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Regulation of cGAS activity by RNA-modulated phase separation

Silian Chen, Miao Rong, Yun Lv, Deyu Zhu, and Ye Xiang
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Corresponding author(s): Ye Xiang (yxiang@mail.tsinghua.edu.cn) , Deyu Zhu (zhudeyu@sdu.edu.cn)

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Dear Dr. Xiang,

Thank you for the submission of your research manuscript to EMBO reports. We have now received reports from the three referees that were asked to evaluate your study, which can be found at the end of this email.

As you will see, all referees think that the findings are of interest, but they also have several comments, concerns and suggestions, indicating that a major revision of the manuscript is necessary to allow publication in EMBO reports. As the reports are below, and I think all points need to be addressed, I will not detail them here.

Given the constructive referee comments, we would like to invite you to revise your manuscript with the understanding that all referee concerns must be addressed in the revised manuscript and in a detailed point-by-point response. Acceptance of your manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

Revised manuscripts should be submitted within three months of a request for revision. We are aware that many laboratories cannot function at full efficiency during the current COVID-19/SARS-CoV-2 pandemic and we have therefore extended our 'scooping protection policy' to cover the period required for full revision. Please contact me to discuss the revision should you need additional time, and also if you see a paper with related content published elsewhere.

When submitting your revised manuscript, please also carefully review the instructions that follow below.

PLEASE NOTE THAT upon resubmission revised manuscripts are subjected to an initial quality control prior to exposition to re-review. Upon failure in the initial quality control, the manuscripts are sent back to the authors, which may lead to delays. Frequent reasons for such a failure are the lack of the data availability section (please see below) and the presence of statistics based on n=2 (the authors are then asked to present scatter plots or provide more data points).

When submitting your revised manuscript, we will require:

1) a .docx formatted version of the final manuscript text (including legends for main figures, EV figures and tables), but without the figures included. Please make sure that changes are highlighted to be clearly visible. Figure legends should be compiled at the end of the manuscript text.

2) individual production quality figure files as .eps, .tif, .jpg (one file per figure), of main figures and EV figures. Please upload these as separate, individual files upon re-submission.

The Expanded View format, which will be displayed in the main HTML of the paper in a collapsible format, has replaced the Supplementary information. You can submit up to 5 images as Expanded View. Please follow the nomenclature Figure EV1, Figure EV2 etc. The figure legend for these should be included in the main manuscript document file in a section called Expanded View Figure Legends after the main Figure Legends section. Additional Supplementary material should be supplied as a single pdf file labeled Appendix. The Appendix should have page numbers and needs to include a table of content on the first page (with page numbers) and legends for all content. Please follow the nomenclature Appendix Figure Sx, Appendix Table Sx etc. throughout the text, and also label the figures and tables according to this nomenclature.

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3) a .docx formatted letter INCLUDING the reviewers' reports and your detailed point-by-point responses to their comments. As part of the EMBO Press transparent editorial process, the point-by-point response is part of the Review Process File (RPF), which will be published alongside your paper.

4) a complete author checklist, which you can download from our author guidelines (https://www.embopress.org/page/journal/14693178/authorguide). Please insert page numbers in the checklist to indicate where the requested information can be found in the manuscript. The completed author checklist will also be part of the RPF.

Please also follow our guidelines for the use of living organisms, and the respective reporting guidelines:
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5) that primary datasets produced in this study (e.g. RNA-seq, ChIP-seq and array data) are deposited in an appropriate public

database. This is now mandatory. If no primary datasets have been deposited in any database, please also state this (e.g. 'No primary datasets have been generated and deposited') in this section (see below).

See also: http://embor.embopress.org/authorguide#datadeposition

Please remember to provide a reviewer password if the datasets are not yet public.

The accession numbers and database should be listed in a formal "Data Availability " section (placed after Materials & Methods) that follows the model below. Please note that the Data Availability Section is restricted to new primary data that are part of this study.

Data availability

The datasets produced in this study are available in the following databases:

- RNA-Seq data: Gene Expression Omnibus GSE46843 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE46843)
- [data type]: [name of the resource] [accession number/identifier/doi] ([URL or identifiers.org/DATABASE:ACCESSION])

*** Note - All links should resolve to a page where the data can be accessed. ***

Moreover, I have these editorial requests:

6) We strongly encourage the publication of original source data with the aim of making primary data more accessible and transparent to the reader. The source data will be published in a separate source data file online along with the accepted manuscript and will be linked to the relevant figure. If you would like to use this opportunity, please submit the source data (for example scans of entire gels or blots, data points of graphs in an excel sheet, additional images, etc.) of your key experiments together with the revised manuscript. If you want to provide source data, please include size markers for scans of entire gels, label the scans with figure and panel number, and send one PDF file per figure.

7) Our journal encourages inclusion of *data citations in the reference list* to directly cite datasets that were re-used and obtained from public databases. Data citations in the article text are distinct from normal bibliographical citations and should directly link to the database records from which the data can be accessed. In the main text, data citations are formatted as follows: "Data ref: Smith et al, 2001" or "Data ref: NCBI Sequence Read Archive PRJNA342805, 2017". In the Reference list, data citations must be labeled with "[DATASET]". A data reference must provide the database name, accession number/identifiers and a resolvable link to the landing page from which the data can be accessed at the end of the reference. Further instructions are available at: http://www.embopress.org/page/journal/14693178/authorguide#referencesformat

8) Regarding data quantification and statistics, can you please specify, where applicable, the number "n" for how many independent experiments (biological replicates) were performed, the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values in the respective figure legends. Please provide statistical testing where applicable, and also add a paragraph detailing this to the methods section. See:
http://www.embopress.org/page/journal/14693178/authorguide#statisticalanalysis

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10) Please add up to five key words to the title page of the manuscript.

I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

Yours sincerely

Achim Breiling
Editor
EMBO Reports

Referee #1:

The manuscript proposed by Chen et al, reports on the phase separation of the protein cGAS, a dsDNA sensor involved in the innate immune response, with cellular RNA and its potential role in regulating immune response in cells. Although the ability of cGAS to phase separate with RNA has been previously shown, the authors provide insights about the physiological significance of this phenomenon. In particular, their data suggest that phase separation of cGAS with RNA enhances immune response

when the concentration of pathogen dsDNA is low, while it inhibits overactivation of cGAS in presence of high concentration of dsDNA. I believe these results could be of interest for the scientific community, however, the data presented by Chen et al, does not fully support their claims and would benefit from additional experiments, statistical analysis and discussions (see below). Moreover, I believe the quality of the data (particularly the microscopy images) should be improved to allow a better appreciation of the results.
Overall, I would recommend publications of this manuscript after major revisions that respond to the comments below:

1) The authors seem to use the terms phase separation and aggregation interchangeably. However, aggregation and phase separation are two mechanistically distinct phenomena. The first one relies on the irreversible misfolding and loss of function of proteins whereas the other one is a reversible thermodynamic process in which macromolecules rearrange in space, without any loss of function. The authors should avoid this confusion, that would be misleading for readers that are not familiar with the field.

2) The authors tend to overinterpret their data and need to add statistical analyses:
a. In many figures, Chen et al compare the ability of different dsDNA and RNAs to promote/trigger cGAS phase separation and often infer that some specific DNA/RNA species are more efficient than others at triggering cGAS phase separation. The images presented by the authors do not allow to support such conclusions. The authors should map the phase diagrams of cGAS with each DNA/RNA specie, and comparison should be driven from the threshold concentration of protein/nucleic acid necessary to trigger phase separation. Indeed, it is hard to tell from Figure S2B for instance, whether the presence of single stranded arms actually influence the phase separation of cGAS, as stated by the authors. The pictures presented in this figure only suggest that 48 bases DNA triggers cGAS phase separation and that partial double strand does not or poorly affect the formation of droplets.
b. In Figure 1, the authors claim that RNA does not activate cGAS, but they only show activity assays involving tRNA. The authors should test whether other RNA species or total RNA have similar effect.
c. In Figure 2: The authors claim that cells overexpressing hcGAS contain phase separated granules. Although the data does indicate the formation of puncta in cells, the authors do not provide any evidence of the phase separated nature of these structures (fusion/dynamism of cGAS when FRAPed/flow in the cytosol). These structures could be amorphous aggregates that form due to overexpression of the protein and that sequester RNA. The authors should provide evidence of their phase separated behavior. Moreover, the authors should test whether endogenous cGAS form puncta in cells under normal conditions. Indeed, the puncta observed by the authors could be artefacts of overexpression.
d. Figure S6: The authors indicate that cGAS binds primarily tRNA and rRNA, the two most abundant RNA species in cells. Although the data do show a slightly higher RNA binding on these populations, the lack of statistical analysis does not permit to establish such strong statement. The experiment should be repeated, and statistical analysis should be performed.

3) Additional explanation or discussion of the results would bring clarity to the manuscript:
a. Why did the authors decided to focus on tRNA? Is cGAS predicted to have low specificity towards RNA?
b. What is the Opti-prep gradient and why did the author use this approach?
c. Figure 3B: could the authors discuss the different behavior of each DNA used in this figure? In particular, 380 bp DNA shows a curious behavior where it first displaces tRNA and promotes recruitment of tRNA to the droplets? Moreover, is there a relationship between length/structure of the DNA species used and their ability to displace tRNA? How does that relate to the function of cGAS in cells and to the immune response?

Other comments:

Figure 1B and 4: hcGAS forms amorphous aggregates with RNA in vitro, at reasonably low concentrations. Is there any physiological relevance for the formation of these structures? Could these aggregates relate to the functions of cGAS in cells?

Figure S6: Could the authors add a pie chart of the relative abundance of each RNA populations in cells to indicate whether there is a preferential binding of cGAS towards certain types of RNA?

The authors use both µM and µg/mL for DNA quantification. The authors should homogenize units to allow comparison between the different figures and conditions.

