nature research

Peer Review File

Manuscript Title: The Molecular Basis of Tight Nuclear Tethering and Inactivation of cGAS

Editorial Notes:

Reviewer Comments & Author Rebuttals

Reviewer Reports on the Initial Version:

Referee #1 (Remarks to the Author):

In this manuscript the authors use biochemical assays and cryo-EM to analyze cGAS interaction with the nucleosome. Although biochemical and structural work is solid, the functional importance of cGAS:Nucleosome complex needs to be better characterized.

1) Several recent studies have addressed cGAS localization in cells with very different outcomes. While Volkman et al, Gentili et al show that cGAS can be nuclear, Barnett et al show very convincingly that cGAS is plasma membrane bound with almost no nuclear localization. Cytosolic localization has been also shown by Sun et al. and several other studies. Barnett et al propose that the plasma membrane localization limits self DNA recognition, and mutations that impair the membrane localization lead to the nuclear localization and cGAS activation.

All these data are contradicting and since determining localization is essential for functional validation of this complex, I would like that the authors show if and how much of cGAS is actually nuclear. Because of conflicting reports, it is essential to show that cGAS can be nuclear and can actually encounter nucleosomes.

2) The authors need to clearly show functional defects in cells when interaction with nucleosome is abolished. The authors need to show if cGAS mutants induce interferon response by looking at RNA or protein levels factors that are expected to be induced (IP-10, IFIT2, IFN-beta...). It is essential to show clear biological consequences when cGAS cannot bind nucleosomes, which according to the model should induce strong response to genomic DNA.

In their previous work (Le et al, 2013) the authors have used some of these mutants in cells and observed IFN-beta induction, however these experiments were done with overexpressed STING and do not show that cGAS mutants that abolish interaction with the nucleosome are constitutively active.

The authors need to show that these mutants will activate interferon response in cells without any over-expressions. Otherwise binding to nucleosome does not seem to be important for normal cells, that do not overexpress STING.

3) The authors should show that interaction with the acidic patch and H2AL1/H2BL2 mutant decreases cGAS interaction with nucleosome to confirm that this is major binding site on the nucleosome side.

4) The authors should test if linker DNA will affect cGAS interaction with the nucleosome. They should use nucleosomes with linker DNA to determine if these mutations affect the binding. This would mimic better the chromatin.

They should also add all components (cGAS, nucleosome and free DNA) at the same time to

determine if cGAS will bind primarily free DNA or nucleosomes.

5) Authors suggest that histone H2A-H2B dimer is the major interaction with cGAS. Since histones are highly abundant, substantial pool of H2A/H2B is also cytoplasmic, especially during S phase. Will cytoplasmic cGAS bind cytoplasmic pool of H2A/H2B? The authors should determine this.

Minor comments

1) It is not clear what is the ratio of nucleosome and cGAS in the activity assays.

Referee #2 (Remarks to the Author):

The molecular basis of tight nuclear tethering and inactivation of cGAS

[Overview]

In this study, Zhao et al. report the use of the cryo-EM electron density map of mcGAS-nucleosome to reconstitute a high-resolution cGAS-nucleosome interface model based on available crystal structures. The authors show that a tight nuclear tethering of cGAS abolishes its catalytic activity by blocking one of the dsDNA binding sites. This work is well performed and presented in general, but the following points should be addressed before further consideration for publication.

[Major comments]

1. The authors mentioned the difference of binding affinity between human and mouse cGAS catalytic domain. How about the affinity difference between full-length and catalytic domain of mouse cGAS? Is it safe to assume that hcGAS-FL has inhibitory effect on the catalytic domain, whereas mcGAS-FL potentially has enhancing effect?

2. In Fig. 1b, is there any possibility that the influence on cGAS activity of fourth chart from the top (light blue) is due to nucleosome competing with mcGAS to bind dsDNA since nucleosome also has high affinity with dsDNA? The authors should exclude this possibility.

