Peer Review File

Manuscript Title: Two cGAS-like receptors induce antiviral immunity in *Drosophila*

Editorial Notes:

Reviewer Comments & Author Rebuttals

Reviewer Reports on the Initial Version:

Referee #1 (Remarks to the Author):

Summary of the key results
In this manuscript, Holleufer et al. characterize two cGAS-like receptors from Drosophila melanogaster. They find that two cGLRs, but not the gene with the most similarity to mammalian cGAS (CG7194), trigger the Drosophila STING pathway in cell lines and adult flies. Knockout of either gene (cGLR1 or cGLR2) leads to a significant drop in STING dependent, virus triggered gene expression, that can be bypassed by direct injection of 2'3'-cGAMP, consistent with the idea that these cGLRs generate signaling cyclic di-nucleotides. Using HEK293 cells as a heterologous system they further find that cGLR2 can activate (human?) STING and generates 2'3'- as well as the novel 3'2'-cGAMP second messengers.

Overall this manuscript is very interesting with tantalizing new findings but the analysis is somewhat superficial.

Originality and significance: if not novel, please include reference
The novelty in this study rests on the discovery of 3'2'-cGAMP generated by cGLR2 (along with "regular" 2'3'-cGAMP). However the importance of this second messenger is not clear.

Data & methodology: validity of approach, quality of data, quality of presentation
Figure 4. Which STING is expressed? Fly or human? I assume human. If fly STING is used, would HEK cells respond to cGLR1, as cGLR1 may only produce 3'2'-cGAMP and perhaps insect STING is uniquely capable of binding 3'2'. This area requires further development

Appropriate use of statistics and treatment of uncertainties
Appropriate statistic tools are used throughout.

Conclusions: robustness, validity, reliability
While the conclusion are solid and are validated with complementary approaches, the advancement is quite limited.

Suggested improvements: experiments, data for possible revision
Survival should be analyzed in the cGLR mutants. It would be valuable to look at multiple viruses, as there may be differences that are informative.
Double cGLR mutants should be analyzed. I.e. why are there two such genes? Is this a straight redundancy or is there some specific role for each
Most critically, the ability of cGLRs to responded to RNA is hinted at by these findings and needs to be experimentally addressed.
It is particularly puzzling that CG7194 shows no STING activating activity. Do the authors have any insight on this finding?

References: appropriate credit to previous work?
All good.

Clarity and context: lucidity of abstract/summary, appropriateness of abstract, introduction and conclusions
The text is lucid and clear, and appropriate for a general audience. No issues.

Referee #2 (Remarks to the Author):

This study further supports evidence of the existence of a functionally relevant cGAS-STING signaling axis in Drosophila. In brief, the authors identify two gene products in Drosophila melanogaster, named cGLR1 and cGLR2, which on overexpression in fly cells and flies induce the upregulation of drosophila Sting and sting-related gene products (Srgs). In addition, overexpression of both cGLRs partially protects flies from mortality following VSV and DCV infection (the latter virus being a natural fly pathogen) with cGLR2 showing more prominent effects. By contrast, deficiency of cGLR1 or cGLR2 abolished Sting and Srg upregulation in the context of DCV infection. The function of STING and Relish, downstream of cGLRs is well established by data obtained from STING KO cells shown in Fig. 3. These results are consistent with a prior report on DCV-induced signaling and host protection via STING and Relish in flies (Goto et al., 2018 (cited)). In addition, the role of cGLR2 as a cyclic dinucleotide synthetase producing two distinct cGAMP isomers, cGAMP2`3`and cGAMP3`2`, is documented, while for the description of the enzymatic product of cGLR1 the authors refer to another study. The mechanism of cGLR activation, though claimed in the abstract, is not presented in the manuscript, instead the authors again refer to another manuscript. The distinctive (in vivo) function of cGLR1/2, which I consider the unique aspect of this manuscript, is currently not established. Here, additional experiments would be required to strengthen the conclusion of the physiological role of cGAS-like receptors in drosophila.

Specific points:
The authors hypothesize that cGLRs promote antiviral immunity in drosophila. This is based on their finding that cGLR1/2 overexpression protect flies from DCV-induced mortality and on the observation that cGLR1/2 deficient flies show decreased upregulation of Sting and Srg products as a surrogate marker for antiviral immunity. However, it would be important to directly demonstrate the impact of cGLR deficiency on virus propagation (viral loads) and overall fly survival. According to a prior study by authors of this work, dSTING plays a critical function in DCV infection in flies in vivo (Goto et al., 2018). Hence, a similar connection between cGLRs and the mentioned parameters, perhaps by using double-knockout lines, should allow the authors to reveal the importance of cGLRs as bona fide antiviral regulator in drosophila. Likewise, S2 cells can be readily infected with DCV (and other viruses), so monitoring the impact of endogenous cGLRs deficiency after viral infection in S2 cells would be important to substantiate the above proposed direct role of cGLRs in antiviral immunity. For this latter point both antiviral gene induction as well as parameters of viral replication must be determined. At present, the natural importance of cGLRs for fly immunity is not demonstrated.