If it has not been done yet, could the authors characterize the droplets formed with RNA? Are they liquid-like/gel like? Are they reversible (meaning if diluted, do they persist or dissolve)? How do they compare to DNA-cGAS droplets?

Why does BSA seem to inhibit phase separation? One would expect that crowder increases interaction and phase separation. Could the authors try a different crowder such as PEG or Ficoll, and discuss these results?

Referee #2:

In this work, Chen et al. study the impact of RNA on liquid phase condensation of cGAS. Through a set of well-designed in vitro experiments with recombinant protein, RNA and/or DNA, the authors demonstrate that cGAS can form liquid phase condensation in the presence of RNA (tRNA or total RNA). While the capacity of RNA to induce such phase separation of cGAS

is not novel per se (this was previously shown in the paper from James Chen [Supplementary Figure S2] with 45bp dsRNA - science.aat1022/DC1), the present study goes much further and suggests that endogenous RNA such as tRNAs are naturally bound to cGAS in steady state in the cytosol, to prevent activation by short DNA. As such, Figure 2 supports the concept that cGAS is naturally bound to RNAs in steady states. Relying on further in vitro analyses, the authors demonstrate that DNA and RNA can substitute each other in the cGAS phase precipitates, and that short DNA cannot displace tRNA-cGAS aggregates, while Y-form and >45 long dsDNA can (Figures 3/4). Finally the authors look at cGAMP production and demonstrate that cGAMP production is increased by tRNA when low levels of dsDNA are present.

Overall, the paper is interesting, well written and well constructed, however the claims need further "in cell" experiments to validate the in vitro findings.

One of the key claims made by the authors revolves around the concept that endogenous cytoplasmic RNAs interacting with cGAS form liquid phase separations which are not functional per se, but facilitate sensing of small amount of cytoplamic dsDNA (provided these are long enough - or with Y-DNA structure).

1) Figure 2 does support the co-localisation of RNA with cGAS (although Figure 2C/D is not very clearly explained in the text - the understanding of this reviewer being that RNase treatment affects the phase in which cGAS is present, but DNAse does not). However, some of the concepts demonstrated in Figs 3, 4 and 5 would need to be supported with cell experiments. One possible descriptive experiment would be to look at the co-localisation of labelled transfected short RNA with cGAS, similar to Figure 2E, and show that potential RNA positive foci can be displaced by transfection of DNA (Cy5-ISD).

2) It would be ideal to support the claims that RNA can functionally modulate DNA sensing by cGAS in cells. For instance, in cells where cytoplasmic DNA is naturally produced (for instance in TREX1-/- cells), transfection of short RNAs may impact cGAS sensing (noting that according to the RNAseq data, short RNAs like miRNAs may be able to do this).
Similarly, based on the concept from the paper that RNA can potentiate dsDNA sensing, one would speculate that co-transfection of dsDNA with RNA should increase cGAMP production.

3) Given that several technologies rely on RNA transfection (siRNA, miRNA, sgRNAs), it would be interesting to define the length requirements for RNA induced cGAS phase separation with short synthetic RNAs (below 45 dsRNA molecules). Assuming that short RNAs can increase cGAS sensing of endogenous cytoplasmic DNA, for instance present in some cancer cells, it may be possible that transfection of short RNAs could boost basal cGAS signalling in these cells, to induce off-target effects (a point worth discussing).

4) It would make sense to try to better support the direct interaction between tRNAs and cGAS, endogenously. For instance, using an anti RNA antibody (for confocal, but possibly also pull down)

Minor points
- Can you indicate for each figure, how many times the experiments have been carried out?
- Figure 2C/D: explain better what the figures are showing in the text (how the impact of the RNase treatment affects cGAS migration in the gradient)
- Figure S2B is hard to see - it would be easier at a table with +++ or + /-...

Referee #3:

In this manuscript, Chen and colleagues propose a model of cGAS regulation in which cellular RNAs thresholds cGAS activation based on dsDNA concentration: RNA is a competitive inhibitor of cGAS at high concentrations of dsDNA, whereas RNA promoted-phase separation of cGAS increases interactions with low levels dsDNA to promote activation.

Consistent with their conclusion that RNA can phase separate with cGAS in vitro, this and prior work has shown cGAS can bind RNA in vitro. This raises two key questions:
1) Does cGAS interact with RNA in cells? The authors' argument and my concerns about this conclusion are as follows:
a) Observation #1:The authors show that over-expressed cGAS forms foci in cells that stain with the RNA dye pyronin Y. However, since these foci are only seen when cGAS is overexpressed, and pyronin Y is not highly specific, I find this argument insufficient to conclude that cGAS forms LLPS with RNA in cells.
b) Observation #2: The authors show the sedimentation of cGAS protein is shifted in an optiprep gradient after RNase treatment. However, I would be nervous about this conclusion since i) the shift is quite small, and ii) the RNase treated gradient appears to have run slightly different examining the lipid bands, which might account for the small shift in the position of the cGAS protein.
All in all, if the authors want to show cGAS binds RNA in cells (even prior to DNA activation) they should work with endogenous protein and see if cGAS has been identified as an RNA-binding protein in global surveys of RNA binding proteins. In addition, if the authors want to make the conclusion that cGAS is forming LLPS in cells with RNA, they should show endogenous protein in foci that are RNase sensitive. Ideally, smFISH to directly show a specific RNA in these foci would be most conclusive.
Taken together, if the authors want to show that cGAS interacts and is modulated by RNA in cells additional experiments will be

required to demonstrate this point.

2) A second key issue is whether the interaction of cGAS with RNA modulates activity of the enzyme.
The authors suggest this is relevant since:
a) In in vitro LLPS experiments, they observe that DNA and RNA can compete for forming LLPS with cGAS protein.
b) That in vitro, RNA can increase the activity of cGAS protein at low DNA concentrations (although this is a very small effect), and at high DNA concentrations can inhibit cGAS activity.
The issue here is whether this is relevant to the situation in cells. This will be a difficult point to prove as it would require identifying mutations in cGAS that alter RNA, but not DNA, binding (if that is even possible), and showing they have an impact on the activity in cells. Alternatively, they could demonstrate that co-transfection with RNA enhances the cGAS response in cells at low DNA concentrations but inhibits at high DNA concentrations.
The bottom line is that additional evidence demonstrating the biological relevance of RNA modulating cGAS activity in cells would be required to make the conclusion the in vitro observations are significant.

Additional Specific comments:

1) Related to Figure 1. The authors should employ a negative control protein and/or hcGAS mutants to show that phase separation with tRNA is specific under their assay conditions.

2) Related to Figure 2A. The authors should use a YFP control or a negative control RNA-binding protein to show that co-localization of these RNA aggregates are specific to cGAS since RNA-containing aggregates appear to be present in cGAS-YFP-null cells. The authors should employ cGAS mutants to identify the regions responsible for this localization and determine if these are required for phase separation in vitro, as suggested above for Figure 1. To show that these structures have properties of phase separations, the authors could measure the dynamics of cGAS exchange by FRAP and perform live-cell imaging to determine if these structures merge.

3) Related to Figure 2E. The authors show that cy5-ISD incorporates into a cGAS aggregate. The authors should use this assay to show that what they observe in vitro in Figure 5 occurs in cells. Specifically, they should stain with RNA, showing that these preformed cGAS granules contain RNA, and that as ISD is incorporated into these cGAS-RNA aggregates, the RNA staining reduces proportionally.

Additional minor comments:
4) The authors should estimate that expression level of their DOX-induced cGAS-YFP and determine if it is overexpressed in comparison to endogenous cGAS, as overexpression of proteins can cause aggregation.
5) Does FL-hcGAS phase separated in vitro with RNA run in fraction 5, similar to endogenous cGAS, when analyzed via Opti-gradient?

6) Figure 2E. Quantification of how often transfected cy5-ISD merges with preformed cGAS granules would strengthen this result.
7) Lines 170-171: "This tRNA mediated formation of phase separation promotes the activation of cGAS with even only a few dsDNA molecules." This line is confusing considering that lines 155-156 state: "tRNA had little or no effect on cGAS activity when the DNA concentration was less than 0.01 mg/mL". Could the authors clarify.
8) Lines 177-179: "At a low dsDNA concentration that is not enough to induce the formation of phase separation, cytoplasmic RNAs, especially tRNAs, form aggregates with cGAS that provide platforms for dsDNA-mediated cGAS activation." The authors should attempt to modulate endogenous RNA levels to actually show their in vitro model is relevant to cells.
9) Lines 180-181: "When the cytoplasmic concentration of dsDNA is high enough to induce phase separation and activate cGAS." This statement is misleading as the author's claim that cGAS is already in phase separation aggregates with RNA under normal conditions. The authors should clarify.
10) The authors claim in lines 182-184 that "given the high concentration of the RNAs in cytoplasm, the RNAs are likely the dominant regulators of cGAS activity." Given this statement, the authors should comment about how changes in cytoplasmic RNAs during the antiviral response, such as during RNase L-mediated RNA decay, may affect cGAS activation.
11) Line 109-111: "hcGAS was detected in fractions from each of these bands by western blot with a cGAS-specific antibody. The endogenous cGAS proteins was located mainly in band 5." The authors should more clearly state in the text what cGAS being in this fraction means. Also, it would be helpful to show which fraction number corresponds to the location in gradient in diagram Figure 2B.
12) Related to Figure 4. Quantification would make the results more convincing.
13) Related to Figure 5. The magnitude of effect of tRNAs promoting cGAMP production in Figure 5C appears to be inconsequential considering the activity is over an order of magnitude lower than with higher dsDNA concentrations, even when being inhibited by tRNAs. Is this difference biologically relevant and meaningful?