3. The authors stated that they used mouse cGAS catalytic domain as human cGAS catalytic domain is not very stable in low salt solution. Why the authors didn't use full-length human cGAS?

4. The authors substituted a positively charged residue (K, R) to a negatively charged residue (E), and this raises concern that the protein may not have correct folding. It is possible that the decrease in the binding affinity and enzyme activity is a result of protein misfolding. The authors should to provide additional information regarding the protein folding states (e.g. circular dichroism spectroscopy). Similarly, Fig. 4a should be provided as an uncropped image to show the unbound mcGAS mutants. The mcGAS should be resolved on the native gels as can be seen from Extended Data Fig. 1e.

5. In Fig. 4a and 4b, the mutant R337E has ds-DNA binding activity but unable to bind to nucleosome. This implies that R337E is an active mutant that is competent of cGAMP generation. However, in Extended Figure 7, there is no production of cGAMP of R337E compared with WT. Likewise, no cGAMP is observed in the constitutive mutants R222E, K240E, and R342E (Extended Figure 7). The authors should explain the discrepancy.

6. The constitutively active R222E, K240E and R342E mutants that the authors report as tethering defective mutants are, at the same time, DNA binding mutants as well. Therefore, a more plausible explanation for the spontaneous activity of the R222E, K240E and R342E mutants would be that mutations in these amino acids trigger a conformational change of cGAS instead of losing affinity to bind nucleosome. The authors should introduce a constitutively active mutant that cannot bind to nucleosomes but still bind to DNA (like R337E).

7. The authors should provide data (e.g. pull-down data) that cGAS does not interact with histones H3, H4 and dsDNA associated with the nucleosome.

8. The authors used the hybrid protein complex between human nucleosome and mouse cGAS for cryo-EM analysis, but essentially nothing is said about the structural similarity and/or difference between the mouse cGAS and human cGAS. They need to include superimposed figures of mcGAS and hcGAS as an Extended Figure and dicuss how two proteins are similar or different in terms of docking to human nucleosome.

9. The authors mentioned that these three cGAS mutations are constitutively active in cells. It would be insightful that the authors discuss the relationship between these mutations and autoimmune diseases or cancers.

10. In Extended data Fig. 6a, mcGAS is not shown in SDS-PAGE gel.

11. In Extended data Fi. 7, the legend should be described in more detail. K238E mutants only have enzyme activity. It is difficult to make a conclusion addressing the significance between nucleosome binding and cGAS enzyme activity. The author can also test in vivo cGAS enzyme activity assay

[Minor comments]

1. The authors need to include catalog numbers for reagents in methods

Referee #3 (Remarks to the Author):

Baoyu et al. present biochemical and structural characterization of the cGAS-nucleosome interaction, revealing the acidic patch formed by H2A-H2B as the high affinity cGAS nuclear tethering surface, which directly blocks its dsDNA interaction through the binding site B on cGAS. This is beautiful work and a big advance in the field. The manuscript is well written and they have done a good job selecting figures that illustrate important features of the structure models. All details leading to the cryo-EM reconstructions, models and fittings are very clearly described. There is simply nothing to be complained about the technical approaches chosen by the authors. I have only very minor comments for the authors that might guide small improvements during preparation of a final version of the manuscript. In summary, this is an impressive piece of work.

1. If possible, please speculate why the affinity of mcGAS catalytic domain for nucleosome is much lower than hcGAS, while the authors decided to use it for high resolution structural studies.
2. It's a good idea to add a panel in Fig. 3 on multi-seq alignment for regions of cGAS that interacts with the acidic patch.
3. In Fig. 2a, slightly increase the threshold of the density map so that high resolution feature will show up more clear. If the authors are concerned about lower resolution regions could blow up, consider showing instead a local resolution filtered density map for this purpose.