Function of cGLR1 versus cGLR2 as pattern recognition receptors: the nature of the ligands for cGLR1 and cGLR2 are not addressed in this manuscript. In addition, the work leaves it unclear whether cGLR1 and cGLR2 carry out redundant roles or whether they function independently of each other as pattern recognition receptors. The authors suggest, but do not provide evidence, for direct recognition of RNA as a means to stimulate cGLR1 and cGLR2, respectively. With both an apparently solid reporter readout at hand and an in vitro cell system amenable for perturbation, one can expect to be able to reveal potential differences or similarities of the two receptors concerning their function in fly immunity. For examples, does RNA immunoprecipitated cGLR1 or cGLR2 in insect cells? I regard experiments into a more complete validation of RNA-binding induced activation as an essential, yet missing piece of the current work. In particular regarding the bold claim on “sensing different types of nucleic acids” further investigations into that direction are necessary. To further address this point, the authors could use of different (RNA) viruses for

infection as previously reported (Goto et al., 2018). Alternatively, the two receptors cGLR1/cGLR2 may trigger distinctive effects downstream of STING, for example autophagy, a previously proposed mechanism of antiviral function of dSTING in flies (Liu et al., 2018). Lastly, the enzymatic product of cGLR1 is not described in this work. Instead, the authors refer in this – and other points (see above) – to another manuscript from the Kranzusch lab. Also the observation that cGLR2 may produce two distinct isomers of cGAMP is not further worked out. Given prior findings on the existence of STING-based antiviral immunity in drosophila, a more complete characterization of the consequences and upstream ligands is a critical.

Referee #3 (Remarks to the Author):

This manuscript identified three novel cGAS-like genes, two of which they characterized as cGLR1 and cGLR2 in Drosophila. Based on the cell biological experiments, they further identified the two cGLRs activate innate immunity via Sting. Using S2 cells,they showed that insect antiviral immunity is established by cGLRs-Sting-NF-kB (Relish) axis, while mammalian antiviral immunity is established by cGAS-Sting-IRF axis to produce interferon and the NF-kB is involved in pro-inflammation via transcription of cytokines. This in intriguing point to deviate insect and mammalian innate immunity. They further challenged the transgenic flies with vesicular stomatitis virus (VSV) and picorna-like Drosophila C virus (DCV) and overexpressed cGLR1 and cGLR2. This important experiments revealed that overexpression of cGLR2 reduced the replication of two viruses, while that of cGLR1 was only effective to combat DCV. Later, the authors identified through overexpressions of the GLRs in S2 and HEK cells and subsequent mass spectrometry that cGLR2 produces a mixture of a novel CDN, 3'2'-cGAMP as well as 2'3'-cGAMP, while cGLR1 only produces 3'2'-cGAMP. Although the authors described that elucidation of biological role of different cGAMP is out of scope, this reviewer strongly recommend the authors to do the virus transgeneic flies experiment with overexpression of cGAS, since co-production of 3'2'-cGAMP and 2'3'-cGAMP comvat both of the VSV and DCV, while production of 3'2'-cGAMP only combat DCV. Based on the transgenic experiments with overexpression of cGAS, the authors should discuss the distinct antiviral functions of 3'2'-cGAMP and 2'3'-cGAMP. Based on the viral transgenic experiments, the authors discussed that the activator of cGLRs are not dsDNA but RNA, like RIG-1 and MDA5. Altogether, this manuscript provides very clear comparisons of innate immunity between insects and mammals, and also identified two new CDN-generating enzymes with well characterization. Furthermore, the pseudo gene-like cGAS candidate CG7194 possibly will provide a new discovery of missing activity and bona fide readout in near future. Therefore, this manuscript provides an excellent new concept of insect innate immunity with comparison with that of mammals, and deserves a wealth of a possible publication in Nature, with the following additional experiments and discussions.

1. Viral transgenic experiment with cGAS overexpression should be performed to uncover the distinct functions of 3'2'-cGAMP and 2'3'-cGAMP.

2. cGAS and cGLRs target dsDNA and RNA. The author possibly should discuss the difference in the substrate nucleic acid binding surface to provide the structural basis for the different substrate recognition.

3. The authors should discuss, if possible, on structural difference of CDN synthetic catalitic site to elucidate the different chemical reactions.

Author Rebuttals to Initial Comments:

Referee #1 (Remarks to the Author):

Summary of the key results.

In this manuscript, Holleufer et al. characterize two cGAS-like receptors from Drosophila melanogaster. They find that two cGLRs, but not the gene with the most similarity to mammalian cGAS (CG7194), trigger the Drosophila STING pathway in cell lines and adult flies. Knockout of either gene (cGLR1 or cGLR2) leads to a significant drop in STING dependent, virus triggered gene expression, that can be bypassed by direct injection of 2'3'-cGAMP, consistent with the idea that these cGLRs generate signaling cyclic di-nucleotides. Using HEK293 cells as a heterologous system they further find that cGLR2 can activate (human?) STING and generates 2'3'- as well as the novel 3'2'-cGAMP second messengers.