Point-by-point responses to the reviewers' comments

Dear Reviewers,

We thank you very much for your thoughtful comments and helpful suggestions for our manuscript. We have revised the manuscript based on your comments and our supplemental experiments. All of the significant revisions in the manuscript were highlighted in red. The point-by-point responses are listed below in blue.

Referee #1:

The manuscript proposed by Chen et al, reports on the phase separation of the protein cGAS, a dsDNA sensor involved in the innate immune response, with cellular RNA and its potential role in regulating immune response in cells. Although the ability of cGAS to phase separate with RNA has been previously shown, the authors provide insights about the physiological significance of this phenomenon. In particular, their data suggest that phase separation of cGAS with RNA enhances immune response when the concentration of pathogen dsDNA is low, while it inhibits overactivation of cGAS in presence of high concentration of dsDNA. I believe these results could be of interest for the scientific community, however, the data presented by Chen et al, does not fully support their claims and would benefit from additional experiments, statistical analysis and discussions (see below). Moreover, I believe the quality of the data (particularly the microscopy images) should be improved to allow a better appreciation of the results.
Overall, I would recommend publications of this manuscript after major revisions that respond to the comments below:

1) *The authors seem to use the terms phase separation and aggregation interchangeably. However, aggregation and phase separation are two mechanistically distinct phenomena. The first one relies on the irreversible misfolding and loss of function of proteins whereas the other one is a reversible thermodynamic process in which macromolecules rearrange in space, without any loss of function. The authors should avoid this confusion, that would be misleading for readers that are not familiar with the field.*

We thank the referee for indicating this. We had changed "aggregation" to "phase-separated granules" or "phase-separated condensate" (Garcia-Jove Navarro et al., 2019, Mehta and Zhang, 2022).

2) The authors tend to overinterpret their data and need to add statistical analyses:

a. *In many figures, Chen et al compare the ability of different dsDNA and RNAs to promote/trigger cGAS phase separation and often infer that some specific DNA/RNA species are more efficient than others at triggering cGAS phase separation. The images presented by the authors do not allow to support such conclusions. The authors should map the phase diagrams of cGAS with each DNA/RNA specie, and comparison should be driven from the threshold concentration of protein/nucleic acid necessary to trigger phase separation. Indeed, it is hard to tell from Figure S2B for instance, whether the presence of single stranded arms actually influence the phase separation of cGAS, as stated by the authors. The pictures presented in this figure only suggest that 48 bases DNA triggers cGAS phase separation and that partial double strand does not or poorly affect the formation of droplets.*

Sorry for the misleading description. We intended to point out that dsDNAs shorter than 20 bp barely activate FL-hcGAS or form phase separated condensates with FL-cGAS. Single stranded DNA of 48 bases can induce phase separation, but can not activate cGAS. However, once attached with one or two ssDNA arms, short double stranded DNAs (6-20 bp) induce phase separations of FL-hcGAS and activate the enzyme. We have reorganized the figure and the text in the figure legend to make it clear. In addition, we have made the phase diagrams to show the formation of phase separation with single stranded DNA of 48 bases and short double stranded DNAs (6-20 bp) with one or two ssDNA arms, but not short dsDNAs, for better comparisons (Figure EV2).

b. *In Figure 1, the authors claim that RNA does not activate cGAS, but they only show activity assays involving tRNA. The authors should test whether other RNA species or total RNA have similar effect.*

We have added the data with extracted total RNA and the results show similar effect (Figure 1B).

c. *In Figure 2: The authors claim that cells overexpressing hcGAS contain phase-separated granules. Although the data does indicate the formation of puncta in cells, the authors do not provide any evidence of the phase separated nature of these structures (fusion/dynamism of cGAS when FRAPed/flow in the cytosol). These structures could be amorphous aggregates that form due to overexpression of the protein and that sequester RNA. The authors should provide evidence of their phase separated behavior. Moreover, the authors should test whether endogenous cGAS form puncta in cells under normal conditions. Indeed, the puncta observed by the authors could be artefacts of overexpression.*

We have done FRAP (Figure 2B-C) and the results show the dynamic nature of the hcGAS granules formed in cells. We have added the data in Figure 2B-C. We also observed the state of endogenous cGAS in HeLa cells stained with a cGAS-specific antibody and the results showed that although cGAS does not form big puncta, cGAS is not uniformly distributed and is in a condensed form (Figure 2E-G). Furthermore, endogenous RNAs labeled through a highly specific click-reaction are well correlated and colocalized with endogenous cGAS (Figure 2G-I).

d. *Figure S6: The authors indicate that cGAS binds primarily tRNA and rRNA, the two most abundant RNA species in cells. Although the data do show a slightly higher RNA binding on these populations, the lack of statistical analysis does not permit to establish such strong statement. The experiment should be repeated, and statistical analysis should be performed.*

We thank the referee for the suggestion. We improved our protocol for the OptiPrep gradient so that the bands with cGAS are well separated from others. We also repeated the RNA sequencing twice. However, the reads for different RNAs can only indicate the RNA species in the condensate, but cannot be used to quantitate the amount of each RNA species. So, we changed the description in the text as “The sequencing results indicated that

cGAS is associated with different RNA species, including mRNA, microRNA, tRNA and rRNA". The results were updated in Figure S4.

3) Additional explanation or discussion of the results would bring clarity to the manuscript:

a. Why did the authors decided to focus on tRNA? Is cGAS predicted to have low specificity towards RNA?

We focused on tRNA because it is the most abundant RNA molecules as reported by other literatures (Palazzo and Lee, 2015). In addition, the tRNA is stable and the results with tRNA are not affected by the degradation problem of other RNAs such as mRNA. We also performed additional assays by using the total RNA extraction. Our EMSA assays and substitution assays do not support that cGAS have lower specificity towards RNA when compared with dsDNA.

b. What is the Opti-prep gradient and why did the author use this approach?

OptiPrep is the trade name of iodixanol by Sigma Aldrich. We have added the details is the manuscript. We used this approach by following a study on the dsDNA induced cGAS phase separation by James Chen et al. Cytoplasmic fractions with different densities can be well separated by using an OptiPrep gradient.

c. Figure 3B: could the authors discuss the different behavior of each DNA used in this figure? In particular, 380 bp DNA shows a curious behavior where it first displaces tRNA and promotes recruitment of tRNA to the droplets? Moreover, is there a relationship between length/structure of the DNA species used and their ability to displace tRNA? How does that relate to the function of cGAS in cells and to the immune response?

We thank the referee for indicating that. We noticed this phenomenon as well. We have carefully reexamined and repeated the experiment with different dsDNA prepares. The 380 bp dsDNA was produced by PCR and was purified by using a PCR product purification kit, while other dsDNAs were prepared by annealing a pair of ssDNAs in a buffer containing 20 HEPES at pH7.5 and 150 mM NaCl. We found that the abnormal behavior of the 380 bp dsDNA showed in the manuscript was caused by the salt contamination during the preparation process. We removed the salt contamination, repeated the assays and corrected the curve in Figure 4B. As for the relationship between length/structure of the DNA species used and their ability to displace tRNA, long dsDNA and DNA with special structure such as the Y-form DNA can easily displace tRNA. Short dsDNA such as the 14 or 20 bp dsDNAs barely displace tRNA. We assumed that this is the reason that long dsDNAs and Y-form DNAs activate cGAS in cells, while short dsDNA such as 14 or 20 bp dsDNAs barely activate cGAS in cells.

Other comments:

Figure 1B and 4: hcGAS forms amorphous aggregates with RNA in vitro, at reasonably low concentrations. Is there any physiological relevance for the formation of these structures? Could these aggregates relate to the functions of cGAS in cells?

To further investigate the physiological relevance of the RNA regulated cGAS activity in cells, we co-transfected RNA with ISD into THP1 cells. We found that co-transfection of RNAs strongly enhanced the production of IFNb (a fold change of over 20) in cells when the dsDNA concentration is low (Figure 5J), indicating that RNA helps to activate cGAS in cells.

Figure S6: Could the authors add a pie chart of the relative abundance of each RNA populations in cells to indicate whether there is a preferential binding of cGAS towards certain types of RNA?

We did a literature search on the abundance of the RNAs in cells. However, there is no consistent conclusion on the relative abundance of each RNA populations in cells. A common conclusion is that tRNA should be relatively abundant. We have changed in the manuscript that cGAS is associated with RNA in cells.

The authors use both μM and μg/mL for DNA quantification. The authors should homogenize units to allow comparison between the different figures and conditions.

We have made the unit consistent.

If it has not been done yet, could the authors characterize the droplets formed with RNA? Are they liquid-like/gel like? Are they reversible (meaning if diluted, do they persist or dissolve)? How do they compare to DNA-cGAS droplets?

Our FRAP assay showed that the RNA-cGAS droplet is liquid-like and reversible (Figure 2B-C). The fluorescence signal of droplet could be recovered after photobleach in ~ 10 seconds. Similar phenomena have also been observed for the dsDNA-cGAS droplets (Du and Chen, 2018).

Why does BSA seem to inhibit phase separation? One would expect that crowder increases interaction and phase separation. Could the authors try a different crowder such as PEG or Ficoll, and discuss these results?

We used PEG as the crowder (Figure S2C). It also slightly diminishes phase separation. The crowd environment may prevent the quick diffusion of the molecules, thus slightly inhibit the formation of the phase-separated droplets.

Referee #2:

In this work, Chen et al. study the impact of RNA on liquid phase condensation of cGAS. Through a set of well-designed in vitro experiments with recombinant protein, RNA and/or DNA, the authors demonstrate that cGAS can form liquid phase condensation in the presence of RNA (tRNA or total RNA). While the capacity of RNA to induce such phase separation of cGAS is not novel per se (this was previously shown in the paper from James Chen [Supplementary Figure S2] with 45bp dsRNA – science.aat1022/DC1), the present study goes much further and suggests that endogenous RNA such as tRNAs are naturally bound to cGAS in steady state in the cytosol, to prevent activation by short DNA. As such, Figure 2 supports the concept that cGAS is naturally bound to RNAs in steady states. Relying on further in vitro analyses, the authors demonstrate that DNA and RNA can substitute each other in the cGAS phase precipitates, and that short DNA cannot displace tRNA-cGAS aggregates, while Y-form and >45 long dsDNA can (Figures 3/4). Finally the authors look at cGAMP production and demonstrate that cGAMP production is increased by tRNA when low levels of dsDNA are present.