Author Rebuttals to Initial Comments:

Response to the Reviewers' Comments

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In this manuscript the authors use biochemical assays and cryo-EM to analyze cGAS

interaction with the nucleosome. Although biochemical and structural work is solid, the functional importance of cGAS:Nucleosome complex needs to be better characterized.

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All these data are contradicting and since determining localization is essential for functional validation of this complex, I would like that the authors show if and how much of cGAS is actually nuclear. Because of conflicting reports, it is essential to show that cGAS can be nuclear and can actually encounter nucleosomes.

We have identified a collaborator and conducted western blot analyses of cellular fractions to demonstrate that cGAS were mostly in the nuclear fractions in HeLa cells and mouse embryonic fibroblast (MEF) cells reconstituted with HA-cGAS (Fig. 1a). We have also conducted immunofluorescence microscopy with these two cell lines and observed that cGAS were mostly localized in the nucleus (Fig. 1b). Quantification from the immunofluorescence images demonstrates that over 75% of cGAS are localized in the nucleus in these two cell lines (Fig. 1c). Overall, these results are consistent with the observations from the eLife paper published by the Stetson lab (Volkman et al. eLife, 2019).

2) The authors need to clearly show functional defects in cells when interaction with nucleosome is abolished. The authors need to show if cGAS mutants induce interferon response by looking at RNA or protein levels factors that are expected to be induced (IP-10, IFIT2, IFN-beta...). It is essential to show clear biological consequences when cGAS cannot bind nuclesomes, which according to the model should induce strong response to genomic DNA.

In their previous work (Le et al, 2013) the authors have used some of these mutants in cells and observed IFN-beta induction, however these experiments were done with overexpressed STING and do not show that cGAS mutants that abolish interaction with the nucleosome are constitutively active.

The authors need to show that these mutants will activate interferon response in cells without any over-expressions. Otherwise binding to nucleosome does not seem to be important for normal cells, that do not overexpress STING.

We have conducted IFN-β luciferase reporter assays in HEK 293T cells transfected with STING with mouse and human cGAS mutants (Fig. 4d, e). The amount of STING plasmid used in the transfections was optimized to ensure that the reporter signals were generated by the transfection of cGAS or its mutants instead of the overexpression of STING. The amount of cGAS plasmids used for the assays were also optimized to avoid saturation of the system. The reporter assays were conducted at two different transfections of cGAS to test how the expression level of cGAS affects signaling (Fig. 4d, e). We have also confirmed the

expression of STING and all the cGAS mutants by western blot. The expression levels of the mcGAS or hcGAS mutants are similar to the WT (Extend Data Fig. 9d,e).

3) The authors should show that interaction with the acidic patch and H2AL1/H2BL2 mutant decreases cGAS interaction with nucleosome to confirm that this is major binding site on the nucleosome side.

We did not have the H2AL1/H2BL2 nucleosome or the genes for these histone proteins to generate nucleosomes for binding studies. Instead, we have obtained the H2A.Bbd nucleosome from Active Motif, which contains mutations of the acidic patch residues compared with regular H2A and conducted cGAS binding study by gel shift assay (Extended Data Fig. 6e, f). We observed that this nucleosome did not bind cGAS, demonstrating that the acidic patch is critical for cGAS binding (Extended Data Fig. 6f).

4) The authors should test if linker DNA will affect cGAS interaction with the nucleosome. They should use nucleosomes with linker DNA to determine if these mutations affect the binding. This would mimic better the chromatin.

They should also add all components (cGAS, nucleosome and free DNA) at the same time to determine if cGAS will bind primarily free DNA or nucleosomes.