Overall this manuscript is very interesting with tantalizing new findings but the analysis is somewhat superficial.

We appreciate the positive view on our novel findings and we have done our best to complete the analysis. This includes (i) characterization of both cGLR1, cGLR2 and cGLR1/2 single and double knockout flies using 4 viruses and (ii) establishment of an *in vitro* system to measure activity of cGLR1 and to characterize both the activator of cGLR1 and its product.

Originality and significance: if not novel, please include reference¨

The novelty in this study rests on the discovery of 3'2'-cGAMP generated by cGLR2 (along with "regular" 2'3'-cGAMP). However the importance of this second messenger is not clear.

We believe that the discovery of the novel pattern recognition receptors (PRRs) cGLR1 and cGLR2 and their importance for antiviral immunity in an insect, also contribute to a significant degree to the novelty of our study. Here, we would like to emphasize that all the antiviral phenotypes we describe are observed in flies with a fully functional RNAi response, thus cGLR1 and cGLR2 are novel antiviral sensors that complement the RNAi system for antiviral

immunity in flies. However, the reviewer rightly points out that we did not establish the function of 3',2'cGAMP in the first version of the manuscript. We have now studied the ability of both the canonical 2',3'-cGAMP and the novel cyclic dinucleotide (CDN) 3',2'-cGAMP to activate both human and *Drosophila* STING. The results are presented in Extended Data Fig. 9f, g, h and discussed further below.

Data & methodology: validity of approach, quality of data, quality of presentation.

Figure 4. Which STING is expressed? Fly or human? I assume human. If fly STING is used, would HEK cells respond to cGLR1, as cGLR1 may only produce 3'2'-cGAMP and perhaps insect STING is uniquely capable of binding 3'2'. This area requires further development.

We apologize for being unclear here, but yes, it is human STING that is expressed in the HEK293 assay and we have made it clearer in the text. To demonstrate that cGLR1 can activate human STING, we first expressed cGLR1 in HEK293T cells and then provided exogenous dsRNA in the form of transfected poly(I:C). This showed that cGLR1 can signal through human STING (Fig. 4e). We have previously shown that 2,'3'-cGAMP activates Drosophila Sting[1] and since cGLR1 predominantly makes 3',2'-cGAMP (Fig. 4f) and gives a Sting-dependent signal in S2 cells (Fig. 2), we infer that 3',2'-cGAMP can also activate *Drosophila* Sting. Yet, we see the shortcomings by this indirect approach and have addressed those below.

To provide a detailed response to the underlying question, namely if 2',3'- and 3',2'-cGAMP activate both human and *Drosophila* STING, we directly monitored the activity of the two CDNs in human and *Drosophila* cells. The data shown in Extended Data Fig. 9f, g, h indicate that both 2',3'- and 3',2'- cGAMP can activate human STING, with 2'3'-cGAMP being the best activator. Like human STING, *Drosophila* Sting is activated by both nucleotides but here the novel CDN, 3,'2'-cGAMP, is the best activator. In summary, cGLR1 produces predominantly 3',2'-cGAMP, which is a strong agonist for *Drosophila* Sting, but can also activate human STING, although less efficiently.

Finally, we have attempted to detect CDNs produced in cGLR1-overexpressing HEK293T cells, but our MS analysis came up negative as well (Fig. 4). We conclude from all these

experiments that cGLR1 is not active in HEK293T cells, most likely because the pattern-associated molecular pattern (PAMP) activating it is absent in these cells.

Appropriate use of statistics and treatment of uncertainties
Appropriate statistic tools are used throughout.

Thank you!

Conclusions: robustness, validity, reliability.

While the conclusion are solid and are validated with complementary approaches, the advancement is quite limited.

With the data added during the revision, we demonstrate that cGLR1 and cGLR2 are novel cGAS-like PRRs in flies. cGLR1 clearly recognizes dsRNA, a novel PAMP for this family, whereas the PAMP for cGLR2 is yet to be discovered, but clearly different form that recognized by cGLR1 and human cGAS. cGLR1 and cGLR2 both produce a novel secondary messenger, 3',2'-cGAMP, and are crucial for an efficient antiviral response to both the RNA virus Drosophila C virus (DCV) and the DNA virus Kallithea virus (KV).

Specific Points Referee 1.

Suggested improvements: experiments, data for possible revision.