Overall, the paper is interesting, well written and well constructed, however the claims need further "in cell" experiments to validate the in vitro findings.

Thanks for the positive comments. We have added the "in cell" experiments. We cotransfected RNA with ISD into THP1 cells. We found that co-transfection of RNAs strongly enhanced the production of IFNb in cells when the dsDNA concentration is low (Figure 5J), indicating that RNA helps to activate cGAS in cells. These results are consistent with our in vitro observations.

One of the key claims made by the authors revolves around the concept that endogenous cytoplasmic RNAs interacting with cGAS form liquid phase separations which are not functional per se, but facilitate sensing of small amount of 5ytoplasmic dsDNA (provided these are long enough – or with Y-DNA structure).

1) Figure 2 does support the co-localisation of RNA with cGAS (although Figure 2C/D is not very clearly explained in the text – the understanding of this reviewer being that Rnase treatment affects the phase in which cGAS is present, but DNAse does not). However, some of the concepts demonstrated in Figs 3, 4 and 5 would need to be supported with cell experiments. One possible descriptive experiment would be to look at the co-localisation of labelled transfected short RNA with cGAS, similar to Figure 2E, and show that potential RNA positive foci can be displaced by transfection of DNA (Cy5-ISD).

We labeled endogenous RNAs with a new approach, which is based on a highly specific click reaction. The results showed that ISD is colocalized with both cGAS and endogenous RNA in cells. The labeling method is descripted in brief as the following: 5-ethynyl uridine was added to the culture medium of HEK293T cells that express cGAS-GFP. 5-ethynyl uridine is incorporated into newly synthesized RNAs as the cell grows. After 18 h, Cy5-ISD was transfected into the cells. After another 20 h, cells were fixed. Newly synthesized endogenous RNAs containing the special uridine with an ethynyl group was stained by azide-fluor 545, a dye only reacts with the ethynyl group in RNAs. By using this specific labeling method, we observed that Cy5-ISD is colocalized with both hcGAS-GFP and endogenous RNAs in cells (Figure 3B, Figure EV4). We also showed that endogenous cGAS and RNA are highly correlated and colocalized in cells (Figure 2G-I).

2) It would be ideal to support the claims that RNA can functionally modulate DNA sensing by cGAS in cells. For instance, in cells where cytoplasmic DNA is naturally produced (for instance in TREX1-/- cells), transfection of short RNAs may impact cGAS sensing (noting that according to the RNAseq data, short RNAs like miRNAs may be able to do this).
Similarly, based on the concept from the paper that RNA can potentiate dsDNA sensing, one would speculate that co-transfection of dsDNA with RNA should increase cGAMP production.

Thank you for your advices. Referred to our responses to reviewer1's comments and reviewer3's comment 10, we further investigated the physiological relevance of the RNA mediated regulation of cGAS activity in cells. We found that it would be difficult to reduce the amount of RNA in cells. However, it would be possible to increase the amount of RNA in cells by transfection as this comment suggested. Thus, we co-transfected RNA with ISD into THP1 cells. We found that the co-transfected RNAs strongly enhance the production of IFNb in cells when dsDNA concentration is low (Figure 5J). We observed a fold change of over 20 when RNA was cotransfected.

3) Given that several technologies rely on RNA transfection (siRNA, miRNA, sgRNAs), it would be interesting to define the length requirements for RNA induced cGAS phase separation with short synthetic RNAs (below 45 dsRNA molecules). Assuming that short RNAs can increase cGAS sensing of endogenous cytoplasmic DNA, for instance present in some cancer cells, it may be possible that transfection of short RNAs could boost basal cGAS signalling in these cells, to induce off-target effects (a point worth discussing).

In our updated cell assay results, tRNA (or dephosphorylated ssRNA) and ISD were co-transfected into THP1 cells, both tRNA, 50 nt ssRNA, and 200 nt ssRNA enhanced the production of IFNb. However, the RNA along did not induce the expression of IFNb (Figure 5J).

4) It would make sense to try to better support the direct interaction between tRNAs and cGAS, endogenously. For instance, using an anti RNA antibody (for confocal, but possibly also pull down)

As mentioned above, we used a new method for specific RNA labeling (Jao and Salic, 2008). The results showed that endogenous cGAS in HeLa cell cytoplasm is colocalized with endogenous RNAs, which is a direct evidence to the direct interaction between tRNAs and cGAS, endogenously (Figure 2G-I).

Minor points
- Can you indicate for each figure, how many times the experiments have been carried out?

Done

- Figure 2C/D: explain better what the figures are showing in the text (how the impact of the RNase treatment affects cGAS migration in the gradient)

Done

- Figure S2B is hard to see - it would be easier at a table with +++ or + /-...

To make it easier for comparison, we made additional phase diagrams with FITC-labeled cGAS using fluorescent microscopy (Figure EV2).

Referee #3:

In this manuscript, Chen and colleagues propose a model of cGAS regulation in which cellular RNAs thresholds cGAS activation based on dsDNA concentration: RNA is a competitive inhibitor of cGAS at high concentrations of dsDNA, whereas RNA promoted-phase separation of cGAS increases interactions with low levels dsDNA to promote activation.

Consistent with their conclusion that RNA can phase separate with cGAS in vitro, this and prior work has shown cGAS can bind RNA in vitro. This raises two key questions:
1) Does cGAS interact with RNA in cells? The authors' argument and my concerns about this conclusion are as follows:
a) Observation #1:The authors show that over-expressed cGAS forms foci in cells that stain with the RNA dye pyronin Y. However, since these foci are only seen when cGAS is overexpressed, and pyronin Y is not highly specific, I find this argument insufficient to conclude that cGAS forms LLPS with RNA in cells.
b) Observation #2: The authors show the sedimentation of cGAS protein is shifted in an optiprep gradient after RNase treatment. However, I would be nervous about this conclusion since i) the shift is quite small, and ii) the RNase treated gradient appears to have run slightly different examining the lipid bands, which might account for the small shift in the position of the cGAS protein.
All in all, if the authors want to show cGAS binds RNA in cells (even prior to DNA activation) they should work with endogenous protein and see if cGAS has been identified as an RNA-binding protein in global surveys of RNA binding proteins. In addition, if the authors want to make the conclusion that cGAS is forming LLPS in cells with RNA, they should show endogenous protein in foci that are RNase sensitive. Ideally, smFISH to directly show a specific RNA in these foci would be most conclusive.
Taken together, if the authors want to show that cGAS interacts and is modulated by RNA in cells additional experiments will be required to demonstrate this point.

Thank you for your advices. To make the pyronin Y staining experiment more reliable, we used the 293T-YFP-null cell as a negative control and added the data in Figure 2A. In addition, we used a highly specific click reaction based approach for RNA labeling (Jao and Salic, 2008) and proved that endogenous RNA is colocalized with endogenous cGAS (Figure 2G-I). See also responses to Referee 2-point1. The labeling method is described briefly as the following: 5-ethynyl uridine was added to the culture medium of HeLa cells and incorporation of 5-ethynyl uridine into newly synthesized RNA enables further specific RNA labeling with the dye Alexa Fluor 488-azide, which only reacts with the 5-ethynyl uridines in the RNAs. Then, cGAS in HeLa cells was stained with a specific antibody. Correlation coefficient analysis showed that endogenous cGAS is colocalized with RNAs (Figure 2G, I). As a control, mcherry in 293T cells is not colocalized with RNA (Figure 2H, I). What's more, using similar RNA labeling method, we proved that Cy5-ISD is colocalized with both hcGAS-GFP and endogenous RNA in 293T cells (Figure 3B, Figure EV4). In addition, we improve our

OptiPrep gradient ultracentrifugation experiment and the band shift is much more obvious, which provides additional solid evidence (Figure 2D-F).

2) A second key issue is whether the interaction of cGAS with RNA modulates activity of the enzyme.
The authors suggest this is relevant since:
a) In in vitro LLPS experiments, they observe that DNA and RNA can compete for forming LLPS with cGAS protein.
b) That in vitro, RNA can increase the activity of cGAS protein at low DNA concentrations (although this is a very small effect), and at high DNA concentrations can inhibit cGAS activity.
The issue here is whether this is relevant to the situation in cells. This will be a difficult point to prove as it would require identifying mutations in cGAS that alter RNA, but not DNA, binding (if that is even possible), and showing they have an impact on the activity in cells. Alternatively, they could demonstrate that co-transfection with RNA enhances the cGAS response in cells at low DNA concentrations but inhibits at high DNA concentrations.
The bottom line is that additional evidence demonstrating the biological relevance of RNA modulating cGAS activity in cells would be required to make the conclusion the in vitro observations are significant.

Thank you for the suggestion. Referred to our responses to the other two reviewers on the biological relevance of the RNA modulated cGAS activity. We co-transfected RNA with ISD into THP1 cells. We found that co-transfected RNAs strongly enhances the production of IFNb (the fold change is over 20) in cells when the amount of the transfected dsDNA is low (0.1 μg) (Figure 5J), which indicates that RNAs can play an important role in regulated the cGAS activity in vivo. We did not observe any inhibition of cGAS activity when transfecting a higher amount of dsDNA into THP1 cells, probably due to the concentration of the transfected ISD has not reach the threshold for the inhibition effect. However, the fold change (~2) decreases significantly when RNA was co-transfected with a high amount of ISD (1 μg). Transfecting the cells with excessive ISD (more than 5 μg) and lipofectamine 2000 caused cell death, which suggests that the inhibition effects of RNA may not be observed under physiological conditions.