We have generated oligonucleosomes by partial digestion of the chromatin from HEK 293T cells (Extended Data Fig. 1f,g). Binding studies showed that the oligonucleosomes also bind cGAS (Extend Data Fig. 1h). We have conducted nucleosome binding studies with mcGAS mutants with the oligonucleosomes. We observed that the mutations of mcGAS affect oligonucleosome binding in a similar fashion as mononucleosome binding (Extended Data. Fig. 7c). In addition, we have conducted cGAS activity assays using the oligonucleosomes and observed that oligonucleosome binding inhibits the activity of cGAS (Extended Data Fig. 2k). Surprisingly, we observed that the cGAS-oligonucleosome complex can activate ligand free cGAS (Extended Data Fig. 2k). To determine if dsDNA compete with nucleosome to bind cGAS, we mixed nucleosome, dsDNA and excess cGAS and analyzed the samples by gel filtration chromatography and SDS-PAGE. We observed that cGAS binds to the nucleosome, but does not bind to the dsDNA efficiently (Extended Data Fig. 2i).

5) Authors suggest that histone H2A-H2B dimer is the major interaction with cGAS. Since histones are highly abundant, substantial pool of H2A/H2B is also cytoplasmic, especially during S phase. Will cytoplasmic cGAS bind cytoplasmic pool of H2A/H2B? The authors should determine this.

We have generated both WT and mutants of H2A/H2B dimers at the acidic patch and conducted Ni-NTA pull-down assays. We observed the WT H2A/H2B dimers bind to mcGAS, while mutations within the acidic patch disrupted cGAS binding (Extended Data Fig. 6d). However, we were unable to investigate if H2A/H2B binds cGAS during the S phase due to the lack of expertise for this study. Based on the in vitro data, we speculate that cGAS should bind the H2A/H2B dimers during the S phase.

Minor comments

1) It is not clear what is the ratio of nucleosome and cGAS in the activity assays.

We have provided details about amount of cGAS and nucleosomes used in the cGAS activity assays in the method section.

Referee #2 (Remarks to the Author):

The molecular basis of tight nuclear tethering and inactivation of cGAS

[Overview]

In this study, Zhao et al. report the use of the cryo-EM electron density map of mcGAS-nucleosome to reconstitute a high-resolution cGAS-nucleosome interface model based on available crystal structures. The authors show that a tight nuclear tethering of cGAS abolishes its catalytic activity by blocking one of the dsDNA binding sites. This work is well performed and presented in general, but the following points should be addressed before further consideration for publication.

[Major comments]

1. The authors mentioned the difference of binding affinity between human and mouse cGAS catalytic domain. How about the affinity difference between full-length and catalytic domain of mouse cGAS? Is it safe to assume that hcGAS-FL has inhibitory effect on the catalytic domain, whereas mcGAS-FL potentially has enhancing effect?

We have purified both full-length and the catalytic domain of mouse cGAS and conducted binding studies by SPR and Bio-layer Interferometry (BLI). Similar binding affinities were obtained for both samples (Extended Data Fig. 2a-h). The newly measured affinities of mouse cGAS and human cGAS are similar (Extended Data Fig. 2a-h). It is obvious that the N-terminal domain of cGAS plays a minor role in nucleosome binding as the binding affinities of full-length cGAS are similar to the catalytic domains. With the data we had, we cannot speculate how the N-terminal domains of cGAS affect nucleosome binding by full-length mouse and human cGAS as the affinities of the full-length proteins and the catalytic domains are very similar.

2. In Fig. 1b, is there any possibility that the influence on cGAS activity of fourth chart from the top (light blue) is due to nucleosome competing with mcGAS to bind dsDNA since nucleosome also has high affinity with dsDNA? The authors should exclude this possibility.

We have mixed nucleosome, cGAS and dsDNA and analyzed the sample by gel filtration chromatography. We observed that cGAS binds to the nucleosome, but does not bind to the dsDNA efficiently (Extended Data Fig. 2i). Since the nucleosomes used in the activity assays already have dsDNA bound to them, it is unlikely that these nucleosomes will bind to the extra dsDNA added to the purified cGAS-nucleosome complex.

3. The authors stated that they used mouse cGAS catalytic domain as human cGAS

catalytic domain is not very stable in low salt solution. Why the authors didn't use full-length human cGAS?