1. Survival should be analyzed in the cGLR mutants. It would be valuable to look at multiple viruses, as there may be differences that are informative. Double cGLR mutants should be analyzed. I.e. why are there two such genes? Is this a straight redundancy or is there some specific role for each

We have isogenized the mutant fly lines with wild-type background and characterized them in terms of infection with 4 different viruses. Those data are shown in Fig. 3 and Extended Data Fig. 6, 7 and 8 and provide a clearer picture of the *in vivo* role of cGLR1 and cGLR2. cGLR1 is required for resistance to DCV as shown by (i) reduced survival, (ii) increased viral load and

(iii) decreased induction of Sting-regulated genes (Srgs). After complete isogenization of cGLR2 mutant flies, we observed no phenotype for DCV, but double KO flies display a markedly increased mortality, compared to both wild-type and cGLR1 single KO flies. This points to a role for cGLR2 in the control of viral infection, but also to some redundancy with cGLR1. Next, we tested KV, a large DNA virus and, like DCV, a natural pathogen of *Drosophila*. Using this virus, both cGLR1 and cGLR2 KO flies exhibited a reduced survival. However, due to time constraints, we have not yet been able to test double KO flies using this virus. We also tested a negative strand RNA virus, vesicular stomatitis virus (VSV), and a DNA virus, invertebrate iridescent virus 6 (IIV6), and saw no clear phenotype in single KO flies for either of those viruses, which are not natural *Drosophila* pathogens.

2. Most critically, the ability of cGLRs to responded to RNA is hinted at by these findings and needs to be experimentally addressed.

This is now done for cGLR1, please see Fig. 4f and g. Despite a significant effort, we have not yet been able to identify the PAMP for cGLR2, although we now present data supporting the claim that cGLR2 is activated by an endogenously expressed ligand that it is different from dsRNA.

3. It is particularly puzzling that CG7194 shows no STING activating activity. Do the authors have any insight on this finding?

Like the reviewer, we are puzzled by this, as this protein shows the highest degree of sequence similarity to human cGAS and was the first protein we identified when we began our work in 2016. We have purified CG7194 from both S2 cells and *E. coli*, but have never been able to detect any activity from this protein, although the protein preparation was well behaved, monodispersed and showed no obvious signs of aggregation or unfolding. We also tested activity by overexpression in both S2 and HEK293T cells (as shown in our paper), but again could not obtain evidence of activity. This work is described in details in the doctoral thesis of Andreas Holleufer, which is publicly available and can be requested form the graduate school of Aarhus University. In addition, we are currently establishing transgenic *Drosophila* lines to overexpress this gene in the hope of observing a phenotype *in vivo*. However, it will take several months before we get any results from those experiments.

References: appropriate credit to previous work?

All good.

Clarity and context: lucidity of abstract/summary, appropriateness of abstract, introduction and conclusions.

The text is lucid and clear, and appropriate for a general audience. No issues.

We are pleased that the reviewers find our paper well written.

Referee #2 (Remarks to the Author):

This study further supports evidence of the existence of a functionally relevant cGAS-STING signaling axis in Drosophila. In brief, the authors identify two gene products in Drosophila melanogaster, named cGLR1 and cGLR2, which on overexpression in fly cells and flies induce the upregulation of drosophila Sting and sting-related gene products (Srgs). In addition, overexpression of both cGLRs partially protects flies from mortality following VSV and DCV infection (the latter virus being a natural fly pathogen) with cGLR2 showing more prominent effects. By contrast, deficiency of cGLR1 or cGLR2 abolished Sting and Srg upregulation in the context of DCV infection. The function of STING and Relish, downstream of cGLRs is well established by data obtained from STING KO cells shown in Fig. 3. These results are consistent with a prior report on DCV-induced signaling and host protection via STING and Relish in flies (Goto et al., 2018 (cited)). In addition, the role of cGLR2 as a cyclic dinucleotide synthetase producing two distinct cGAMP isomers, cGAMP2`3`and cGAMP3`2`, is documented, while for the description of the enzymatic product of cGLR1 the authors refer to another study. The mechanism of cGLR activation, though claimed in the abstract, is not presented in the manuscript, instead the authors again refer to another manuscript. The distinctive (in vivo) function of cGLR1/2, which I consider the unique aspect of this manuscript, is currently not established. Here, additional experiments would be required to strengthen the conclusion of the physiological role of cGAS-like receptors in drosophila.

Specific points Referee 2:

1. The authors hypothesize that cGLRs promote antiviral immunity in drosophila. This is based on their finding that cGLR1/2 overexpression protect flies from DCV-induced mortality and on the observation that cGLR1/2 deficient flies show decreased upregulation of Sting and Srg products as a surrogate marker for antiviral immunity. However, it would be important to directly demonstrate the impact of cGLR deficiency on virus propagation (viral loads) and overall fly survival. According to a prior study by authors of this work, dSTING plays a critical function in DCV infection in flies in vivo (Goto et al., 2018). Hence, a similar connection between cGLRs and the mentioned parameters, perhaps by using double-knockout lines, should allow the authors to reveal the importance of cGLRs as bona fide antiviral regulator in drosophila. Likewise, S2 cells can be readily infected with DCV (and other viruses), so monitoring the impact of endogenous cGLRs deficiency after viral infection in S2 cells would be important to substantiate the above proposed direct role of cGLRs in antiviral immunity. For this latter point both antiviral gene induction as well as parameters of viral replication must be determined. At present, the natural importance of cGLRs for fly immunity is not demonstrated.