Additional Specific comments:

1) Related to Figure 1. The authors should employ a negative control protein and/or hcGAS mutants to show that phase separation with tRNA is specific under their assay conditions.

We have added a negative control by using FITC labeled mcGAS-CTD (Figure1C, D).

2) Related to Figure 2A. The authors should use a YFP control or a negative control RNA-binding protein to show that co-localization of these RNA aggregates are specific to cGAS since RNA-containing aggregates appear to be present in cGAS-YFP-null cells. The authors should employ cGAS mutants to identify the regions responsible for this localization and determine if these are required for phase separation in vitro, as suggested above for Figure 1. To show that these structures have properties of phase separations, the authors could measure the dynamics of cGAS exchange by FRAP and perform live-cell imaging to determine if these structures merge.

We updated our experiments and shows that no RNA-containing condensates present in cGAS-YFP-null cells (Figure 2A). Also, FRAP experiment was performed to prove that the condensates have the dynamic properties of phase separations (Figure 2B-C).

3) Related to Figure 2E. The authors show that cy5-ISD incorporates into a cGAS aggregate. The authors should use this assay to show that what they observe in vitro in Figure 5 occurs in cells. Specifically, they should stain with RNA, showing that these preformed cGAS granules contain RNA, and that as ISD is incorporated into these cGAS-RNA aggregates, the RNA staining reduces proportionally.

Technically, it is difficult to specifically stain or label RNA in live cells for imaging experiment. We did alternative experiments to prove that the transfected ISD is colocalized with both endogenous cGAS and RNA. We added 5-ethynyl uridine in the cell cultures of HEK293T cells that express cGAS-GFP. The 5-ethynyl uridine is incorporated into newly synthesized RNA when the cell grows. Cy5-ISD was then transfected into the cells. After then, cells were fixed and newly synthesized endogenous RNAs were labeled by azide-fluor 545, which can have a highly specific click reaction with the ethynyl group of the 5-ethynyl uridine insertions in RNAs. By using this specific RNA labeling method, colocalization of Cy5-ISD with both cGAS-GFP and endogenous RNA was observed by using a confocal microscope (Figure 3B, Figure EV4).

Additional minor comments:
4) The authors should estimate that expression level of their DOX-induced cGAS-YFP and determine if it is overexpressed in comparison to endogenous cGAS, as overexpression of proteins can cause aggregation.

To address the concern about the overexpression, we used a new specific approach to label endogenous RNAs and the results showed that endogenous RNA and cGAS are colocalized in cells (Jao and Salic, 2008). Please see the details in our responses to point 3 and to similar points raised by other referees. The results are updated in Figure 2G-I.

5) Does FL-hcGAS phase separated in vitro with RNA run in fraction 5, similar to endogenous cGAS, when analyzed via Opti-gradient?

We further analyzed FL-hcGAS and tRNA mixture via the OptiPrep gradient ultracentrifugation and find that the FL-hcGAS-tRNA complex is located at the bottom of the gradient. As a control, FL-hcGAS without RNA stay on the top of the gradient (Figure 2D-F).

6) Figure 2E. Quantification of how often transfected cy5-ISD merges with preformed cGAS granules would strengthen this result.

We have quantified the colocalized cy5-ISD, cGAS-GFP and RNA (Figure 3B, Figure EV4).

7) Lines 170-171: "This tRNA mediated formation of phase separation promotes the activation of cGAS with even only a few dsDNA molecules." This line is confusing considering that lines 155-156 state: "tRNA had little or no effect on cGAS activity when the DNA concentration was less than 0.01 mg/mL". Could the authors clarify.

We corrected the description as “tRNA enhanced cGAS activity when the DNA concentration is low and in the range of 0.0034 and 0.0136 mg/mL (Figure 5A).”

8) Lines 177-179: "At a low dsDNA concentration that is not enough to induce the formation of phase separation, cytoplasmic RNAs, especially tRNAs, form aggregates with cGAS that provide platforms for dsDNA-mediated cGAS activation." The authors should attempt to modulate endogenous RNA levels to actually show their in vitro model is relevant to cells.

We further performed experiments in cells by co-transfecting RNA with ISD into THP1 cells. We found that co-transfected RNAs strongly enhances cGAS activity in cells when dsDNA concentration is low (Figure 5J).

9) Lines 180-181: "When the cytoplasmic concentration of dsDNA is high enough to induce phase separation and activate cGAS." This statement is misleading as the author's claim that cGAS is already in phase separation aggregates with RNA under normal conditions. The authors should clarify.

Sorry for the misleading description. We have changed this sentence to “when the concentration of dsDNA is high enough to induce strong phase separations and to replace the RNAs in the preformed condensates of cGAS-RNA, dsDNAs and RNAs could be in a dynamic equilibration, through which the RNAs can compete with the dsDNAs and presumably limit over activation of the enzyme.”.

10) The authors claim in lines 182-184 that "given the high concentration of the RNAs in cytoplasm, the RNAs are likely the dominant regulators of cGAS activity." Given this statement, the authors should comment about how changes in cytoplasmic RNAs during the antiviral response, such as during RNase L-mediated RNA decay, may affect cGAS activation.

Thank you for your advice. We made a stable THP1 cell line that enable inducible expression of RNase L, and analyzed the IFNb mRNA level after transfection of ISD or co-transfection of ISD and tRNA. We found that expression of RNase L enhanced the IFNb mRNA level. RNase L can not diminish RNA but cleave the RNAs in to short self-RNAs (6). We showed that short ssRNA of 50 nts can also enhance the IFNb production together with ISD. Furthermore, it has been reported that small self-RNA generated by RNase L can stimulate the antiviral innate immunity via RIG-I, MDA5 and IPS-1 (Malathi et al., 2007). So, we feel that it is not possible to differentiate the role of RNase L in its indirect effects on cGAS activation or in stimulating the RIG-I based pathway.

11) Line 109-111: "hcGAS was detected in fractions from each of these bands by western blot with a cGAS-specific antibody. The endogenous cGAS proteins was located mainly in band 5." The authors should more clearly state in the text what cGAS being in this fraction means. Also, it would be helpful to show which fraction number corresponds to the location in gradient in diagram Figure 2B.

We improved our OptiPrep gradient ultracentrifugation experiment and cGAS proteins was located mainly in band 3 and band 4, where the opti-prep concentration is between 20%-25% and the density is between 1.107-1.133 g/mL, which means that cGAS protein is in a heavy and condensed form. We have labeled the positions of the bands in the gradient.

12) Related to Figure 4. Quantification would make the results more convincing.

We have added two new panels (Figure 4F-G) to show the quantification results.

13) Related to Figure 5. The magnitude of effect of tRNAs promoting cGAMP production in Figure 5C appears to be inconsequential considering the activity is over an order of magnitude lower than with higher dsDNA concentrations, even when being inhibited by tRNAs. Is this difference biologically relevant and meaningful?

We found that co-transfected RNAs with ISD into THP1 cells strongly enhances cGAS activity in cells with a fold change of over 20 when the amount of dsDNA used is low (Figure 5J). So, we think this difference is biologically relevant and meaningful.

DU, M. & CHEN, Z. J. 2018. DNA-induced liquid phase condensation of cGAS activates innate immune signaling. *Science,* 361**,** eaat1022.

GARCIA-JOVE NAVARRO, M., KASHIDA, S., CHOUAIB, R., SOUQUERE, S., PIERRON, G., WEIL, D. & GUEROUI, Z. 2019. RNA is a critical element for the sizing and the composition of phase-separated RNA-protein condensates. *Nat Commun,* 10**,** 3230.

JAO, C. Y. & SALIC, A. 2008. Exploring RNA transcription and turnover in vivo by using click chemistry. *Proc Natl Acad Sci U S A,* 105**,** 15779-84.

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MEHTA, S. & ZHANG, J. 2022. Liquid-liquid phase separation drives cellular function and dysfunction in cancer. *Nat Rev Cancer,* 22**,** 239-252.

PALAZZO, A. F. & LEE, E. S. 2015. Non-coding RNA: what is functional and what is junk? *Frontiers in genetics,* 6**,** 2.

Dear Dr. Xiang,

Thank you for the submission of your revised manuscript to our editorial offices. I have received the reports from the three referees that were asked to re-evaluate your study that I have already forwarded to you, you will also find again below. Looking through the preliminary point-by-point response you sent, I think that most remaining points of the referees will be adequately addressed during a further revision as indicated in your letter. I thus ask you to address these comments in a final revised manuscript as suggested.

Nevertheless, referees indicated during cross-commenting (and after looking through your p-b-p-response):

Referee #2:
I recommend that the authors do the following experiments to support their claims (and rule out a feedback of RNA sensing [albeit very weakly activated] on cGAS sensing - which is very possible): Do co-transfection of DNA and RNA using cGAS KO THP-1 (as a control) AND measure the levels of cGAMP in cell lysates from WT cells co-transfected with DNA and RNA.

Referee #3:
My comment about the no RNA control has been addressed in the previous figure. My apologies for not noticing the controls. My comment about the formation of condensates as visualized by microscopy holds. I don't think that the comments the authors made address the issue.

Please also address these points in your final revised manuscript.

Moreover, I have these editorial requests I also ask you to address:

- Please provide the abstract written in present tense throughout and with not more than 175 words.

- Please add up to 5 keywords to the title page.

- We plan to publish your manuscript in the Report format. For a Scientific Report we require that results and discussion sections are combined in a single chapter called "Results & Discussion". Please do this for your manuscript. For more details please refer to our guide to authors: http://www.embopress.org/page/journal/14693178/authorguide#researcharticleguide

- We updated our journal's competing interests policy in January 2022 and request authors to consider both actual and perceived competing interests. Please review the policy https://www.embopress.org/competing-interests and update your competing interests if necessary. Please name this section 'Disclosure and Competing Interests Statement' and put it after the Acknowledgements section.

