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ZDHHC18 negatively regulates cGAS-mediated innate immunity through palmitoylation

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Dear Dr. Yin,

Thank you for submitting your manuscript to The EMBO Journal. Your study has now been seen by two referees and I am afraid that the overall recommendation is not a positive one.

As you can see below, the referees find certain aspects of the paper interesting, but also find that we gain too limited support for how cGAS is palmitoylation is regulated and as well as the physiological relevance of the reported findings. Given these comments by key experts in the field, I am afraid that I can't offer to consider publication here.

I thank you for the opportunity to consider this manuscript. I am sorry that we cannot be more positive on this occasion, but I hope nevertheless that you will find our referees' comments helpful.

Yours sincerely,

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

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Referee #1:

In this work Shi et al report that cGAS is palmitoylated by ZDHHC18 to exert negative regulation of its activity by impairing DNA binding. The data presented are generally strong, and appear convincing. However, the work is mainly based on overexpression in HEK293T cells, and hence it is very difficult to assess the regulation of the proposed mechanism as well as the physiological relevance.

1. One essential unresolved question is whether palmitoylation and ZDHHC18 are part of the constitutive suppression of cGAS activity or an activation-induced negative feed-back. This is important to resolve. This should include (but not be limited to) detailed kinetics of DNA-induced IFNb expression in wt versus KO cells.

2. If it turns out to be a negative feed-back loop, some initial characterization of what induces ZDHHC18 activation / cGAS palmitoylation should be performed. For instance, is cGAS palmitoylation dependent on STING or TBK1?

3. The infection phenotype presented in Fig. 6A is rather weak. Different doses of virus should be tested, or other infection/disease models should be tested. In the absence of a clear phenotype in mice or a primary human cell system, this reviewer is not convinced about the physiological relevance.

4. In line with the modest phenotype, the data in Fig 6G does not show a strong effect on pSTING in KO cells.

5. One important question is whether ZDHHC18 is specific for cGAS. It should be tested in details whether ZDHHC18 KO cells exhibit different response to other PRR agonists (RIG-I and TLR pathways).

Referee #2:

The authors demonstrated that cGAS undergoes palmitoylation, which restricts its activity. They identified the responsible palmitoyltransferase ZDHHC18, and further demonstrated the significance of this post-translational modification in KO mice. The work presented here is potentially interesting, but some experiments, especially the ones regarding subcellular localization, were underdeveloped.

Major critiques:

Figure 1:
* In addition to C474S, the degree of palmitoylation appeared to be drastically reduced in C405S and C409S. The corresponding texts in "Results" is misleading and should be amended.
* The amount of cGAMP should be examined with/without palmitic acid.

Figure 2:
* The image of ZDHHC18 in Figure 2K and Figure S2D appeared very different, even in the same cells (HeLa cells) used. Figure 2K showed the punctate localization (what kind of organelles?), in contrast, Figure S2D showed the typical Golgi localization. Given this discrepancy, it is impossible to interpret the results shown in Figure 2K (a partial co-localization between cGAS puncta and ZDHHC18 after DNA transfection). In Figure S2F, ZDHHC18 appeared to show the typical ER localization.

Figure 3:
* Figure 3A: It is very confusing that they used GFP-cGAS in this particular experiment. Why was HA-cGAS not found in the nucleus in Figure 2K? If the tagging anything to cGAS interferes its localization, the authors should examine the localization of endogenous cGAS throughout the experiments (as they stained in Figure S2F). In Figure S3A, the stain of Rab7 is not convincing. The authors should carefully pay attention to the previous literatures that never describe the cGAS localization to late endosomes. Again, the endogenous cGAS localization should be examined.

Figure 5:
* Figure 5B: ZHHC18 (CS) should be examined.
* Figure 5C: Does KD of ZHHC18 affect the amount of cGAMP?

Technical critiques:
(1) In Figure 1, having the schematic for acyl-RAC assay would be helpful for general readers.

(2) All of the WB lack the molecular weight indicators. Some WB lack the appropriate legends (blotted with what antibody? such as Fig. 1G).

First of all, thank you very much for your note with reviewers' comments for our manuscript entitled ZDHHC18 negatively regulates cGAS-mediated innate immune immunity through palmitoylation (manuscript #: 2021-109272). I would like to reiterate that the EMBO Journal is a favorite venue dear to my heart, which is the reason why we sent to you the best of our works. While we believe that these comments are hugely helpful for the further improvement of our manuscript, I would like to point out that the reviewers may also miss some information from our manuscript due to confusion and factual errors. Furthermore, we have collected substantial additional data since the submission of our original manuscript. We would like to discuss with you about a potential resubmission given that we can address almost ALL concerns raised by reviewers.

As a cytosolic DNA sensor, cGAS plays a central role in innate immune activation and has been linked to various autoimmune diseases. Especially, understanding the regulatory mechanism of cGAS activation is an intriguing and challenging task that has draw significant attention recently (Ablasser & Chen, Science, 2019, 1055).In this work, we focus on a palmitoylation modification that tightly controls the activity of cGAS as a specific mechanism in cGAS regulation, which is the first in its kind. In addition, our findings uncovered a new protein machinery that is responsible for this process and demonstrate its biological relevance both in vitro and in vivo. Both reviewers regarded our work as "generally significant, convincing, and interesting".

We definitely committed to further improvement of the current manuscript to meet your high standard of publishing for the EMBO Journal. Given the opportunity to revise, we are confident to address two major questions: 1) the specific regulatory mechanism and) the biological relevance of cGAS palmitoylation by ZDHHC18. We have detailed these in the attached point-to-point response to the reviewers' comments.

Referee #1:

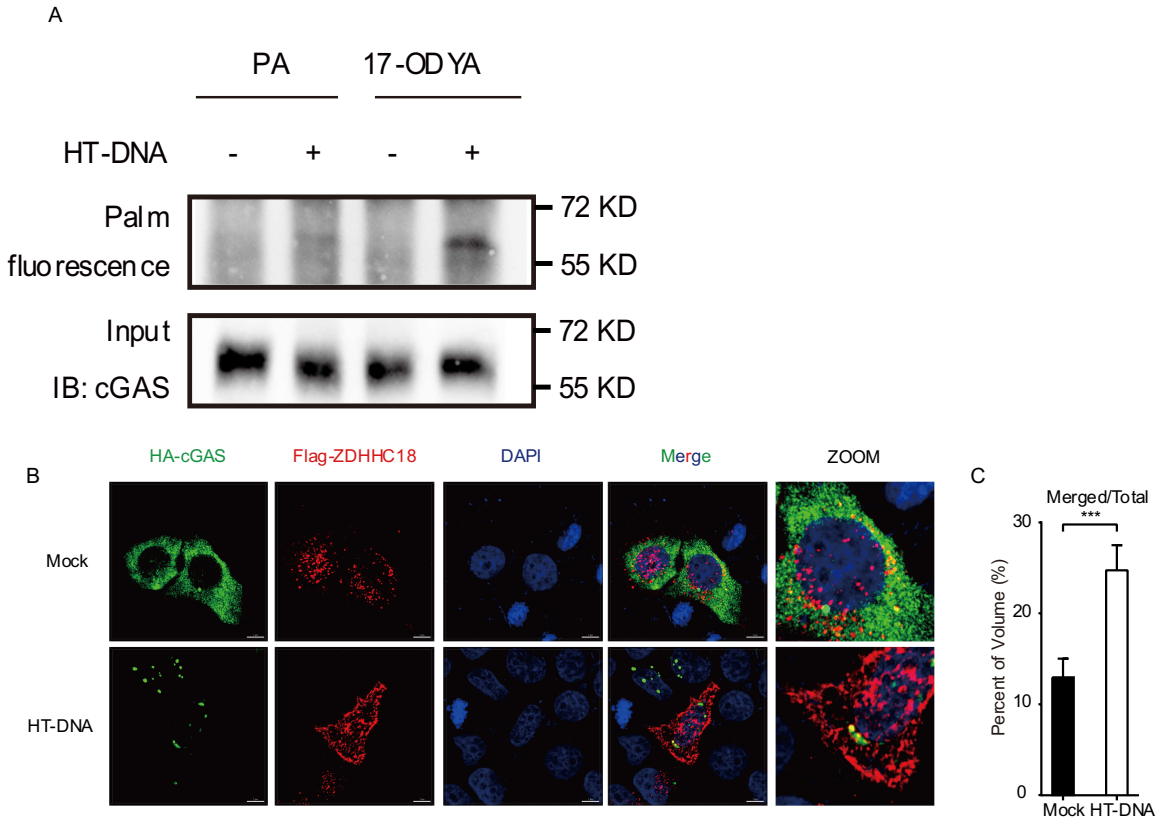
This reviewer fully recognized the significance of our data: *"In this work Shi et al report that cGAS is palmitoylated by ZDHHC18 to exert negative regulation of its activity by impairing DNA binding. The data presented are generally strong, and appear convincing."*