Regarding S2 cells, both cGLR1 and cGLR2 are activated in these cells upon overexpression without the addition of exogenous PAMPs, thus we are assuming that their PAMPs already exist within these *Drosophila* cells. Accordingly, we observed a significant drop in the background activity of the Sting-driven luciferase reporter gene in Sting KO S2 cells (remember that activated Sting drives its own expression in *Drosophila* but not in mammals). Finally, we have been unable to observe any significant induction of STing or Srg1-3 by DCV infection in S2 cells. Overall, our data indicate that there is a certain degree of constant activation of Sting in S2 cells, which prevents us from using them to monitor activation of the Sting pathway by viruses.

Therefore, we focused our efforts on *in vivo* experiments and we performed a thorough characterization of cGLR1, cGLR2 and cGLR1/2 double KO flies, please see answer to Referee 1, specific point 1 above.

2. Function of cGLR1 versus cGLR2 as pattern recognition receptors: the nature of the ligands for cGLR1 and cGLR2 are not addressed in this manuscript. In addition, the work leaves it unclear whether cGLR1 and cGLR2 carry out redundant roles or whether they function independently of each other as pattern recognition receptors.

Regarding the identification of the PAMP for cGLR1 and cGLR2, please see point 3 below.

Concerning the respective roles of cGLR1 and cGLR2, we show that single KO flies for cGLR1 have both reduced survival as well as increased viral load and reduced expression of Srg1-3 upon DCV infection. In contrast, single KO of cGLR2 have no clear phenotype when infected with DCV. Nevertheless, when comparing single cGLR1 KO to cGLR1/2 double KO, a much more severe phenotype is observed in the double KO flies, in particular when looking at survival. Thus, cGLR1 can only partly compensate for the lack of a functional cGLR2 gene in the case of DCV infection. In the case of the DNA virus KV, which was previously shown to induce expression of Srgs[2], we observe a clear reduction in survival upon infection in both cGLR1 and cGLR2 KO flies, indicating that they are non-redundant.

Based on these new data, we favor a scenario where cGLR1 and cGLR2 are independent PRRs recognizing different PAMPs, but both acting through Sting. This would be similar to what is seen for MDA-5 and RIG-I (upstream of MAVS) or TLR7 and TLR9 (upstream of MyD88) in the mammalian system.

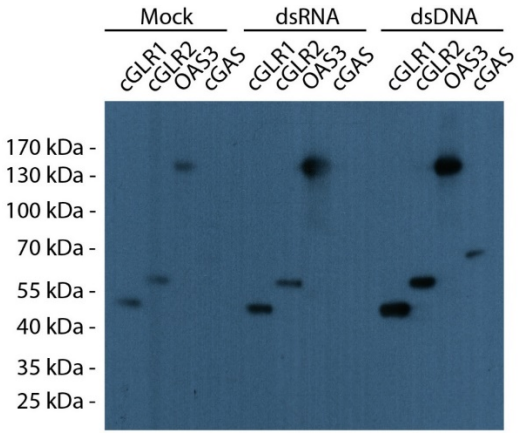
3. The authors suggest, but do not provide evidence, for direct recognition of RNA as a means to stimulate cGLR1 and cGLR2, respectively. With both an apparently solid reporter readout at hand and an in vitro cell system amenable for perturbation, one can expect to be able to reveal potential differences or similarities of the two receptors concerning their function in fly immunity. For examples, does RNA immunoprecipitated cGLR1 or cGLR2 in insect cells? I regard experiments into a more complete validation of RNA-binding induced activation as an essential, yet missing piece of the current work. In particular regarding the bold claim on "sensing different types of nucleic acids" further investigations into that direction are necessary.

To address this point, we purified recombinant cGLR1 from *E. coli* and performed *in vitro* activity assays. We show that cGLR1 is activated by both the synthetic dsRNA analogue poly(IC) and by a 100 bp long dsRNA oligo made by T7 transcription of the individual strands and subsequent annealing, as described elsewhere[3]. Importantly, dsDNA showed no activation in this assay. Upon activation by dsRNA, recombinant cGLR1 predominantly produced the novel secondary messenger 3',2'-cGAMP. We attempted the same experiment

with cGLR2, however, the quality of the purified recombinant protein was poor and the results were non-conclusive.

The activation of cGLR1 by dsRNA also explains why this protein was not active in HEK293T cells, as these cells do not contain free dsRNA. Indeed, we were able to activate cGLR1 in HEK293T cells by transfection with poly(I:C) (Fig. 4e). This is reminiscent of human cGAS, an enzyme that requires transfection with DNA upon expression in HEK293T cells for activity. Because cGLR2 is active in HEK293T cells without addition of a PAMP, in contrast to either cGLR1 or human cGAS, we hypothesize that the PAMP for cGLR2 is different from the ones activating cGLR1 (dsRNA) and human cGAS (dsDNA). To support this hypothesis, we introduced mutations in positively charged residues in the putative nucleic acid binding domain of cGLR2. These mutants strongly reduced the signaling activity of the receptor, indirectly supporting the claim that cGLR2 requires interaction with a negatively charged ligand to become active.

