed in triplicates and are shown with mean $\pm$ s.d. ($n = 3$) **b**, Immunoblot showing the expression of the V5-tagged proteins from panel (**a**). cGLR2 appears to be rapidly degraded in S2 cells, whereas it could easily be detected in HEK293T cells following transfection (Extended Data Fig. 10a). For gel source data, see Supplementary Fig. 1.

Extended Data Fig. 3 | Ectopic expression of *cGLR1* and *cGLR2* in transgenic flies induces *Srg2* and *Srg3*.

Extended Data Fig. 3 | Ectopic expression of *cGLR1* or *cGLR2* in transgenic flies induces expression of *srg2* and *srg3*. **a, b, c, d,** Expression of *cGLR1* (**a**), *cGLR2* (**b**), *Srg2* (**c**) and *Srg3* (**d**) was monitored by RT-qPCR in transgenic flies ectopically expressing wild-type or mutant *cGLRs*. Expression was normalized to the housekeeping gene *RpL32*. Data are from three independent experiments (red, blue and green, each performed in triplicate) and shown with mean ($n = 9$). **e,** Flies of the indicated genotypes were injected with Tris and survival was monitored daily. Data are from 3 independent experiments, each with 30 flies ($n = 90$). In **a, b, c,** and **d**, data were analysed using two-way ANOVA and Holm-Šídák post hoc test and compared to relevant AFA mutants. In **e**, log-rank test was used to compare *cGLR1*

604 vs. cGLR1 AFA (ns) and cGLR2 vs. cGLR2 AFA (ns). ns not significant, * $P < 0.05$. ** $P <$
605 0.01. *** $P < 0.001$.**** $P < 0.0001$.

Extended Data Fig. 4 | Verification of Sting knockout S2 cells. S2 cells transfected with expression vectors for cGAS, cGLR1 or cGLR2 and plasmids encoding firefly or *Renilla* luciferase under transcriptional control of the *Sting* or *Actin5C* promoter, respectively, were used to monitor activation of the Sting pathway. **a**, S2 cells with or without co-transfection with an expression vector for Sting. **b**, Sting knockout (KO) S2 cells with or without co-transfection with an expression vector for Sting. **c**, S2 cells transfected with expression vectors for cGAS, cGLR1 or cGLR2 and plasmid encoding firefly luciferase under transcriptional control of the *Sting* promoter or a mutated version containing two point mutations in the Relish binding site. Data are from three independent experiments (blue, red, and green, each performed in triplicates) and are shown with mean ($n = 9$). Data were analyzed using two-way ANOVA and Holm-Šidák post hoc test and compared to mock. ns not significant, **** $P < 0.0001$. **d**, Sanger sequencing showing the 2 bp deletion in the *Sting* gene in Sting knockout (KO) S2 cells. **e**, Immunoblot showing the lack of expression of Sting in Sting KO S2 cells. The arrow indicates the position of the Sting band. For gel source data, see Supplementary Fig. 1.

Extended Data Fig. 5 | Generation of *cGLR1* and *cGLR2* knockout flies

Extended Data Fig. 5 | Generation of *cGLR1* and *cGLR2* knockout flies. The *cGLR1* and *cGLR2* genes, both located on the right arm of the second chromosome, are shown together with their annotated transcripts. Open reading frames are indicated in green. For *cGLR1*, an 8 bp deletion was introduced in the first exon using CRISPR/Cas9 technology. The deletion creates a frameshift after the asparagine residue at position 31, leading to termination of translation after insertion of single glycine residue. For *cGLR2*, a 5 bp deletion was created in exon 3, which is shared by all isoforms. The deletion results in a frameshift after the glutamate residue at position 338, leading to termination of translation after insertion of a 32 amino acid extension (HDDRIDPGSSLGNVPVRAKDSKRPEGRRDQPE).