1. One essential unresolved question is whether palmitoylation and ZDHHC18 are part of the constitutive suppression of cGAS activity or an activation-induced negative feed-back. This is important to resolve. This should include (but not be limited to) detailed kinetics of DNA-induced IFNb expression in wt versus KO cells. 2. If it turns out to be a negative feed-back loop, some initial characterization of what induces ZDHHC18 activation / cGAS palmitoylation should be performed. For instance, is cGAS palmitoylation dependent on STING or TBK1?

Response: We thank this reviewer's comment. As we mentioned in the Discussion section, we believe that the suppression of cGAS avtivity by palmitoylation and ZDHHC18 is a DNA-induced negative feed-back. There are also three pieces of key evidence:1, using click chemistry to detect endogenous cGAS palmitoylation in mouse macrophages, we found that cGAS is not palmitoylated in resting state, while palmitoylation of cGAS appears when cGAS is activated by DNA (Response fig. 1A); 2, using acyl-RAC assay to detect cGAS palmitoylation in cells, cGAS is overexpressed through transfecting expressing plasmids, thus cGAS is activated by plasmid DNA and its palmitoylation is detected (Figure 1A, 1B, 1G, 2A and 2C); 3, since ZDHHC18 is the major palmitoyl-transferase of cGAS, the association between cGAS and ZDHHC18 is increased in the presence of DNA, agreeing with the activation-induced negative feed-back model of cGAS palmitoylation (Response fig. 1B and 1C). Therefore, we concluded that in the resting state, cGAS is not palmitoylated, which primed cGAS for rapid activation to initiate immune responses; after cGAS is activated by DNA, ZDHHC18 closely interacts with cGAS and promotes its palmitoylation, functioning as a negative feed-back to reduce cGAS signaling and prevent its overactivation.

Furthermore, based on our data, the cGAS palmitoylation is not dependent on STING or TBK1. As we carried out cGAS palmitoylation experiments in HEK293T cells which lack the endogenous expression of STING, TBK1 and downstream signaling pathway is not activated. Thus, cGAS palmitoylation happens independent of STING and TBK1.

Finally, we agree with the reviewer that kinetics studies would be helpful. Given the chance to revise, we will be happy to perform detailed kinetics of DNA-induced IFNb expression in ZDHHC18- wt versus ZDHHC18- KO cells and the effect of palmitoylation inhibitors.



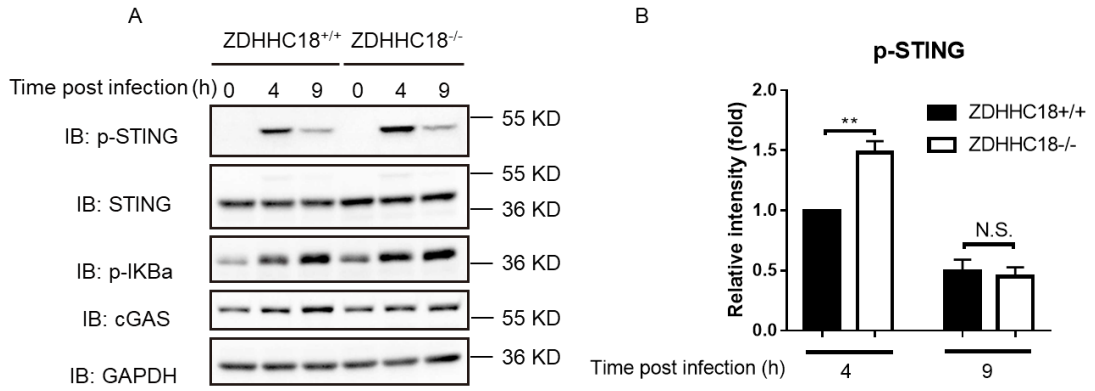
Response fig. 1 (A) This is data of Figure 1C in manuscript. Click chemistry applied for detecting endogenous cGAS palmitoylation in RAW264.7 cells. PA: palmitic acid; 17-ODYA: 17-octadecanoic acid. (B) This is data of Figure 2K in manuscript. Immunofluorescence analysis of HA-cGAS (green) and Flag-ZDHHC18 (red) in HT-DNA-stimulated (or not) HeLa cells. Scale bars: 7 μm. (C) This is data of Figure 2L in manuscript. Colocalization (merged volume of total cGAS signal) of cGAS and ZDHHC18 in (B). ***, P<0.001.

3. The infection phenotype presented in Fig. 6A is rather weak. Different doses of virus should be tested, or other infection/disease models should be tested. In the absence of a clear phenotype in mice or a primary human cell system, this reviewer is not convinced about the physiological relevance.

Response: These are good points. Given the chance to resubmit, we plan to test different doses of HSV-1 infection in mice. Moreover, we are going to test another DNA virus, VACV, which is generally used to activate cGAS in mice models. Further, we will use siRNA to detect the phenotype of ZDHHC18 and cGAS palmitoylation in human peripheral blood mononuclear cells (PBMCs).

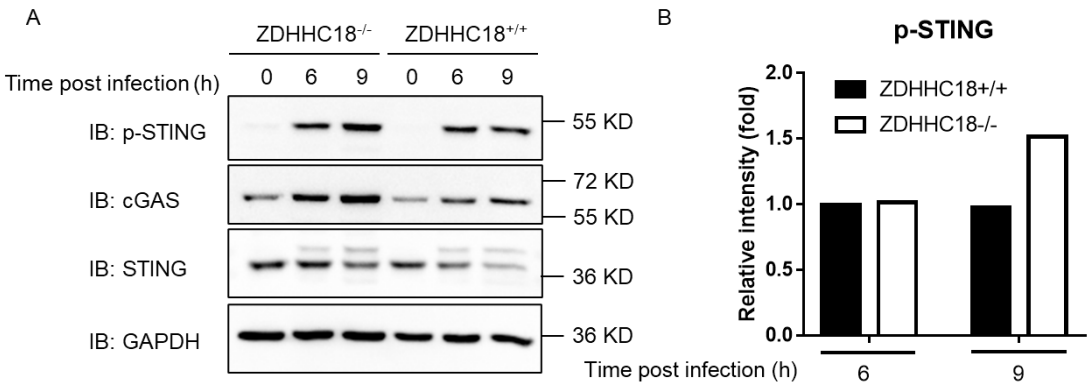
4. In line with the modest phenotype, the data in Fig 6G does not show a strong effect on pSTING in KO cells.