We can only purify full-length hcGAS using a buffer containing 0.5 M NaCl. The protein aggregates upon dilution into low salt (150 mM NaCl) buffers. The purity and stability of FL-hcGAS is also not as good as the mouse cGAS catalytic domain, so we did not use full-length hcGAS for the structural studies.

4. The authors substituted a positively charged residue (K, R) to a negatively charged residue (E), and this raises concern that the protein may not have correct folding. It is possible that the decrease in the binding affinity and enzyme activity is a result of protein misfolding. The authors should to provide additional information regarding the protein folding states (e.g. circular dichroism spectroscopy). Similarly, Fig. 4a should be provided as an uncropped image to show the unbound mcGAS mutants. The mcGAS should be resolved on the native gels as can be seen from Extended Data Fig. 1e.

We have analyzed all the mouse cGAS mutants by CD and observed that the spectra of all the mutants are almost the same as the WT proteins (Extended Data Fig. 7d). Since most of the cGAS mutants either retained catalytic activity or nucleosome binding, this also reflects the cGAS mutants are folded properly.

We have conducted the gel shift assays under several different cGAS concentrations, but we still could not see cGAS on the native gels. To resolve this issue, we provided SDS-PAGE analyses of all samples used for the gel shift assays. We can see both cGAS and the histone proteins on these SDS-PAGE gels (Extended Data Fig. 7b, c).

5. In Fig. 4a and 4b, the mutant R337E has ds-DNA binding activity but unable to bind to nucleosome. This implies that R337E is an active mutant that is competent of cGAMP generation. However, in Extended Figure 7, there is no production of cGAMP of R337E compared with WT. Likewise, no cGAMP is observed in the constitutive mutants R222E, K240E, and R342E (Extended Figure 7). The authors should explain the discrepancy.

We have optimized our cGAS activity assays to increase the sensitivity of the assay by using higher concentrations of cGAS and salmon sperm DNA in the assays. We still did not observe any activity for the R337E mutant of mcGAS (Extended Data Fig. 9b). The corresponding human cGAS mutant R349E is also not active (Extended Data Fig. 9c). The R222E only showed very weak activity (1% of WT, Extended Data Fig. 9b). The R342E mutant is still not active under the new assay condition (Extended Data Fig. 9b). However, the K240E showed about 9% activity relative to WT mcGAS (Extended Data Fig. 9b). Overall, these data agree with the data presented previously.

6. The constitutively active R222E, K240E and R342E mutants that the authors report as tethering defective mutants are, at the same time, DNA binding mutants as well. Therefore, a more plausible explanation for the spontaneous activity of the R222E, K240E and R342E mutants would be that mutations in these amino acids trigger a conformational change of cGAS instead of losing affinity to bind nucleosome. The authors should introduce a constitutively active mutant that cannot bind to nucleosomes but still bind to DNA (like R337E).

We agree with the reviewer that mutations of cGAS that disrupt nucleosome binding is likely due to conformational changes of the mutants. However, it is also likely due to the loss of specific electrostatic interactions mediated by these residues. The R337E mutation significantly reduced both DNA and nucleosome binding and abolished the catalytic activity of cGAS (Fig. 4a, c, Extended Data Fig. 9b). For all the mcGAS and hcGAS mutants we have tested, we did not observe any mutation that disrupts nucleosome binding but does not affect DNA binding. We also observed that DNA binding of cGAS does not correlate well with the catalytic activity.

7. The authors should provide data (e.g. pull-down data) that cGAS does not interact with histones H3, H4 and dsDNA associated with the nucleosome.