Extended Data Fig. 6 | Expression of *Sting*, *Srg2* and *Srg3* in flies upon infection with DCV. **a**, Corresponding control to Fig. 2a. w^{1118} , cGLR1 KO, cGLR2 KO or cGLR1/2 KO flies were injected with Tris and survival was monitored daily. Data are from three independent experiments, each with three groups of around 10 flies. **b**, **c**, **d**, w^{1118} , cGLR1 KO, cGLR2 KO or cGLR1/2 KO flies were injected with DCV or Tris and expression of *Sting* (**a**), *Srg2* (**b**) and *Srg3* (**c**) was monitored by RT-qPCR at 2 and 3 days post-infection (dpi). Expression was normalized to the housekeeping gene *RpL32*. Data are from three independent experiments, each performed in triplicates (n = 9). In **a**, log-rank test was used to test if the survival curves differed. In **b**, **c** and **d**, data were analyzed using two-way ANOVA and Holm-Šidák post hoc test and compared to w^{1118} flies. ns not significant, ** $P < 0.01$. *** $P < 0.001$. **** $P < 0.0001$.

Extended Data Fig. 7 | Expression of *Sting*, *Srg2* and *Srg3* in flies upon infection with KV. **a**, Corresponding control to Fig. 2b. w^{1118} , cGLR1 KO or cGLR2 KO flies were injected with Tris and survival was monitored daily. Data are from three independent experiments, each with three groups of around 10 flies. **b**, **c**, **d**, w^{1118} , cGLR1 KO or cGLR2 KO flies were injected with KV or Tris and expression of *Sting* (**a**), *Srg2* (**b**) and *Srg3* (**c**) was monitored by RT-qPCR at 5 and 10 days post-infection (dpi). Expression was normalized to the housekeeping gene *Rpl32*. Data are from three independent experiments, each performed in triplicates (n = 9). **e**, **f**, w^{1118} , cGLR1 KO, cGLR2 KO or cGLR1/2 KO flies were injected with KV (**e**) or Tris (**f**) and survival was monitored daily. Data are from three independent experiments, each with three groups of around 10 flies. **g**, w^{1118} , cGLR1 KO, cGLR2 or cGLR1/2 KO flies were injected with KV and viral load was monitored by RT-qPCR at 5 days post-infection (dpi). Expression was normalized to the housekeeping gene *Rpl32*. Data are from three independent experiments, each performed in triplicates (n = 9). In **a**, **e**, **f**, log-

rank test was used to compare the survival curves pairwise followed by a Holm-Šídák multiple comparison correction. In **b**, **c** and **d**, data were analyzed using two-way ANOVA and Holm-Šídák post hoc test and compared to w^{1118} flies. In **g**, Log transformed data were analyzed using one-way ANOVA and Dunnett T3 post hoc test and compared to w^{1118} flies. ns not significant, ** $P < 0.01$. *** $P < 0.001$. **** $P < 0.0001$.

Extended Data Fig 7 | Loss of cGLR1 or cGLR2 has a limited effect on VSV infection in flies

Extended Data Fig. 8 | Loss of cGLR1 or cGLR2 has a limited effect on VSV infection in flies. **a**, **b**, w^{1118} , cGLR1 KO or cGLR2 KO flies were injected with VSV (**a**) or Tris (**b**) and

survival was monitored daily. Data are from three independent experiments, each with three groups of around 10 flies. **c, d, e, f, g**, w^{1118} , cGLR1 KO, cGLR2 KO or cGLR1/2 KO flies were injected with VSV or Tris and viral load (**c**) as well as expression of *Sting* (**d**), *Srg1* (**e**), *Srg2* (**f**) and *Srg3* (**g**) were monitored by RT-qPCR at 4 and 5 days post-infection (dpi). Expression was normalized to the housekeeping gene *RpL32*. Data are from three independent experiments, each performed in triplicates (n = 9). In **a** and **b**, log-rank test was used to test if the survival curves differed. In **c**, Log transformed data were analyzed using one-way ANOVA and Dunnett T3 post hoc test and compared to w^{1118} flies. In **d, e, f** and **g**, data were analyzed using two-way ANOVA and Holm-Šídák post hoc test and compared to w^{1118} flies. ns not significant, * $P < 0.05$. *** $P < 0.001$. **** $P < 0.0001$.