- We now use CRediT to specify the contributions of each author in the journal submission system. CRediT replaces the author contribution section. Please use the free text box to provide more detailed descriptions. Thus, please remove the author contributions section from the manuscript text file. See also guide to authors:
https://www.embopress.org/page/journal/14693178/authorguide#authorshipguidelines

- Please add a formal "Data Availability section" (DAS - placed after Materials and Methods) to the manuscript. This is now mandatory (like the COI statement). If no primary datasets have been deposited in any database, please state this in this section (e.g. 'No primary datasets have been generated and deposited').

Please order the manuscript sections like this (using these names):
Title page - Abstract - Keywords - Introduction - Results & Discussion - Materials & Methods - DAS - Acknowledgements - Disclosure and Competing Interests Statement - References - Figure legends - Expanded View Figure legends

- Please make sure that the number "n" for how many independent experiments were performed, their nature (biological versus technical replicates), the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values is indicated in the respective figure legends (main, EV and Appendix figures), and that statistical testing has been done where applicable. Please avoid phrases like 'independent experiment', but clearly state if these were biological or technical replicates. Please add complete statistical testing to all diagrams (main, EV and Appendix figures). Please also indicate (e.g. with n.s.) if testing was performed, but the differences are not significant.

- Please add scale bars of similar style and thickness to all the microscopic images (main, EV and Appendix figures), using clearly visible black or white bars (depending on the background). Please place these in the lower right corner of the images. Please do not write on or near the bars in the image but define the size in the respective figure legend.

- Please make sure that all the funding information is also entered into the online submission system and that it is complete and

similar to the one in the acknowledgement section of the manuscript text file.

- It seems there are no panel callouts for Fig. 1, EV1, EV2, EV3, Appendix Figs S2, S5 and S6. Please check and make sure that figure panels are called our separately and sequentially.

- For the callouts of the Appendix Figures, please use "Appendix Figure Sx".

- Also in the Appendix file legends, please name the figures "Appendix Figure Sx".

- In the Appendix, please move the legends directly below the figure on the same page (one figure plus legend per page). I think this is much more comprehensive.

- Please use our reference format:
http://www.embopress.org/page/journal/14693178/authorguide#referencesformat

- Finally, please find attached a word file of the manuscript text (provided by our publisher) with changes we ask you to include in your final manuscript text, and some queries, we ask you to address. Please provide your final manuscript file (using the attached file as basis) with track changes, in order that we can see any modifications done.

In addition, I would need from you:
- a short, two-sentence summary of the manuscript (not more than 35 words).
- two to four short bullet points highlighting the key findings of your study (two lines each).
- a schematic summary figure (in jpeg or tiff format with the exact width of 550 pixels and a height of not more than 400 pixels) that can be used as a visual synopsis on our website.

I look forward to seeing the final revised version of your manuscript when it is ready. Please let me know if you have questions regarding the revision.

Please use this link to submit your revision: https://embor.msubmit.net/cgi-bin/main.plex

Yours sincerely,

Achim Breiling
Senior Editor
EMBO Reports

Referee #1:

The manuscript re-submitted by Chen et al, explores the role of RNA in the phase separation of cGAS and the regulation of the immune response. In this manuscript, the authors show that, similar to various dsDNA species, RNA can trigger phase separation of cGAS in vitro, although these droplets to do activate the enzyme. Instead, RNA appears to regulate cGAS phase separation with DNA and subsequent cGAS activation. Indeed, the authors found that RNA increases cGAS activity when DNA concentrations are low, and decreases cGAS activity when DNA concentration are high, thus allowing a tight control of cGAS activity in response to pathogens.

In this revised manuscript, the authors provided excellent additional data that strengthened the manuscript message overall and answered all previous comments and concerns.

In addition, this manuscript is very well organized, well written and clear, and I believe that, overall, the presented data and conclusions will be of great interest for the fields of innate immune response and for the field of condensate biology.
Nevertheless, I must express some reserves regarding the interpretations and conclusions provided by the authors on Figure 2D-I and cannot support the publication of this manuscript in its present form.

Indeed, in figure 2, the authors evaluate whether endogenous hcGAS forms condensates with RNA in Hela cells, using an optiprep gradient analysis and a staining of cGAS/RNA and Pearson's correlation analysis.
Although the results clearly demonstrate that cGAS forms large complexes with RNA and colocalize with RNA in cells, this data does not fully support the authors conclusion that cGAS forms condensates with RNA.
Especially:
• No clear foci are visible in cells after staining of cGAS
• The complexes found in fraction 3 and 4 of the optiprep gradient are much less dense than the purified cGAS/tRNA droplets (which result in cGAS signal in fraction 6).
• The authors state that the density measurements are indicative of a condensed state -Such statement is incorrect.
As explained in the excellent review recently published by Julie Forman-Kay et al, (https://pubmed.ncbi.nlm.nih.gov/34772786/)

many types of complexes including molecular machines and supramolecular complexes (e.g. proteasome, ribosomes), are not condensates, and condensates may span a large array of sizes, ranging from a few nanometer in size to a micrometer-sized structures.
Therefore, density or size does not allow to differentiate a condensate from a large non-phase separated complexe or macro-structure, and the provided data does not support the formation of cGAS/RNA condensates in cells.

Nevertheless, the data does not disprove the formation of nanoscopic condensates that cannot be resolved in the provided images, nor the ability of cGAS to form large condensates with RNA, which may occur under conditions that have not been tested by the authors (for instance, infection by RNA viruses, or following translation arrest during a stress).

It also does not contradict the rest of the manuscript, especially regarding the ability of cGAS to undergo LLPS with RNA, as well as the role of RNA in regulating cGAS LLPS and activation.

Therefore, I strongly recommend revisiting this section before publication.

Referee #2:

The addition of the co-transfected DNA and RNA experiment in THP-1, along with other new experiments (such as new gradient purification and use of ethynyl RNA for co-localisation) have definitely increased the strength of the claims about the role of RNA in DNA sensing of cGAS. However it would have been ideal to have controlled for the need for cGAS in this response to co-transfected DNA and RNA (using cGAS KO THP-1) and also to measure the levels of cGAMP in cell lysates (which was requested in my original review). As it stands the THP-1 experiment is not unequivocal (i.e. the result of increase IFNb production could be due to another pathway/mechanism).

In addition, this reviewer specifically asked for details about the amount of independent repeats of the experiments shown - but this is only indicated on a few figure panels (noting that the authors responded "Done" to this point). Are we meant to understand that the data is often from single experiments?

The authors should also put their results in the context of the new literature on the topic - particularly the paper showing that G3BP1 (which binds dsRNA) binds cGAS to help form cGAS primary condensates.

Finally, the authors also need to explain the FRAP result - what was actually shown and how this "Proves" that we are dealing with LLPS.

Referee #3:

This revised manuscript is improved and the authors provide strong data for cGAS interacting with RNA in vitro and being able to form "phase-separated" assemblies in vitro with RNA. This is consistent with earlier work.
In my prior comments, I had raised
1) Whether there was sufficient data that endogenous cGAS forms condensates in cells with RNA.
This data has been improved somewhat but is still limited in that no condensates are seen with endogenous levels of cGAS. This would be the data in Figure 2G/H/I where there is a fair overlap of the signals, but they both appear diffuse. This could be consistent with cGAS biochemically interacting with RNA in cells but not forming higher level assemblies. At a minimum, the authors would need to acknowledge the limitations of their data and be explicit that the phase separation assemblies might be an artifact of in vitro experiments and/or over-expression.

2) Whether there was sufficient data that RNA enhanced cGAS activation in cells. The experiment presented, adding RNA to DNA during transfections and seeing an increase in the interferon response is flawed in that the effect of the RNA alone was never examined. Thus, the synergy of RNA on DNA signaling might be because the exogenous RNA is triggering an RNA based response leading to interferon induction. Thus, the data presented so far do not allow a strong conclusion that RNA can affect cGAS activation in cells.

Overall, this is a difficult manuscript to make a final decision on. The in vitro work on phase separation and the interaction of RNA with cGAS is well done and provides some understanding of what these molecules can do. The in vivo work is weak, and while it support that hypothesis that RNA can enhance cGAS signaling in cells under some conditions, it is not definitive.

Referee #1

The manuscript re-submitted by Chen et al, explores the role of RNA in the phase separation of cGAS and the regulation of the immune response. In this manuscript, the authors show that, similar to various dsDNA species, RNA can trigger phase separation of cGAS in vitro, although these droplets to do activate the enzyme. Instead, RNA appears to regulate cGAS phase separation with DNA and subsequent cGAS activation. Indeed, the authors found that RNA increases cGAS activity when DNA concentrations are low, and decreases cGAS activity when DNA concentration are high, thus allowing a tight control of cGAS activity in response to pathogens.

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• The complexes found in fraction 3 and 4 of the optiprep gradient are much less dense than the purified cGAS/tRNA droplets (which result in cGAS signal in fraction 6).
• The authors state that the density measurements are indicative of a condensed state -Such statement is incorrect.
As explained in the excellent review recently published by Julie Forman-Kay et al, (https://pubmed.ncbi.nlm.nih.gov/34772786/) many types of complexes including molecular machines and supramolecular complexes (e.g. proteasome, ribosomes), are not condensates, and condensates may span a large array of sizes, ranging from a few nanometer in size to a micrometer-sized structures.
Therefore, density or size does not allow to differentiate a condensate from a large non-phase separated complexe or macro-structure, and the provided data does not support the formation of cGAS/RNA condensates in cells.