Response: In order to clarify the effect of phosphorylated STING in KO cells, we calculated the relative intensity of phosphorylated STING in figure 6G (Response fig. 2A) and its replicates. We found that at 9 hours post HSV-1 infection when the level of phosphorylated STING is low, the effect in KO cells is indeed not obvious (Response fig. 2B right two columns). However, at 4 hours post HSV-1 infection when the level of phosphorylated STING is high, the effect in KO cells is much more apparent (about 1.5-fold intensity relative to WT control) (Response fig. 2B left two columns).
Furthermore, we plan to examine the kinetics of phosphorylated STING in KO cells.



Response fig. 2 (A) This is data of Figure 6G in manuscript. BMDMs (Zdhhc18+/+ or Zdhhc18-/-) were infected with HSV-1 (MOI=2) for the indicated times before immunoblot analysis, as shown. (B) Relative intensity of phosphorylated STING in (A) and replicates. N.S., no significance. **, P<0.005.

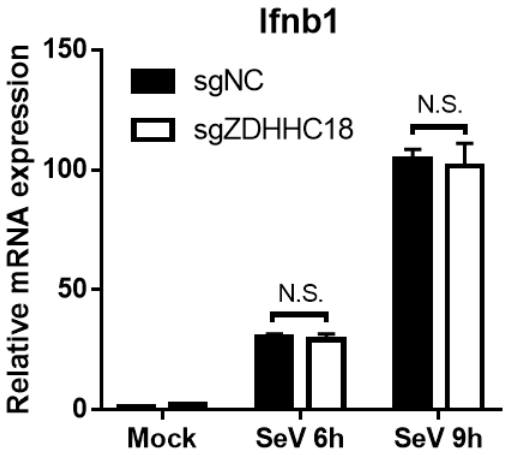
Furthermore, we have also infected HSV-1 in ZDHHC18-/- primary MEFs. The phosphorylated STING after HSV-1 infection in MEFs showed difference compared with BMDMs (Response fig. 3A). We found that at 6 hours post HSV-1 infection, the effect of phosphorylated STING in KO MEFs was not obvious (Response fig. 3B left two columns). Whereas at 9 hours post HSV-1 infection, the intensity of phosphorylated STING in KO MEFs was about 1.5-fold to WT cells (Response fig. 3B right two columns). Taken together, these results suggested that in different ZDHHC18 KO primary cells, phosphorylated level of STING increased to ~1.5-fold at a specific time point.



Response fig. 3 (A) MEFs (Zdhhc18+/+ or Zdhhc18-/-) were infected with HSV-1 (MOI=5) for the indicated times before immunoblot analysis, as shown. (B) Relative intensity of phosphorylated STING in (A).

5. One important question is whether ZDHHC18 is specific for cGAS. It should be tested in detail whether ZDHHC18 KO cells exhibit different response to other PRR agonists (RIG-I and TLR pathways).

Response: We agree that it's important to know whether ZDHHC18 is specific for cGAS. In order to answer the question, we have used CRISPR to KO ZDHHC18 in HEK293T cells and infected cells with Sendi virus (SeV) which can effectively activate RIG-I pathways. As shown here, ZDHHC18 KO had no effects on Sendi virus induced type I interferon expression (Response fig. 4), indicating ZDHHC18 did not affect RIG-I pathway. Next, we plan to test the downstream signals response of ZDHHC18 KO cells to other PRR agonists in TLR pathway.



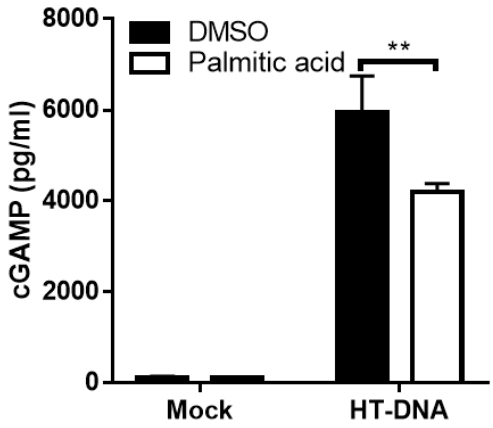
Response fig. 4 HEK293T cells were transfected with px458 (500 ng), px458-sgZDHHC18 (500 ng) plasmids for 24 h. Cells were treated with SeV (1:200) for 6 h and 9h before RT-qPCR analysis of IFNb1 expression. N.S., no significance.

Referee #2:

This reviewer commented favorably on our manuscript: *"The authors demonstrated that cGAS undergoes palmitoylation, which restricts its activity. They identified the responsible palmitoyltransferase ZDHHC18, and further demonstrated the significance of this post-translational modification in KO mice. The work presented here is potentially interesting."*

Major critiques:
Figure 1:
** In addition to C474S, the degree of palmitoylation appeared to be drastically reduced in C405S and C409S. The corresponding texts in "Results" is misleading and should be amended.*
** The amount of cGAMP should be examined with/without palmitic acid.*

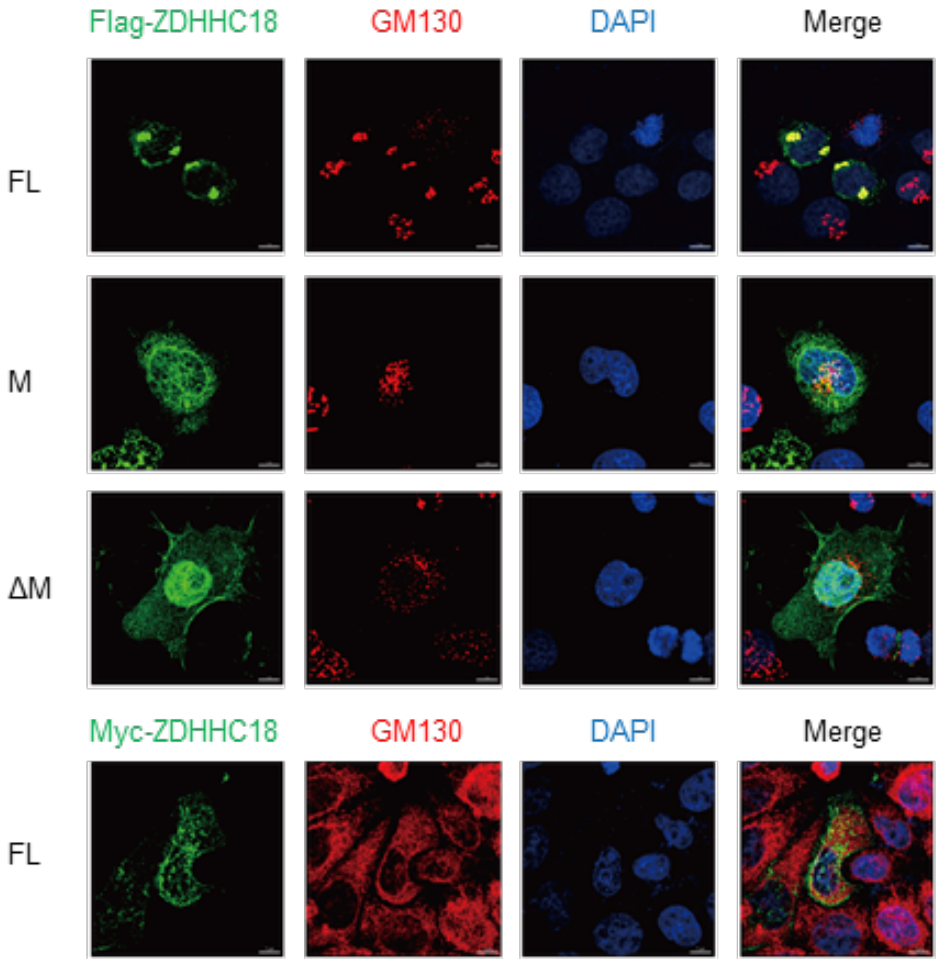
Response: We thank the suggestion by the reviewer. We examined the amount of cGAMP with/without the addition of palmitic acid in THP-1 cells. The results showed that palmitic acid which promoted palmitoylation of cGAS reduced the production of cGAMP in the presence of double-stranded DNA (Response fig. 5), which is consistent with our conclusion that palmitoylation impaired the enzymatic activity of cGAS.