Extended Data Fig. 9 | Loss of cGLR1 or cGLR2 has a limited effect on IIV6 infection in flies. **a, b**, w^{1118} , cGLR1 KO or cGLR2 KO flies were injected with IIV6 (**a**) or Tris (**b**) and survival was monitored daily. Data are from three independent experiments, each with three groups of around 10 flies. **c, d, e, f, g**, w^{1118} , cGLR1 KO or cGLR2 KO flies were injected with IIV6 or Tris and viral load (**c**) as well as expression of *Sting* (**d**), *Srg1* (**e**), *Srg2* (**f**) and *Srg3* (**g**) were monitored by RT-qPCR at 5 and 10 days post-infection (dpi). Expression was normalized to the housekeeping gene *Rpl32*. Data are from three independent experiments, each performed in triplicates ($n = 9$). In **a** and **b**, log-rank test was used to test if the survival curves differed. In **c**, Log transformed data were analyzed using one-way ANOVA and Dunnett T3 post hoc test and compared to w^{1118} flies. In **d, e, f** and **g**, data were analyzed using two-way ANOVA and Holm-Šidák post hoc test and compared to w^{1118} flies. ns not significant, * $P < 0.05$. ** $P < 0.01$. *** $P < 0.001$. **** $P < 0.0001$.

Extended Data Fig. 10 | cGRL2 produce 2'3'-cGAMP and 3'2'-cGAMP, which can activate human and *Drosophila* STING. **a**, Immunoblot showing the expression of STING and FLAG-tagged nucleotidyltransferases from Fig. 3a. **b**, HEK293T cells transfected with cGAS, cGAS AFA, cGRL2, cGRL2 AFA, and STING as indicated and plasmids encoding firefly or *Renilla* luciferase under transcriptional control of the *IFN β 1* or a constitutive promoter, respectively. Data are from three independent experiments (blue, red, and green, each performed in triplicates) and shown with mean (n = 9). **c**, Immunoblot showing the expression of STING and FLAG-tagged nucleotidyltransferases from panel (b). **d**, Representative chromatograms from mass spectrometry analysis of 2'3'-cGAMP or 3'2'-cGAMP spiked lysates from GFP transfected cells. **e**, Representative chromatogram from mass spectrometry analysis of cGAS transfected cells. **f**, Representative chromatograms from mass spectrometry analysis of GFP or cGRL2 transfected cells. **g**, **h**, **i**, HT-1080 (**g**) or S2

cells (**h** and **i**) permeabilized using digitonin and treated with the indicated cGAMPs. Expression of *IFNB1* (**g**), *Sting* (**h**) or *Srg1* (**i**) were monitored by RT-qPCR at 6 and 8 h post treatment for S2 and HT-1080 cells, respectively. Expression was normalized to the housekeeping genes GAPDH (**g**) and *RpL32* (**h** and **i**). Data are shown with mean $\pm$ s.e.m. (n = 4 for **g** and n = 3 for **h** and **i**). In **b**, data were analyzed using two-way ANOVA and Holm-Šídák and compared to mock. In **g**, **h**, **i**, data were analyzed using one-way ANOVA and Dunnett T3 post hoc test and compared to mock. ns not significant, ** $P < 0.01$. *** $P < 0.001$. **** $P < 0.0001$.

Extended Data Fig. 11 | cGRL1 produces 3'2'-cGAMP in response to dsRNA. **a**, Representative chromatograms from anion exchange chromatography analysis of different nucleotides. **b**, Representative chromatograms from anion exchange chromatography analysis of reaction products from activity assays with recombinant cGRL1 in the presence of different nucleic acids. **c**, Immunoblot showing the expression of FLAG-tagged cGRL2 and mutants thereof from Fig. 3f. **d**, S2 cells transfected with cGRL2 or mutants thereof as well as plasmids encoding firefly or *Renilla* luciferase under transcriptional control of the *Sting* or *Actin5C* promoter, respectively. Data are from three independent experiments (blue, red, and green, each performed in triplicates) and shown with mean ($n = 9$). In **d**, data were analyzed using two-way ANOVA and Holm-Šídák post hoc test and compared to cGRL2. ns not significant, **** $P < 0.0001$.

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