Nevertheless, the data does not disprove the formation of nanoscopic condensates that cannot be resolved in the provided images, nor the ability of cGAS to form large condensates with RNA,

which may occur under conditions that have not been tested by the authors (for instance, infection by RNA viruses, or following translation arrest during a stress).

It also does not contradict the rest of the manuscript, especially regarding the ability of cGAS to undergo LLPS with RNA, as well as the role of RNA in regulating cGAS LLPS and activation.

Therefore, I strongly recommend revisiting this section before publication.

We fully agree with the referee that the size alone can not prove or disprove the condensate state of endogenous cGAS. However, in combination with our in vitro data and the data from overexpressed cGAS-GFP, we think that the endogenous cGAS is highly likely in a condensed form. Our new data showed that the overexpressed recombinant cGAS-GFP in HEK293 cells is located at a similar position in the OptiPrep gradient as that of the endogenous cGAS of HeLa cells (Appendix Fig S5A, bands 3 and 4 contain the cGAS-GFP-RNA complex), while foci formed by cGAS-GFP could be clearly observed in Fig 2A and has been proved to be condensates with FRAP (Fig. 2B-C). Furthermore, it is technically impossible to prove the state of endogenous cGAS since these can only be labeled with specific antibodies. Antibody labeling can not reflect the internal properties of nanoscopic condensates as does FRAP, even if they could be observed with super resolution microscopy. As for "The complexes found in fraction 3 and 4 of the OptiPrep gradient are much less dense than the purified cGAS/tRNA droplets", we think the density could be influenced by many other factors in cytoplasm, including nucleic acid binding proteins such as G3BP1. The complexes found in fraction 3 and 4 should be a mixture of many different proteins and RNAs which is different from purified cGAS mixed with tRNA.

We revised Figure 2G-I related part as the following:

Before:
"The OptiPrep concentration at band 3 and 4 is between 20%-25% with a corresponding density of 1.107-1.133 g/mL, which indicate that cGAS is in a condensed form (Figure 2E-F). "

After:
"The OptiPrep concentration at band 3 and 4 is between 20%-25% with a corresponding density of 1.107-1.133 g/mL, which indicate that cGAS is most likely in a condensed form (Figure 2E-F). "

Insert:
Line140: "We further analyzed the cytoplasmic extact of HEK293T cells that overexpress the hcGAS-GFP. Similar bands were observed in the OptiPrep gradient and western blot analysis of the bands showed that hcGAS-GFP is located at similar positions as these of the endogenous cGAS (Appendix Fig S5). Our FRAP results have showed that hcGAS-GFP in the cells forms phase separated granules with cytoplasmic RNAs (Fig 2A-C). The OptiPrep gradient results of cytoplasmic hcGAS-GFP further supported the condensate state of endogenous cGAS."

Line 152: "The difference between the in vitro and the in vivo data reflects complex states of the endogenous cGAS, which probably directly or indirectly binds many other factors as shown in previous studies (Andreeva et al., 2017, Zhao et al., 2022)."

Referee #2

The addition of the co-transfected DNA and RNA experiment in THP-1, along with other new experiments (such as new gradient purification and use of ethynyl RNA for co-localisation) have definitely increased the strength of the claims about the role of RNA in DNA sensing of cGAS. However it would have been ideal to have controlled for the need for cGAS in this response to co-transfected DNA and RNA (using cGAS KO THP-1) and also to measure the levels of cGAMP in cell lysates (which was requested in my original review). As it stands the THP-1 experiment is not unequivocal (i.e. the result of increase IFNb production could be due to another pathway/mechanism).

The RNAs we used were treated with CIAP (calf intestinal alkaline phosphatase, thermo, 18009019), and purified with a clean-up kit (Thermo, AM1908) according to the manuals provided by the vendor. Thus, the RNAs should not be a strong stimulator of the RNA-related pathways. In addition, we have the controls that has the RNA or dsDNA alone. Furthermore, we have added new data with G150, a specific inhibitor of cGAS (Lama et al., 2019). The results showed that G150 completely impaired the enhanced production of IFNb induced by co-transfecting DNA and RNA (Fig EV5A-B). As a control, G150 have little influence on the poly(I:C) induced IFNb production (Fig EV5C). Thus, in presence of dsDNA, the RNA mediated enhancement of IFNb production is through the cGAS dependent pathway

.

In addition, this reviewer specifically asked for details about the amount of independent repeats of the experiments shown - but this is only indicated on a few figure panels (noting that the authors responded "Done" to this point). Are we meant to understand that the data is often from single experiments?

We have added the repeat numbers in the figure legends.

The authors should also put their results in the context of the new literature on the topic - particularly the paper showing that G3BP1 (which binds dsRNA) binds cGAS to help form cGAS primary condensates.

We have added in the discussion "Nucleic acid binding proteins in cytoplasm such as G3BP1 might also contribute to the formation of the granules (Zhao et al., 2022)."

Finally, the authors also need to explain the FRAP result - what was actually shown and how this "Proves" that we are dealing with LLPS.

We have changed the FRAP related part.

Before:
"To further confirm the liquid-liquid phase separation nature of the cGAS involved granules, a fluorescence recovery after photo-bleach (FRAP) assay was performed to measure the fluidity of the granules (Figure 2B & 2C). The results proved that the cytoplasmic cGAS-RNA granules are liquid-like droplets rather than insoluble aggregates."

After:
"To further confirm the liquid-liquid phase separation nature of the hcGAS-GFP involved granules, a fluorescence recovery after photo-bleach (FRAP) assay was performed to measure the fluidity of the granules (Figure 2B & 2C). The fluorescence in the photobleached region could be recovered shortly after the bleach. The results indicated frequent exchange of the cGAS molecules inside the granules with the cGAS molecules in the environment, which is indicative of liquid-like phase separation (Brangwynne et al., 2009, Alberti et al., 2019, Hyman et al., 2014). The results combined proved that the cytoplasmic hcGAS-GFP-RNA granules are liquid-like droplets rather than insoluble aggregates."

Referee #3

This revised manuscript is improved and the authors provide strong data for cGAS interacting with RNA in vitro and being able to form "phase-separated" assemblies in vitro with RNA. This is consistent with earlier work.
In my prior comments, I had raised
1) Whether there was sufficient data that endogenous cGAS forms condensates in cells with RNA.
This data has been improved somewhat but is still limited in that no condensates are seen with endogenous levels of cGAS. This would be the data in Figure 2G/H/I where there is a fair overlap of the signals, but they both appear diffuse. This could be consistent with cGAS biochemically interacting with RNA in cells but not forming higher level assemblies. At a minimum, the authors would need to acknowledge the limitations of their data and be explicit that the phase separation assemblies might be an artifact of in vitro experiments and/or over-expression.

Please see our response to Referee 1

2) Whether there was sufficient data that RNA enhanced cGAS activation in cells. The experiment presented, adding RNA to DNA during transfections and seeing an increase in the interferon response is flawed in that the effect of the RNA alone was never examined. Thus, the synergy of RNA on DNA signaling might be because the exogenous RNA is triggering an RNA based response leading to interferon induction. Thus, the data presented so far do not allow a strong conclusion that RNA can affect cGAS activation in cells.

We have the RNA control in the data, please check figure 5J. The RNA control labels were placed vertically.

Overall, this is a difficult manuscript to make a final decision on. The in vitro work on phase separation and the interaction of RNA with cGAS is well done and provides some understanding of what these molecules can do. The in vivo work is weak, and while it support that hypothesis that RNA can enhance cGAS signaling in cells under some conditions, it is not definitive.

ALBERTI, S., GLADFELTER, A. & MITTAG, T. 2019. Considerations and Challenges in Studying Liquid-Liquid Phase Separation and Biomolecular Condensates. *Cell,* 176**,** 419-434.

ANDREEVA, L., HILLER, B., KOSTREWA, D., LÄSSIG, C., MANN, C. C. D. O., DREXLER, D. J., MAISER, A., GAIDT, M., LEONHARDT, H., HORNUNG, V. & HOPFNER, K.-P. 2017. cGAS senses long and HMGB/TFAM-bound U-turn DNA by forming protein–DNA ladders. *Nature,* 549**,** 394-398.

BRANGWYNNE, C. P., ECKMANN, C. R., COURSON, D. S., RYBARSKA, A., HOEGE, C., GHARAKHANI, J., JULICHER, F. & HYMAN, A. A. 2009. Germline P Granules Are Liquid Droplets That Localize by Controlled Dissolution/Condensation. *Science,* 324**,** 1729-1732.

HYMAN, A. A., WEBER, C. A. & JULICHER, F. 2014. Liquid-liquid phase separation in biology. *Annu Rev Cell Dev Biol,* 30**,** 39-58.

LAMA, L., ADURA, C., XIE, W., TOMITA, D., KAMEI, T., KURYAVYI, V., GOGAKOS, T., STEINBERG, J. I., MILLER, M., RAMOS-ESPIRITU, L., ASANO, Y., HASHIZUME, S., AIDA, J., IMAEDA, T., OKAMOTO, R., JENNINGS, A. J., MICHINO, M., KUROITA, T., STAMFORD, A., GAO, P., MEINKE, P., GLICKMAN, J. F., PATEL, D. J. & TUSCHL, T. 2019. Development of human cGAS-specific small-molecule inhibitors for repression of dsDNA-triggered interferon expression. *Nat Commun,* 10**,** 2261.

ZHAO, M., XIA, T., XING, J. Q., YIN, L. H., LI, X. W., PAN, J., LIU, J. Y., SUN, L. M., WANG, M., LI, T., MAO, J., HAN, Q. Y., XUE, W., CAI, H., WANG, K., XU, X., LI, T., HE, K., WANG, N., LI, A. L., ZHOU, T., ZHANG, X. M., LI, W. H. & LI, T. 2022. The stress granule protein G3BP1 promotes pre-condensation of cGAS to allow rapid responses to DNA. *EMBO Rep,* 23**,** e53166.