We have conducted gel shift assay to show that the H2A.Bbd nucleosome does not bind cGAS (Extended Data Fig. 6f). Since this kind of nucleosome contains H2A chains with mutations in the acidic patch, and contains regular H2B, H3, H4 proteins, indicating H3 or H4 is not involved in cGAS binding. Studies by Zierhut et al. (Cell, 2019) also showed H3-H4 did not bind cGAS directly. Although we could not determine directly if the nucleosome DNA binds cGAS or not, our cGAS activity assays showed that dsDNA bound to mononucleosomes could not activate ligand free cGAS effectively (Fig. 1e). By contrast, oligonucleosomes could activate ligand free cGAS (Extended Data Fig. 2k), likely through the linker dsDNA. Based on these observations, we speculate that most of the dsDNA associated with the nucleosome could not bind cGAS, while the linker DNA could bind and activate cGAS.

8. The authors used the hybrid protein complex between human nucleosome and mouse cGAS for cryo-EM analysis, but essentially nothing is said about the structural similarity and/or difference between the mouse cGAS and human cGAS. They need to include superimposed figures of mcGAS and hcGAS as an Extended Figure and discuss how two proteins are similar or different in terms of docking to human nucleosome.

We have included a superposition of ligand free human and mouse cGAS structures (Extended Data Fig. 6c). The two structures are very similar and the nucleosome binding residues are highly conserved (Extended Data Fig. 6b,c). We speculate that hcGAS would bind to the nucleosome in a similar fashion as mcGAS. Our mutagenesis and nucleosome binding studies confirmed this hypothesis (Fig. 4a,b). Mutations of these conserved residues have similar effects on mcGAS and hcGAS mediated signaling in cells as well (Fig 4d,e).

9. The authors mentioned that these three cGAS mutations are constitutively active in cells. It would be insightful that the authors discuss the relationship between these mutations and autoimmune diseases or cancers.

We speculate in the discussion section that perturbation of nuclear tethering of cGAS may boost antiviral and antitumor response, but this may also have the risk of causing autoimmunity. The cGAS mutants identified in this study will be valuable to test these hypotheses in the future.

10. In Extended data Fig. 6a, mcGAS is not shown in SDS-PAGE gel.

We have included SDS-PAGE analyses of all the mcGAS mutants as suggested (Extended Data Fig. 7a).

11. In Extended data Fi. 7, the legend should be described in more detail. K238E mutants only have enzyme activity. It is difficult to make a conclusion addressing the significance between nucleosome binding and cGAS enzyme activity. The author can also test in vivo cGAS enzyme activity assay

We have optimized our cGAS activity assays so that we can resolve the small differences between the activities of the mcGAS mutants (Extended Data Fig. 9b). We observed significant activity of the K240E, R244E, K315E, and K323E mutants (Extended Data Fig. 9b). However, the other mutants still showed very low or nearly no activities (Extended Data Fig. 9b), which agrees with results from our previous activity assays. In addition, we have analyzed the enzyme activities of all the human cGAS mutants under similar assay conditions. Similar results were obtained as the mcGAS mutants (Extended Data Fig. 9c). We have conducted IFN-β luciferase reporter assays of the mouse and human cGAS mutants to test how these mutations affect cGAS mediated signaling in cells (Fig. 4d,e).

[Minor comments]

1. The authors need to include catalog numbers for reagents in methods

We have provided catalog numbers for critical reagents used in this study.

Referee #3 (Remarks to the Author):

Baoyu et al. present biochemical and structural characterization of the cGAS-nucleosome interaction, revealing the acidic patch formed by H2A-H2B as the high affinity cGAS nuclear tethering surface, which directly blocks its dsDNA interaction through the binding site B on cGAS. This is beautiful work and a big advance in the field. The manuscript is well written and they have done a good job selecting figures that illustrate important features of the structure models. All details leading to the cryo-EM reconstructions, models and fittings are very clearly described. There is simply nothing to be complained about the technical approaches chosen by the authors. I have only very minor comments for the authors that might guide small improvements during preparation of a final version of the manuscript. In summary, this is an impressive piece of work.

1. If possible, please speculate why the affinity of mcGAS catalytic domain for nucleosome is much lower than hcGAS, while the authors decided to use it for high resolution structural studies.