Response fig. 5 THP-1 cells were treated with DMSO or palmitic acid (100 μM) for 12 h. Six hours after transfection with HT-DNA (2 μg/mL), cGAMP was extracted and quantified by cGAMP ELISA. **, P<0.005.

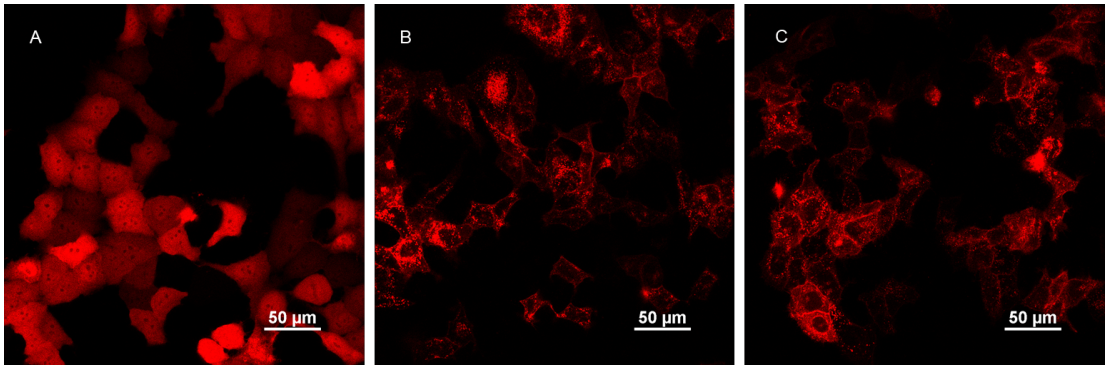
*Figure 2: * The image of ZDHHC18 in Figure 2K and Figure S2D appeared very different, even in the same cells (HeLa cells) used. Figure 2K showed the punctate localization (what kind of organelles?), in contrast, Figure S2D showed the typical Golgi localization. Given this discrepancy, it is impossible to interpret the results shown in Figure 2K (a partial co-localization between cGAS puncta and ZDHHC18 after DNA transfection). In Figure S2F, ZDHHC18 appeared to show the typical ER localization.*

Response: We thank the reviewer to point this out. It is worthy to note that the state of cGAS overexpression is different between Figure 2K (Response fig. 1B) and Figure S2D (Response fig. 6): in Figure 2K, we overexpressed Flag-ZDHHC18 together with HA-cGAS and found that ZDHHC18 showed the punctate localization (Response fig. 1B); whereas in Figure 2D, we overexpressed Flag-ZDHHC18 without overexpressing cGAS and found that ZDHHC18 showed typical Golgi localization (Response fig. 6).



Response fig. 6 This is data of Figure S2D in manuscript. Immunofluorescence analysis of Flag-ZDHHC18 (FL or truncated mutants) or Myc-ZDHHC18 and GM130 or calnexin in HeLa cells. FL: full length. Scale bars: 7 μm.

Moreover, we also detected the localization of ZDHHC18 in cGAS KO HeLa cells (Response fig. 7). We found that after cGAS was deleted, ZDHHC18 showed spots-like localization which was different from that in Figure 2K (Response fig. 7). One possible reason for these results is that different concentration and aggregation state of cGAS in cytoplasm (depletion, endogenous expression and overexpression) may alter the localization pattern of ZDHHC18.

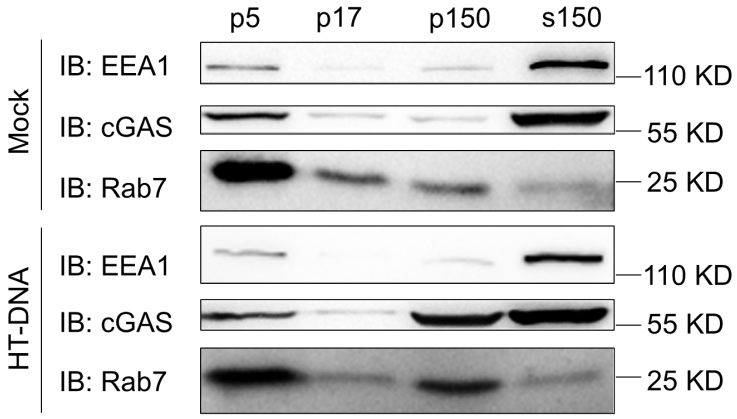


Response fig. 7 Immunofluorescence analysis of mCherry (A), ZDHHC18-mCherry and ZDHHC18(CS)-mCherry in cGAS-/- HeLa cells. ZDHHC18(CS): a catalytic mutant with a cysteine-to-serine substitution in the DHHC motif of ZDHHC18.

Figure 3: Figure 3A: It is very confusing that they used GFP-cGAS in this particular experiment. Why was HA-cGAS not found in the nucleus in Figure 2K? If the tagging anything to cGAS interferes its localization, the authors should examine the localization of endogenous cGAS throughout the experiments (as they stained in Figure S2F). In Figure S3A, the stain of Rab7 is not convincing. The authors should carefully pay attention to the previous literatures that never describe the cGAS localization to late endosomes. Again, the endogenous cGAS localization should be examined.*

Response: We would like to point out that during our experiments, we found that when cells are growing at low density, overexpressed cGAS was mainly detected in the nucleus, whereas when cells reached at high density, overexpressed cGAS was predominantly localized in the cytoplasm, which was consistent with the results reported by experts of this field (Yang *et al*., *PNAS*, 2017, E4612-E4620).

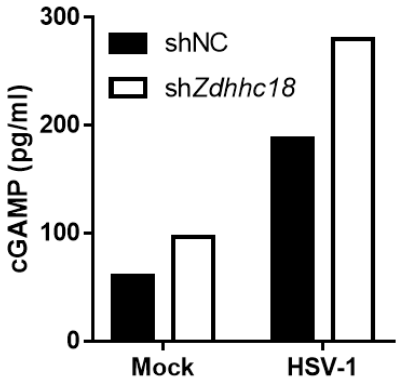
We would like to point out that some literatures described some fraction of cGAS was associated with light vesicles or organelles (Sun *et al*., *Science*, 2013, 786-791; Barnett *et al*., *Cell*, 2019, 1432-1446). However, direct evidence of the localization of cGAS to late endosomes remains obscure. Meanwhile, in our fraction experiments (Response fig. 8), we also found that both cGAS and Rab7 were present in the P150 fraction, which indicates an association of cGAS and Rab7. Together with the cell staining results in Figure S3A in manuscript, we speculate an association between cGAS and Rab7, but more direct evidence is needed. Based on these points, we are much interested to collect more detailed data about the cellular localization of cGAS with late endosomes.



Response fig. 8 THP-1 cells were homogenized in hypotonic buffer and subjected to differential centrifugation. Pellets at different speeds of centrifugation (e.g., P150: pellets after 150,000g) and S150 were immunoblotted with the indicated antibodies.