Finally, we also attempted to perform the pull down experiments suggested by the reviewer. We used biotinylated dsRNA and dsDNA (same 100 bp as used for activity studies) which were coupled to streptavidin-coated magnetic beads. This experiment suggest that both cGLR1 and cGLR2 bind to dsRNA and dsDNA. This agrees with previous data from both OAS and cGAS, two enzymes that can bind to a variety of oligonucleotides without this necessarily leading to activation [4,5]. In addition, we observe some background binding to the beads, despite efforts to coat them using nuclease-free BSA. Thus, at present, those data are of insufficient quality to make any robust conclusions and we do not plan on including them in the manuscript.

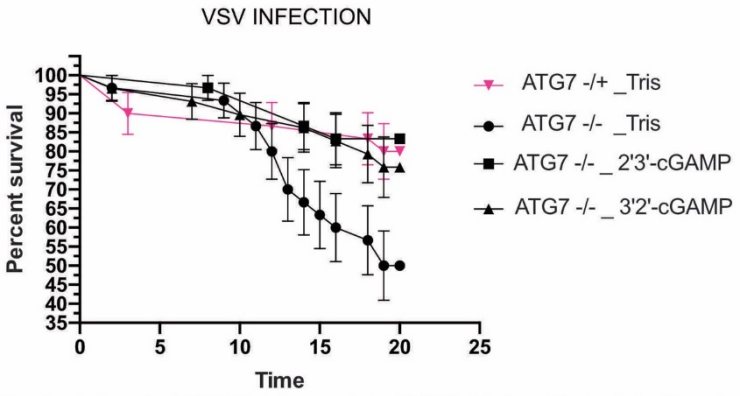


4. To further address this point, the authors could use of different (RNA) viruses for infection as previously reported (Goto et al., 2018).

This has been done, please see answer to Referee 1, specific point 1.

5. Alternatively, the two receptors cGLR1/cGLR2 may trigger distinctive effects downstream of STING, for example autophagy, a previously proposed mechanism of antiviral function of dSTING in flies (Liu et al., 2018).

We previously reported that the antiviral effect of 2',3'-cGAMP on DCV was not dependent on Atg7, a component of the canonical autophagy pathway[1]. We have now performed the experiment with both 2',3'- and 3',2'-cGAMP and VSV, a virus that was shown to be restricted by autophagy[6,7] . Our results shown below indicate that both CDNs improve resistance to VSV in Atg7 mutant flies. We have not included these data in the revised version of the manuscript, but we could do so if the reviewer and the editor deem it necessary.



Survival of control (ATG7 -/+) or mutant (ATG7 -/-) flies after coinjection of VSV with Tris or the indicated cyclic dinucleotides. The time is indicated in days. Flies mutant for the gene Atg7 are less resistant to VSV infection than the heterozygote controls and a clear protective effect of 2'3'- and 3'2'-cGAMP can be observed in the absence of canonical autophagy.

6. Lastly, the enzymatic product of cGLR1 is not described in this work. Instead, the authors refer in this - and other points (see above) - to another manuscript from the Kranzusch lab. Also the observation that cGLR2 may produce two distinct isomers of cGAMP is not further worked out. Given prior findings on the existence of STING-

based antiviral immunity in drosophila, a more complete characterization of the consequences and upstream ligands is a critical.

We have now established an *in vitro* system, showing that cGLR1 produces 3',2'-cGAMP upon stimulation by the synthetic dsRNA analogue poly(I:C), please see Fig. 4f.

We have also reproduced our MS data showing that the cGLR2-expressing HEK293T cell do produce both 2',3'-cGAMP and 3',2'-cGAMP. Transfection of either dsDNA or poly(I:C) into these cells did not increase cGAMP production, in contrast to transfection of dsDNA into HEK293T cells overexpressing human cGAS, which triggers production of 2,'3'-cGAMP.

Referee #3 (Remarks to the Author):