We have conducted SPR and BLI based binding studies that showed both human and mouse cGAS bind nucleosome with nanomolar affinities (Extended Data Fig. 2a-h). The reason that we decided to use mcGAS catalytic domain for the structural studies is because mcGAS catalytic domain is stable at low salt condition, while both full-length mouse and human cGAS and human cGAS catalytic domain are not stable in low salt condition and could only be purified in the presence of 0.5 M NaCl, which would interfere with nucleosome

binding. The better yield and purity of mcGAS catalytic domain was the other reasons why we used it for the structural studies.

2. It's a good idea to add a panel in Fig. 3 on multi-seq alignment for regions of cGAS that interacts with the acidic patch.

Yes, we have included a sequence alignment of mouse and human cGAS to show the residues involved in nucleosome binding (Extended Data Fig. 6b).

3. In Fig. 2a, slightly increase the threshold of the density map so that high resolution feature will show up more clear. If the authors are concerned about lower resolution regions could blow up, consider showing instead a local resolution filtered density map for this purpose.

We have generated the figures by slightly increasing the threshold of the density map. Indeed, the high-resolution details showed up more clearly than the previous figure (Fig. 2).

Reviewer Reports on the First Revision:

Referee #1 (Remarks to the Author):

In the revised version the authors have nicely addressed several of my concerns, however, the in vivo significance of this interaction is still not clear. Before publication, the authors should address the remaining issues.

1) I am not quite sure how the authors have optimized the amount of STING plasmid used in the transfections. Why is it even necessary to over-express STING? STING over-expression suggests that observed in vivo activity does not happen in normal cells that do not over-express STING. If cGAS is only active when STING is over-expressed, why is it necessary to bind nucleosome to prevent autoreactivity?

2) The data presented in Figures 4d and e are not really convincing. The authors draw conclusions from indirect results that in vivo activity of certain mutants is higher than in vitro. The authors conclude that cGAS having mutations in residues that bind nucleosome is constitutively active in vivo. Unfortunately the current data do not really show this. Mutants that are constitutively active should be active without a stimulus, while wild type should be only active upon stimulus. In the assay presented in Figure 4d, e, both wild type and mutant cGAS are active.
The authors need to show that the interaction with nucleosome prevents autoreactivity on endogeneous DNA. If this is true, cGAS mutants that do not bind nucleosome should be activated by the host DNA, without external stimulus. The current in vivo data are indirect and do not show this.

Minor comments

1) The authors could make a table similar to table 4b for all the mutants and assays that have done to make them more accessible to readers.

Referee #2 (Remarks to the Author):

no further comment. authors thoroughly responded the previous comments.

Referee #3 (Remarks to the Author):

The authors have addressed all my questions. The manuscript is ready for publication.

Author Rebuttals to First Revision:

Response to the Reviewer's Comments

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HEK 293T cells do not express cGAS or STING, making this cell line very useful for studying how mutations of cGAS or STING affect their functions in cells by transfection- based studies such as IFN-β luciferase reporter assays. To test how mutations in cGAS affect cGAMP signaling to STING and IRF3 or to examine how STING mutants affect the recruitment of TBK1 and IRF3 (Zhao et al, PNAS, 2016, Zhao et al, Nature 2019), we have to transfect 293T cells with plasmids encoding STING. Since the over-expression of STING alone can activate the IFN-β luciferase reporter independently of cGAS, we first optimized the assay by transfecting the cells with several concentrations of STING plasmid. Based on these studies, we determined that transfecting 293T cells with 0.4 ng of STING in each assay does not constitutively activate the IFN-β luciferase reporter (Fig. 4d and Extended Data Fig. 9d). Therefore, we conclude that this concentration is sufficient to re-introduce STING, but not overexpress the protein. Indeed, only co-expression of WT cGAS and STING stimulates the reporter activation. We also optimized the amount of cGAS plasmids used for the assays to make sure the signal is not too low or saturated. Furthermore, we have transfected the cells with two different concentrations of cGAS plasmids to fully reflect the effects of cGAS mutations on downstream STING signaling and IFN-β luciferase reporter activity (Fig. 4d and Extended Data Fig. 9d). In many other cell lines, such as THP1 cells, the cGAS-STING pathway is intact, so our data and data from the Stetson group (Volkman et al. eLife, 2019) indicate that tight nucleosome binding serves as a means to suppress constitutive cGAS activity and perhaps dampen autoreactivity.