Figure 5:
** Figure 5B: ZHHC18 (CS) should be examined.*
** Figure 5C: Does KD of ZHHC18 affect the amount of cGAMP?*

Response: We have measured the amount of cGAMP in ZDHHC18 KD L929 cells with the infection of HSV-1 to activate cGAS and found that KD of ZDHHC18 promoted the production of cGAMP, which further confirmed the negative effect of ZDHHC18 in regulating cGAS activity (Response fig. 9). Moreover, we plan to examine the phenotype of ZDHHC18(CS) when overexpressed in HEK293T cells.



Response fig. 9 L929 cells stably transfected with control shRNA or ZDHHC18 shRNA were infected with HSV-1 (MOI=5) for the indicated times, and the amount of cGAMP in the lysates was quantified by LC-MS/MS. LC-MS/MS: liquid chromatography-tandem mass spectrometry.

Technical critiques:
(1) In Figure 1, having the schematic for acyl-RAC assay would be helpful for

general readers.

Response: We have drawn a schematic for acyl-RAC.

(2) All of the WB lack the molecular weight indicators. Some WB lack the appropriate legends (blotted with what antibody? such as Fig. 1G).

Response: We have modified the related content.

Dear Hubert,

Thank you for sending me your response to the referees' comments. I have now had a chance to take a look at it.

I appreciate the comments and your response. However, we would need further insight into

- How ZDHHC18 is activated and the cGas/ZDHHC28 interaction is promoted in the presence of DNA. Your proposed kinetics studies go towards to addressing his point, but this is not enough. We need some insight into how cGAS palmitoylation is regulated and it looks like this comes down to ZDHHC18, but how and why is not clear

- We need strong support for that this regulation is physiological relevant (ref #1 - point 3). This is key for consideration of the paper here.

In the absence of such data, I can't offer to consider a revised version. If you have further data along those lines then I can offer to look at another version. Please note that a re-submission will be considered a new submission and that novelty will be taken into consideration at time of submission.

With best wishes

Karin

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

Referee #1:

This reviewer fully recognized the significance of our data. *"In this work Shi et al report that cGAS is palmitoylated by ZDHHC18 to exert negative regulation of its activity by impairing DNA binding. The data presented are generally strong, and appear convincing."* We appreciate this reviewer's comment.

He or she raised a number of concerns that can be fully addressed.

1. One essential unresolved question is whether palmitoylation and ZDHHC18 are part of the constitutive suppression of cGAS activity or an activation-induced negative feed-back. This is important to resolve. This should include (but not be limited to) detailed kinetics of DNA-induced IFNb expression in wt versus KO cells. 2. If it turns out to be a negative feed-back loop, some initial characterization of what induces ZDHHC18 activation / cGAS palmitoylation should be performed. For instance, is cGAS palmitoylation dependent on STING or TBK1?

Response:

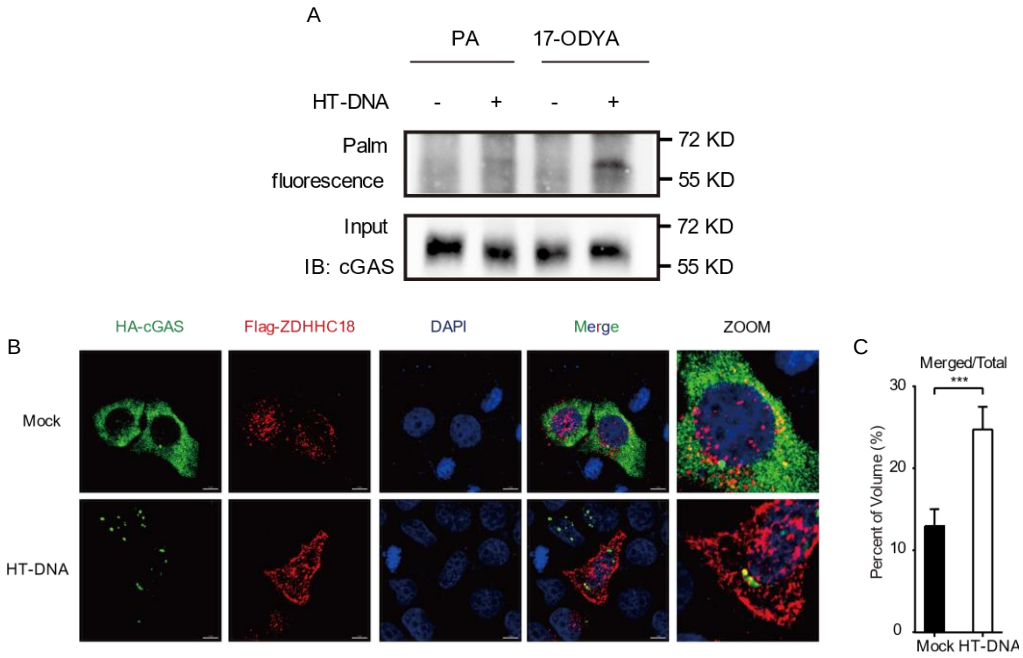
We thank this reviewer's comment.

* We believe that the suppression of cGAS activity by palmitoylation and ZDHHC18 occurs in a DNA-induced manner. There are three pieces of key evidence. First, by using click chemistry to detect endogenous cGAS palmitoylation in mouse macrophages, we found that cGAS is not palmitoylated in the resting state, while palmitoylation of cGAS appears when cGAS is activated by DNA (Response fig. 1A; or Fig 1*C* in the MS). Second, since ZDHHC18 is the major palmitoyl-transferase of cGAS, the association between cGAS and ZDHHC18 is increased in the presence of DNA, agreeing with the DNA-induced palmitoylation of cGAS (Response fig. 1B and 1C; or Fig 2*K* and *L* in the MS); data from the MD analysis also showed that the presence of DNA promoted the formation of the cGAS-ZDHHC18 complex (Response fig. 1D and 1E; or Fig 2*M* and *N* in the MS). Third, by performing a kinetics study of DNA-induced IFNb expression in wt and *Zdhhc18* KO MEFs, we found that IFNb and *Cxcl10* expression in Zdhhc18 deficient MEFs was higher than that in WT MEFs as soon as cGAS was activated by VACV infection (2-6 h) (Response fig. 1, F and G; or Fig 6*H* and *I* in the MS). Moreover, the results were consistent when we detected the phosphorylation level of TBK1 in *Zdhhc18*-deficient MEFs versus WT MEFs (Response fig. 1H; or Fig 6*G* in the MS).

Based on these results, we concluded why and how cGAS was regulated by ZDHHC18-mediated palmitoylation: in the resting state, cGAS is not palmitoylated, which primes cGAS for rapid activation to initiate immune responses; as soon as cGAS recognizes and binds DNA, ZDHHC18 closely interacts with cGAS and promotes its palmitoylation, impairing its DNA binding and dimerization of cGAS, functioning as a negative regulator of cGAS

signaling and prevention of its overactivation.

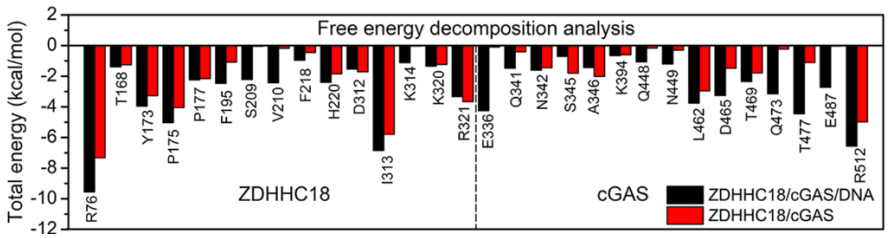
* Furthermore, based on our data, we believe that the cGAS palmitoylation is not dependent on STING or TBK1. When we carried out an acyl-RAC assay to detect cGAS palmitoylation in HEK293T cells that lacked endogenous expression of STING, cGAS was palmitoylated without activation of TBK1, STING or downstream signaling pathways. Thus, cGAS palmitoylation occurs independent of STING and TBK1 (Response Fig. 1I; or Fig 1*A* in the MS).