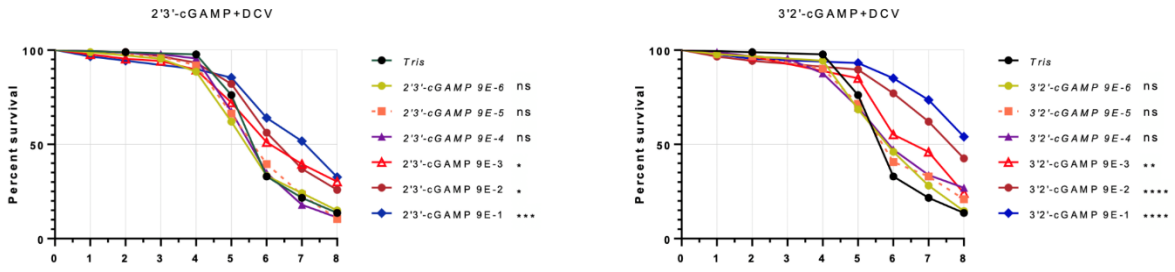
This manuscript identified three novel cGAS-like genes, two of which they characterized as cGLR1 and cGLR2 in Drosophila. Based on the cell biological experiments, they further identified the two cGLRs activate innate immunity via Sting. Using S2 cells, they showed that insect antiviral immunity is established by cGLRs-Sting-NF-kB (Relish) axis, while mammalian antiviral immunity is established by cGAS-Sting-IRF axis to produce interferon and the NF-kB is involved in pro-inflammation via transcription of cytokines. This in intriguing point to deviate insect and mammalian innate immunity. They further challenged the transgenic flies with vesicular stomatitis virus (VSV) and picorna-like Drosophila C virus (DCV) and overexpressed cGLR1 and cGLR2. This important experiments revealed that overexpression of cGLR2 reduced the replication of two viruses, while that of cGLR1 was only effective to combat DCV. Later, the authors identified through overexpressions of the GLRs in S2 and HEK cells and subsequent mass spectrometry that cGLR2 produces a mixture of a novel CDN, 3'2'-cGAMP as well as 2'3'-cGAMP, while cGLR1 only produces 3'2'-cGAMP. Although the authors described that elucidation of biological role of different cGAMP is out of scope, this reviewer strongly recommend the authors to do the virus transgeneic flies experiment with overexpression of cGAS, since co-production of 3'2'-cGAMP and 2'3'-cGAMP combat both of the VSV and DCV, while production of 3'2'-cGAMP only combat DCV. Based on the transgenic experiments with overexpression of cGAS, the authors should discuss the distinct antiviral functions of 3'2'-cGAMP and 2'3'-cGAMP. Based on the viral transgenic experiments, the authors discussed that the activator of cGLRs are not dsDNA but RNA, like RIG-1 and MDA5. Altogether, this manuscript provides very clear

comparisons of innate immunity between insects and mammals, and also identified two new CDN-generating enzymes with well characterization. Furthermore, the pseudo gene-like cGAS candidate CG7194 possibly will provide a new discovery of missing activity and bona fide readout in near future. Therefore, this manuscript provides an excellent new concept of insect innate immunity with comparison with that of mammals, and deserves a wealth of a possible publication in Nature, with the following additional experiments and discussions.

1. Viral transgenic experiment with cGAS overexpression should be performed to uncover the distinct functions of 3'2'-cGAMP and 2'3'-cGAMP.

We understand the concern of the referee. We have compared the antiviral effect of 3',2'-cGAMP versus 2',3'-cGAMP on DCV and, as shown in the figure below, have observed that 3',2'-cGAMP protects against DCV infection (these data obtained by the Cai and Imler lab are included in the paper by Kranzusch and colleagues). Therefore, we do not believe that the lack of activity of cGLR1 (which produces mostly 3',2'-cGAMP, whereas cGLR2 produces equal amounts of 2',3'-cGAMP and 3',2'-cGAMP) on DCV in the transgenic flies reflects distinct functions of the two CDNs. Rather, we think that they reflect the presence of ligands able to activate the two cGLRs in the flies. In this regard, we note that the activity of cGAS in our tissue culture cell experiments is dependent on the presence of the transfected DNA (see Fig. 4). It is therefore likely that cGAS would not be active upon ectopic expression in transgenic flies.

To address the point raised by the referee, we performed experiment testing the ability of both 2',3'-cGAMP and 3',2'-cGAMP to activate both human and *Drosophila* STING, please see our response to Referee 1 on page 2.



Survival after DCV infection and coinjection with Tris or indicated dose of 2'3'-cGAMP or 3' 2' -cGAMP. Data were collected from three independent experiments. Each has three groups of 10 flies (5 males and 5 females). The comparisons of survival were performed by Log-rank (Mantel-Cox) test.

2. cGAS and cGLRs target dsDNA and RNA. The author possibly should discuss the difference in the substrate nucleic acid binding surface to provide the structural basis for the different substrate recognition.

The Zn finger that is present in vertebrate cGAS, but is absent in cGLRs from insects, is the main structural element responsible for the DNA specificity of cGAS. The cGLRs appear to bind to nucleic acids in a manner similar to what is seen for the related oligoadenylate synthetase (OAS) proteins, using a positively charged grove positioned on the interface of the N- and C-terminal domains, and opposite the active site. This positive grove is also present in cGAS and does contribute to DNA binding through electrostatic contacts, but the DNA specificity originates from the Zn finger. Sequence alignment and phylogenetic studies suggest that cGLRs are more closely related to human cGAS than to the OAS proteins. Thus, we view cGLRs as the ancestral form of cGAS and the insertion of the Zn finger as a later evolutionary event occurring approximately at the time of the radiation of vertebrates. We provide evidence in Fig. 4i that positively charged residues in the nucleic acid binding interface of cGLR2 are required for its STING signaling activity. This suggests that this cGLR is also activated by a negatively charged ligand.

3. The authors should discuss, if possible, on structural difference of CDN synthetic catalitic site to elucidate the different chemical reactions.