2) The data presented in Figures 4d and e are not really convincing. The authors draw conclusions from indirect results that in vivo activity of certain mutants is higher than in vitro. The authors conclude that cGAS having mutations in residues that bind nucleosome is constitutively active in vivo. Unfortunately the current data do not really show this. Mutants that are constitutively active should be active without a stimulus, while wild type should be only active upon stimulus. In the assay presented in Figure 4d, e, both wild type and mutant

cGAS are active.
The authors need to show that the interaction with nucleosome prevents autoreactivity on endogeneous DNA. If this is true, cGAS mutants that do not bind nucleosome should be activated by the host DNA, without external stimulus. The current in vivo data are indirect and do not show this.

A recent study from the Stetson lab (Volkman et al. eLife, 2019) using HeLa cells stably transduced with Dox-inducible lentiviruses encoding key cGAS mutants has elegantly demonstrated that mutations of cGAS that disrupt nuclear tethering render cGAS constitutively active in cells. These studies showed that mutations R222E, K240E, and R241E of mouse cGAS induce the production of cGAMP without exogenous DNA transfection. Mutation R255E of human cGAS, which corresponds to mutation R241E of mcGAS, also induced spontaneous production of cGAMP in cells. To confirm these findings, we have conducted cGAMP assays in HEK 293T cells transfected with several mouse and human cGAS mutants that disrupt nucleosome binding. Consistent with results from the Stetson lab, we also observed that these mutants of cGAS induced higher levels of cGAMP in the cells without DNA transfection (Fig. 4e, f).

We agree with the reviewer that our data presented in Fig. 4d and Extended Data Fig. 9d do not provide direct evidence that key cGAS mutants, which do not bind nucleosome, are constitutively active. Since we used a transfection-based assay instead of the inducible lentiviral system as described by the Stetson lab, it is most likely the newly synthesized cGAS protein detects residual plasmid cGAS and/or STING DNA to drive the IFN-β luciferase reporter activity. The significant reporter signal from mutants of cGAS with disrupted nucleosome binding can be best explained by the constitutive activity of cGAS since DNA binding and catalytic activity of these mutants are severely compromised. As WT cGAS transfection induces a reporter signal without DNA stimulation, we cannot draw a clear-cut conclusion that these cGAS mutants are constitutively active in cells. We have revised the manuscript to state that mutations of cGAS that disrupt nucleosome binding affect cGAS-mediated signaling in cells instead of saying that those mutants are constitutively active in cells.

The study by the Stetson lab already showed that WT cGAS is not active without DNA stimulation in cells (Volkman et al. eLife, 2019). By contrast, several mouse and human cGAS mutants with disrupted nuclear tethering are constitutively active even without exogenous DNA transfection into the cytosol (Volkman et al. eLife, 2019). Since the activity of cGAS in vivo also depends on DNA stimulation, the hyperactive cGAS mutants are likely activated by endogenous DNA such as micronuclei or DNA leaked from the mitochondria. The exact role of nuclear tethering on the regulation of cGAS activity needs to be addressed with appropriate CRISPR-knockin cell-based and transgenic mouse approaches to introduce mutations in cGAS that disrupt nucleosome binding. Building upon our current study, we plan to perform experiments in the future to address these important open questions.

Minor comments

1) The authors could make a table similar to table 4b for all the mutants and assays that have done to make them more accessible to readers.

As suggested by the reviewer, we have provided a table (Fig. 4b) to include the relative catalytic activities of the mouse and human cGAS mutants.