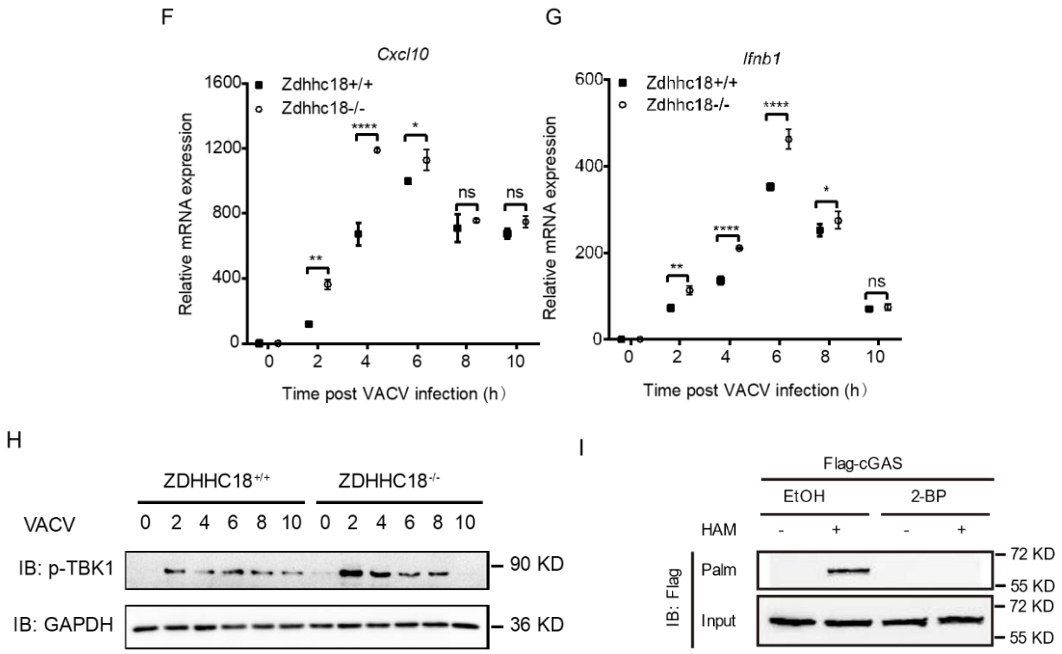


D

Binding free energy between ZDHHC18 and cGAS (Kcal/mmol)		
	Total	STD
ZDHHC18/cGAS/DNA	-48.38	11.41
ZDHHC18/cGAS	-33.88	14.50

E





Response fig. 1 (A) Click chemistry was applied to detect endogenous cGAS palmitoylation in RAW264.7 cells. PA: palmitic acid; 17-ODYA: 17-octadecanoic acid. (B-C) Immunofluorescence analysis of HA-cGAS (green) and Flag-ZDHHC18 (red) in HT-DNA-stimulated (or not) HeLa cells (B) and the colocalization (merged volume of total cGAS signal) of cGAS and ZDHHC18 (C). (D-E) Comparison of the binding free energy (D) and binding energy decomposition of a per-residue to the binding affinity between ZDHHC18 and cGAS (E) in different complexes. (F-G) MEFs (*Zdhhc18*$^{+/+}$ or *Zdhhc18*$^{-/-}$) were infected with VACV (1:200) for the indicated times before RT-qPCR analysis of *Cxcl10* (F) and *Ifnb1* (G) expression. (H) MEFs (*Zdhhc18*$^{+/+}$ or *Zdhhc18*$^{-/-}$) were infected with VACV (1:200) for the indicated times before immunoblot analysis. (I) Acyl-RAC assay of HEK293T cells transfected with the indicated plasmids for 24 h. EtOH: ethanol; HAM: hydroxylamine; 2-BP: 2-bromopalmitate. Scale bars: 7 μm; *, P<0.01; **, P<0.005; ***, P<0.001; ****, P<0.0001.

3. The infection phenotype presented in Fig. 6A is rather weak. Different doses of virus should be tested, or other infection/disease models should be tested. In the absence of a clear phenotype in mice or a primary human cell system, this reviewer is not convinced about the physiological relevance. 4. In line with the modest phenotype, the data in Fig 6G does not show a strong effect on pSTING in KO cells.

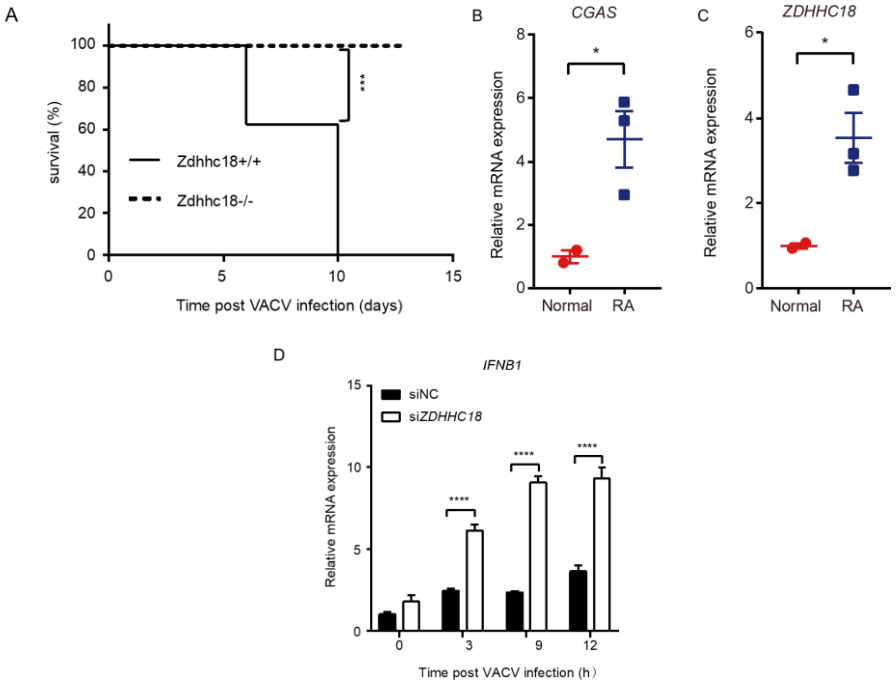
Response:

We thank the reviewer's suggestion.

* We infected *Zdhhc18* KO mice with the DNA virus VACV, which is generally used to activate cGAS in mouse models. As shown in Response Fig. 2A (or Fig 6*A* in the MS), Zdhhc18 depletion dramatically increased the survival rate of

VACV infection (p<0.001). Moreover, we found that the expression of both cGAS and ZDHHC18 was higher in human peripheral blood mononuclear cells (PBMCs) of RA patients than in controls, which indicated a relationship between cGAS and ZDHHC18 in the human primary cell system (Response fig. 2B and C; or *Appendix*, Fig S6, *G* and *H* in the MS). Therefore, we used siRNA to knockdown ZDHHC18 in PBMCs and found that the expression of *IFNB1* became strongly higher after infection with VACV (Response fig. 2D; or Fig 6*J* in the MS). These results indicated that ZDHHC18 also regulated DNA induced cGAS activation in the human primary cell system.

* In addition, the phenotype of the VACV-induced kinetics of Ifnb and Cxcl10 expression in *Zdhhc18* KO MEFs was also clear. The expression of Ifnb and Cxcl10 was dramatically higher in *Zdhhc18* KO MEFs at the early phase of VACV infection (2-6 h), whereas the difference was not significant at the later phase of VACV infection (8-10 h) (Response fig. 1, F and G; or Fig 6*H* and *I* in the MS). Consistently, the VACV-induced phosphorylation level of TBK1 was also substaintially higher in *Zdhhc18* KO MEFs after VACV infection (2-4 h) (Response fig. 1H; or Fig 6*G* in the MS).