- Please shorten the abstract to not more than 175 words.
We have shortened the abstract to not more than 175 words.

- add up to 5 keywords to the title page.

We have added the keywords to the title page.

Innate immunity, cGAS, RNA, phase separation, regulation

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- Please add a formal "Data Availability section" (DAS - placed after Materials and Methods) to the manuscript. This is now mandatory (like the COI statement). If no primary datasets have been deposited in any database, please state this in this section (e.g. 'No primary datasets have been generated and deposited').

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- Please add the Heading 'Expanded View figure legends'.
We have added the Heading 'Expanded View figure legends'.

- Please make sure that the number "n" for how many independent experiments were performed, their nature (biological versus technical replicates), the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values is indicated in the respective figure legends (main, EV and Appendix figures), and that statistical testing has been done where applicable. Please avoid phrases like 'independent experiment', but clearly state if these were biological or technical replicates. Please add complete statistical testing to all diagrams (main, EV and Appendix figures). Please also indicate (e.g. with n.s.) if testing was performed, but the differences are not significant.
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We have indicated the number "n" and their nature in the figure cations for each panel. The bars and error bars and the test used to calculate p-values have been indicated too. Statistical testing has been done where applicable. Representative images from n replicates have been stated. Replicates in 2I were mislabeled and have been corrected. 4F and 4G show the quantification of 4D and 4E (one experiment representative of the 2 biological replicates). The error bars in 4F and 4G represent technical replicates and have been removed. Old version of Fig 5J, EV4, and EV5 only showed one biological replicate from 2. However, we found that n=2 is too enough for the statistics. So we did more biological replicates. The results in Fig 5J, EV4, and EV5 have been updated.

- Please add scale bars of similar style and thickness to all the microscopic images (main, EV and Appendix figures), using clearly visible black or white bars (depending on the background). Please place these in the lower right corner of the images. Please do not write => Please add a scale bar to Fig EV1B. You need to define the scale bars in the legends and remove the size/text from the scale bars in the images.

Scale bars have been adjusted and defined in the legends. We have added scale bars to Fig EV1B. Size/text from the scale bars in the images have been removed.

- Please make sure that all the funding information is also entered into the online submission system and that it is complete and similar to the one in the acknowledgement section of the manuscript text file.
=> Funders "the National Natural Science Foundation of China (grant numbers: 31861143027, & 31470721), the Spring Breeze Fund of Tsinghua University, the Beijing Frontier Research Center for Biological Structure, and the Beijing Advanced Innovation Center for Structure Biology to Y.X. and the Key R & D Project of Shandong Province (grant number: 2019GSF108212) to D.Z" are missing from the manuscript management system.

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We have added panel callouts for Fig. 1, EV1, EV2, EV3, Appendix Figs S2, S5.

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Dear Dr. Xiang

Thank you for the submission of your further revised manuscript to our editorial offices. I have now received the reports from the two referees I asked to re-assess the study, which I include again below for your information. As you know, referee #2 now fully supports the publication of your manuscript in EMBO reports.

However, referee #1 has remaining concerns I ask you, as discussed, to address in a final revised version by text changes (as indicated by the referee). Please provide your final manuscript text with track changes, in order that we can see the modifications done, and provide a final point-by-point-response addressing the remaining points of referee #2 and explaining the final modifications of the manuscript text.

Moreover I have these editorial requests:

- I would suggest this slightly modified title:
Regulation of cGAS activity by RNA-mediated phase separation

- Please provide the abstract written in present tense throughout (with not more than 175 words).

- Please have your final manuscript text be carefully proofread by a native speaker. There are a few typos and grammatical errors present.

- Could statistical testing be provided for the diagrams shown in panels 4B, 4C, 5A, 5H and 5I? Please add that and provide all the information on the statistical test used and p-values in the figure legends.

I look forward to seeing the final revised version of your manuscript when it is ready. Please let me know if you have questions regarding the revision.

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Achim Breiling
Senior Editor
EMBO Reports

Referee #1:

The revised manuscript submitted by the authors provided strong in vitro work, especially regarding the ability of RNA to induce cGAS LLPS in vitro and the subsequent regulation of cGAS activity. Moreover, additional data that further support the role of RNA in regulating cGAS activity in cells was provided, reinforcing the physiological/functional relevance of this regulation.

However, as it was raised by all three referees, clear and strong evidence that cGAS undergoes LLPS with RNA at physiological concentrations in cells is lacking. Indeed, although in vitro and overexpression data clearly show that cGAS can undergo LLPS and form condensates with RNA, it is also highly possible that both cGAS, RNA, along with other components, do not reach the threshold concentration and form condensate in cells, at physiological concentrations. Consistent with this is the lack of visible condensates inside cells.

In their revised manuscript, the authors compared the density of endogenous cGAS-RNA complexes with cGAS-RNA complexes resulting from overexpression. This data is hard to interpret since:
• This involves a lysis step that likely disrupts the condensates formed upon overexpression of cGAS
• There is no control condensate (such as a stress granule marker in presence or absence of stress) that show that this technic allows to distinguish between a large complex and a condensate.

Consequently, the data presented by the authors do support a possible formation of large complexes. However, I believe the authors cannot conclude that cGAS forms phase separated granules with RNA in the cytoplasm and these results remain unconclusive.

The authors should revise their interpretation, acknowledge the limitations of their data, and provide possible explanations about the lack of visible substructures in cells, as it was also suggested by Reviewer 3 in their previous comments.

Minor point:
Line 152: Factions should be replaced by Fractions

Referee #2:

The Authors have now addressed all my previous concerns.
The addition of the G150 data helps support the main claim that the RNA facilitates cGAS sensing of DNA.

Referee #1

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The authors should revise their interpretation, acknowledge the limitations of their data, and provide possible explanations about the lack of visible substructures in cells, as it was also suggested by Reviewer 3 in their previous comments.

Thanks for your comments and advices. We have revised our interpretation, provided possible explanations about the lack of visible substructures in cells, and acknowledged the limitations of our data.

Minor point:
Line 152: Factions should be replaced by Fractions

We have corrected this typo. Thanks!

Editorial requests:

- I would suggest this slightly modified title:
Regulation of cGAS activity by RNA-mediated phase separation

We have modified title to “Regulation of cGAS activity by RNA-modulated phase separation”

- Please provide the abstract written in present tense throughout (with not more than 175 words).

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- Please have your final manuscript text be carefully proofread by a native speaker. There are a few typos and grammatical errors present.

The manuscript was proofread by a professional scientific editor who speaks English as a first language. All the changes are tracked in the revised manuscript.

- Could statistical testing be provided for the diagrams shown in panels 4B, 4C, 5A, 5H and 5I? Please add that and provide all the information on the statistical test used and p-values in the figure legends.

We have added statistical testing for diagrams shown in panels 4B, 4C, 5A, 5H and 5I and provide the information on the statistical test used and p-values in the figure legends.

Ye Xiang
Tsinghua University
China

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- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
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Laboratory protocol	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Provide DOI OR other citation details if **external detailed step-by-step protocols** are available.	Not Applicable	

Experimental study design and statistics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Include a statement about **sample size** estimate even if no statistical methods were used.	Yes	Figures
Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. **randomization procedure**)? If yes, have they been described?	Not Applicable	
Include a statement about **blinding** even if no blinding was done.	Not Applicable	
Describe **inclusion/exclusion criteria** if samples or animals were excluded from the analysis. Were the criteria pre-established? If sample or data points were omitted from analysis, report if this was due to attrition or intentional exclusion and provide justification.	Not Applicable	
For every figure, are **statistical tests** justified as appropriate? Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. Is there an estimate of variation within each group of data? Is the variance similar between the groups that are being statistically compared?	Yes	Figures

Sample definition and in-laboratory replication	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
In the figure legends: state number of times the experiment was **replicated** in laboratory.	Yes	Figures
In the figure legends: define whether data describe **technical or biological replicates**.	Yes	Figures

Ethics

Ethics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Studies involving **human participants**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval.	Not Applicable	
Studies involving **human participants**: Include a statement confirming that **informed consent** was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	Not Applicable	
Studies involving **human participants:** For publication of **patient photos**, include a statement confirming that consent to publish was obtained.	Not Applicable	
Studies involving experimental **animals**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval. Include a statement of compliance with ethical regulations.	Not Applicable	
Studies involving **specimen and field samples:** State if relevant **permits** obtained, provide details of authority approving study; if none were required, explain why.	Not Applicable	

Dual Use Research of Concern (DURC)	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Could your study fall under dual use research restrictions? Please check biosecurity documents and list of **select agents and toxins** (CDC): https://www.selectagents.gov/sat/list.htm	Not Applicable	
If you used a select agent, is the security level of the lab appropriate and reported in the manuscript?	Not Applicable	
If a study is subject to dual use research of concern regulations, is the name of the **authority granting approval and reference number** for the regulatory approval provided in the manuscript?	Not Applicable	

Reporting

The MDAR framework recommends adoption of discipline-specific guidelines, established and endorsed through community initiatives. Journals have their own policy about requiring specific guidelines and recommendations to complement MDAR.

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For **tumor marker prognostic studies,** we recommend that you follow the **REMARK** reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	Not Applicable	
For **phase II and III randomized controlled trials**, please refer to the **CONSORT** flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	Not Applicable	

Data Availability

Data availability	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Have **primary datasets** been deposited according to the journal's guidelines (see 'Data Deposition' section) and the respective accession numbers provided in the Data Availability Section?	Not Applicable	
Were **human clinical and genomic datasets** deposited in a public access-controlled repository in accordance to ethical obligations to the patients and to the applicable consent agreement?	Not Applicable	
Are **computational models** that are central and integral to a study available without restrictions in a machine-readable form? Were the relevant accession numbers or links provided?	Not Applicable	
If publicly available data were reused, provide the respective **data citations in the reference list**.	Not Applicable	