In mammalian cGAS, the first reaction is the transfer of the donor ATP onto the 2´-OH of the acceptor GTP. The resulting pppG(2´–5´)pA is then cyclized by an additional link between the α-phosphate of GTP and the 3´-OH of ATP[8]. We assume that catalytic mechanism is the same for insect cGLRs. The simplest way to change between the synthesis of 2',3'-cGAMP and 3',2'-cGAMP is to allow GTP to bind to the donor site and ATP to the acceptor site, thereby switching the order of the links.

1 Cai, H. *et al.* 2'3'-cGAMP triggers a STING- and NF-kappaB-dependent broad antiviral response in Drosophila. *Sci Signal* **13**, doi:10.1126/scisignal.abc4537 (2020).

2 Palmer, W. H., Medd, N. C., Beard, P. M. & Obbard, D. J. Isolation of a natural DNA virus of Drosophila melanogaster, and characterisation of host resistance and immune responses. *PLoS pathogens* **14**, e1007050, doi:10.1371/journal.ppat.1007050 (2018).

3 Wang, Y., Holleufer, A., Gad, H. H. & Hartmann, R. Length dependent activation of OAS proteins by dsRNA. *Cytokine* **126**, 154867, doi:10.1016/j.cyto.2019.154867 (2020).
4 Civril, F. *et al.* Structural mechanism of cytosolic DNA sensing by cGAS. *Nature* **498**, 332-337, doi:10.1038/nature12305 (2013).
5 Hartmann, R., Justesen, J., Sarkar, S. N., Sen, G. C. & Yee, V. C. Crystal structure of the 2'-specific and double-stranded RNA-activated interferon-induced antiviral protein 2'-5'-oligoadenylate synthetase. *Molecular cell* **12**, 1173-1185 (2003).
6 Lamiable, O. *et al.* Analysis of the Contribution of Hemocytes and Autophagy to Drosophila Antiviral Immunity. *Journal of virology* **90**, 5415-5426, doi:10.1128/JVI.00238-16 (2016).
7 Shelly, S., Lukinova, N., Bambina, S., Berman, A. & Cherry, S. Autophagy is an essential component of Drosophila immunity against vesicular stomatitis virus. *Immunity* **30**, 588-598, doi:10.1016/j.immuni.2009.02.009 (2009).
8 Hornung, V., Hartmann, R., Ablasser, A. & Hopfner, K. P. OAS proteins and cGAS: unifying concepts in sensing and responding to cytosolic nucleic acids. *Nature Reviews Immunology* **14**, 521-528, doi:10.1038/nri3719 (2014).

Reviewer Reports on the First Revision:

Referee #1 (Remarks to the Author):

In this revised manuscript, the authors have provided important new data that more thorough investigates the activity and function of cGLR1 and cGLR2. Most of the important critiques from the earlier round have review have been thoroughly addressed.

based on the new data, two issues remain:

1. The double cGLR should be analyzed with all the viruses - KV, VSV, IIV6, in order to properly interpret the role of these factors in defense of this collection of viruses. In particular, the conclusion that the cGLRs respond to natural viral pathogens is not convincing and there is no possible mechanisms even suggested. Analysis of the double mutant will shed important light on this issue. It could be that it is more a case of straight redundancy for these viruses.

2. Although it is mentioned in the point-by-point, the actual text does not include a comment about the weaker activity of 3'2' with hSTING, and vice versa with dSTING.

Minor comments:
I think the legend to 4i is missing.
RECON is mentioned as a possible precedent, but it is unexplained and unclear.

UW: Further comments in response to additional data:

I am all set with this revision.

This is a very exciting story and all critiques have been addressed.

Referee #2 (Remarks to the Author):

With their revised manuscript, the authors have addressed my previous comments.
The manuscript demonstrates the unique roles of cGLR1 and cGLR2 in drosophila antiviral immunity and defines RNA as a bona fide viral ligand for cGLR1.

I recommend the paper for publication.

Two minor aspects should be corrected:
1) p. 5, line 119-121: This sentence seemingly contains the same information as the previous one.
2) Fig. 3a: The colors haven been wrongly assigned to the genotypes.

Referee #3 (Remarks to the Author):

The authors satisfactorily addressed my concerns, and this reviewer is now positive to publish the paper in Nature.

Author Rebuttals to First Revision:

Referee #1 (Remarks to the Author):

I am all set with this revision.

This is a very exciting story and all critiques have been

addressed. Thank you

Referee #2 (Remarks to the Author):

With their revised manuscript, the authors have addressed my previous comments.
The manuscript demonstrates the unique roles of cGLR1 and cGLR2 in drosophila antiviral immunity and defines RNA as a bona fide viral ligand for cGLR1.

I recommend the paper for

publication. Thank you

Two minor aspects should be corrected:
1) p. 5, line 119-121: This sentence seemingly contains the same information as the previous one.

We agree with referee #2 and have deleted the redundant sentence.

2) Fig. 3a: The colors haven been wrongly assigned to the genotypes.

Thank you for pointing this out. We have corrected the colors in Fig. 3a.

Referee #3 (Remarks to the Author):

The authors satisfactorily addressed my concerns, and this reviewer is now positive to publish the paper in Nature.

Thank you