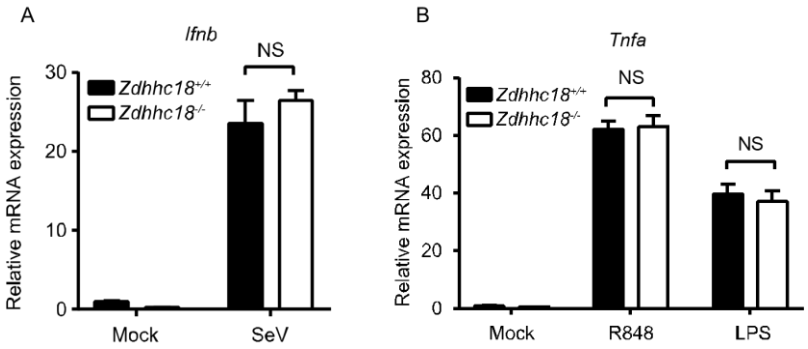


Response fig. 2 (A) Survival analysis of *Zdhhc18*$^{+/+}$ and *Zdhhc18*$^{-/-}$ mice (n = 8 each, 6-8 weeks old) intranasally injected with VACV (5 × 10^7 PFU per mouse) and monitored daily for survival for 13 days using Kaplan–Meier curves and a log-rank (Mantel-Cox) test. PFU: plaque-forming units. (B-C) PBMCs from two healthy people and three RA patients were analyzed by RT-qPCR for the expression of *cGAS* (B) and *ZDHHC18* (C). (D) PBMCs from healthy people were transfected with scrambled siRNA or ZDHHC18 siRNA (cocktail) for 48 h and then infected with VACV (1:200) for the indicated times before RT-qPCR analysis of *IFNB1* expression. *, P<0.01; ***, P<0.001; ****, P<0.0001.

5. One important question is whether ZDHHC18 is specific for cGAS. It should be tested in detail whether ZDHHC18 KO cells exhibit different response to other PRR agonists (RIG-I and TLR pathways).

Response:

We thank for the reviewer's suggestion. We truly agree that it is important to know whether ZDHHC18 is specific for cGAS. To test the downstream signal responses of ZDHHC18 to other PRR agonists, *Zdhhc18* KO MEFs were treated with SeV, R848 and LPS, which can effectively activate RIG-I, TLR7/8 and TLR4, respectively. There were no significant differences between the *IFNB1* expression induced by SeV and *TNFA* induced by R848 and LPS, indicating that ZDHHC18 did not affect the RIG-I, TLR4 or TLR7/8 pathways (Response fig. 3, A and B; or *Appendix*, Fig S5, *D* and *E* in the MS).



Response fig. 3 (A) MEFs (Zdhhc18+/+ or Zdhhc18-/-) were infected with SeV (1:500) (A) or R848 (100 ng/ml) and LPS (100 ng/ml) (B). After six hours, the expression of interferon beta and TNF alpha was analyzed by RT-qPCR. Ns: no significance.

Referee #2:

This reviewer commented favorably on our manuscript. *"The authors demonstrated that cGAS undergoes palmitoylation, which restricts its activity. They identified the responsible palmitoyltransferase ZDHHC18, and further demonstrated the significance of this post-translational modification in KO mice. The work presented here is potentially interesting."* We truly appreciate this reviewer's comment.

He or she raised a number of specific points that we can fully address with our new data.

Major critiques:

Figure 1:

** In addition to C474S, the degree of palmitoylation appeared to be drastically reduced in C405S and C409S. The corresponding texts in "Results" is misleading and should be amended.*

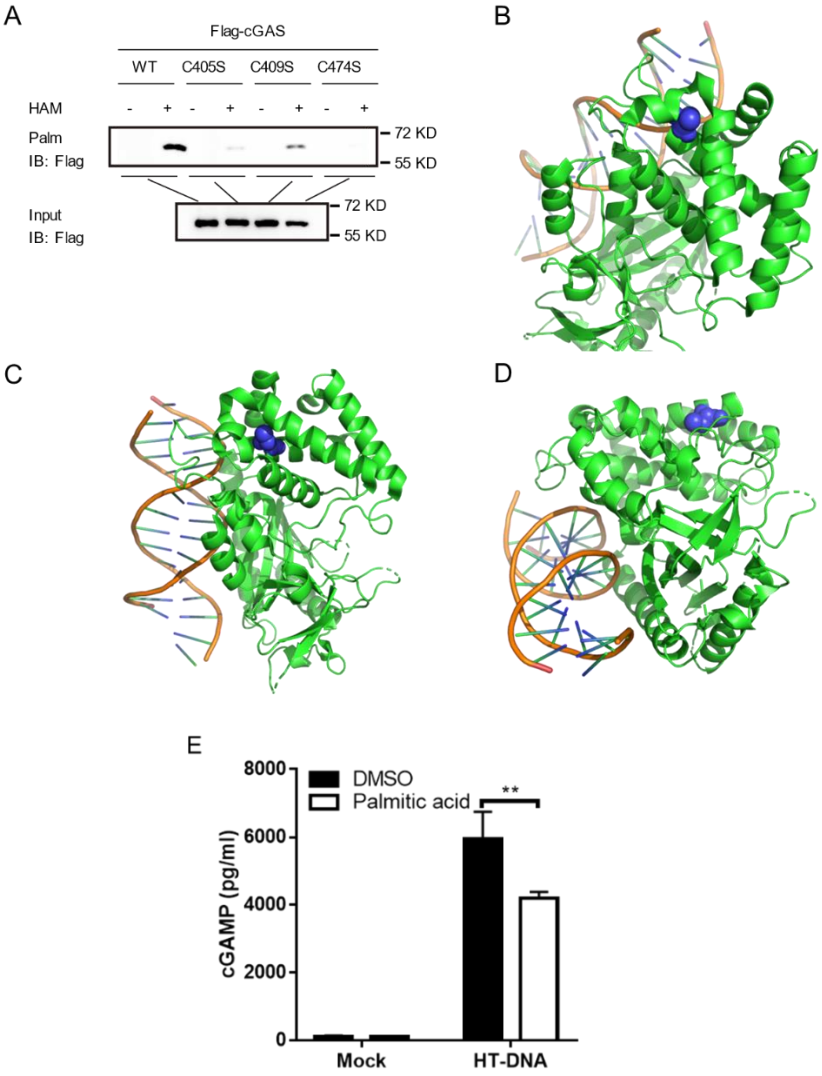
** The amount of cGAMP should be examined with/without palmitic acid.*

Response:

We thank the suggestion by the reviewer.

* We revised the corresponding statement in "Results" about the palmitoylation sites of cGAS. We found that compared to wild-type (WT) cGAS, the C405S, C409S and C474S mutants had drastically reduced palmitoylation signals (Response fig. 4A; or Fig 1*G* in the MS). Since sites C405 and C409 localized toward the inner side of the protein, these two sites were hardly to be palmitoylated structurally (Response fig. 4, B to D; or *Appendix*, Fig S1, *B* to *D* in the MS). The reduced palmitoylation signal of C405S and C409S could be caused by a conformational change, further confirming that the palmitoylated residue of cGAS is C474, which is easily structurally modified (Response fig. 4, B to D; or *Appendix*, Fig S1, *B* to *D* in the MS).

* We examined the amount of cGAMP with/without the addition of palmitic acid in THP-1 cells. The results showed that palmitic acid, which promoted palmitoylation of cGAS, reduced the production of cGAMP in the presence of double-stranded DNA (Response fig. 4E; or Fig 1*I* in the MS), which is consistent with our conclusion that palmitoylation impaired the enzymatic activity of cGAS.



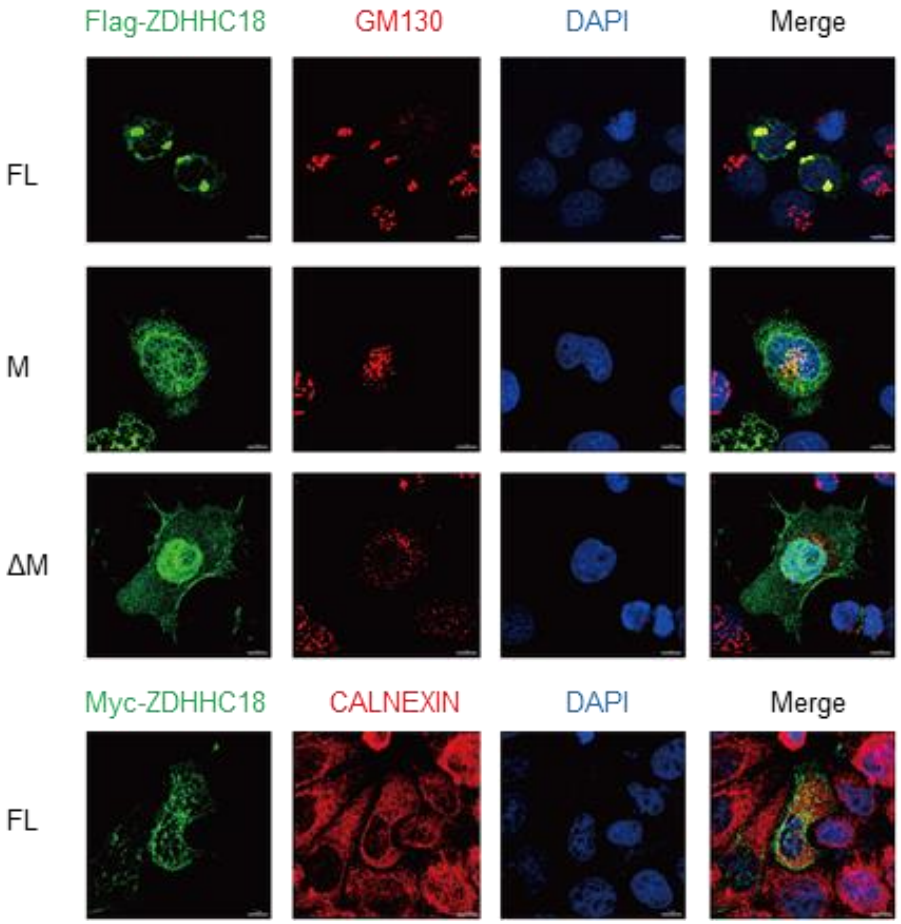
Response fig. 4 (A) Acyl-RAC assay of HEK293T cells transfected with the indicated plasmids for 24 h. HAM: hydroxylamine. (B-D) The positions of C405 (B), C409 (C) and C474 (D) in the human cGAS-DNA complex. The blue sphere indicates cysteines and the yellow helix indicates DNA. (E) THP-1 cells were treated with DMSO or palmitic acid (100 μM) for 12 h. Six hours after transfection with HT-DNA (2 μg/mL), cGAMP was extracted and quantified by cGAMP ELISA. **, P<0.005.

Figure 2:

** The image of ZDHHC18 in Figure 2K and Figure S2D appeared very different, even in the same cells (HeLa cells) used. Figure 2K showed the punctate localization (what kind of organelles?), in contrast, Figure S2D showed the typical Golgi localization. Given this discrepancy, it is impossible to interpret the results shown in Figure 2K (a partial co-localization between cGAS puncta and ZDHHC18 after DNA transfection). In Figure S2F, ZDHHC18 appeared to show the typical ER localization.*

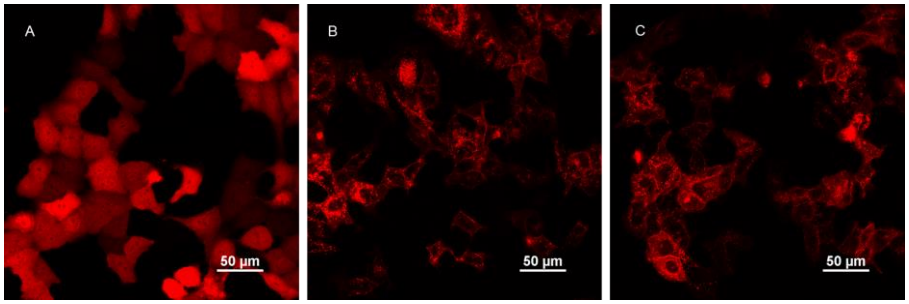
Response:

We thank the reviewer to point this out. It is worth noting that the state of cGAS overexpression is different between Figure 2K (Response fig. 1B; or Fig 2*K* in the MS) and Figure S2D (Response fig. 5; or *Appendix*, Fig S2*D* in the MS): in Figure 2K, we overexpressed Flag-ZDHHC18 together with HA-cGAS and found that ZDHHC18 showed punctate localization (Response fig. 1B; or Fig 2*K* in the MS), whereas in Figure S2D, we overexpressed Flag-ZDHHC18 without overexpressing cGAS and found that ZDHHC18 showed typical Golgi localization (Response fig. 5; or *Appendix*, Fig S2*D* in the MS).



Response fig. 5 Immunofluorescence analysis of Flag-ZDHHC18 (FL or truncated mutants) or Myc-ZDHHC18 and GM130 or calnexin in HeLa cells. FL: full length. Scale bars: 7 μm.

Moreover, we also detected the localization of ZDHHC18 in cGAS KO HeLa cells (Response fig. 6; we did not show it in the MS). We found that after cGAS was deleted, ZDHHC18 showed spot-like localization which was different from that in Response Fig. 6. One possible reason for these results is that different concentrations and aggregation states of cGAS in the cytoplasm (depletion, endogenous expression and overexpression) may alter the localization pattern of ZDHHC18.



Response fig. 6 Immunofluorescence analysis of mCherry (A), ZDHHC18-mCherry and ZDHHC18(CS)-mCherry in cGAS-/- HeLa cells. ZDHHC18(CS): a catalytic mutant with a cysteine-to-serine substitution in the DHHC motif of ZDHHC18.

Figure 3:

** Figure 3A: It is very confusing that they used GFP-cGAS in this particular experiment. Why was HA-cGAS not found in the nucleus in Figure 2K? If the tagging anything to cGAS interferes its localization, the authors should examine the localization of endogenous cGAS throughout the experiments (as they stained in Figure S2F). In Figure S3A, the stain of Rab7 is not convincing. The authors should carefully pay attention to the previous literatures that never describe the cGAS localization to late endosomes. Again, the endogenous cGAS localization should be examined.*

Response:

We thank the reviewer's detailed comment. We would like to point out that during our experiments, we found that when cells were growing at low density, overexpressed cGAS was mainly detected in the nucleus, whereas when cells reached at high density, overexpressed cGAS was predominantly localized in the cytoplasm, which was consistent with the results reported by experts in this field (Yang *et al*., *PNAS*, 2017, E4612-E4620).

After careful consideration, we removed the related content about the localization of cGAS and Rab7.

Figure 5:

** Figure 5B: ZHHC18 (CS) should be examined.*

** Figure 5C: Does KD of ZHHC18 affect the amount of cGAMP?*

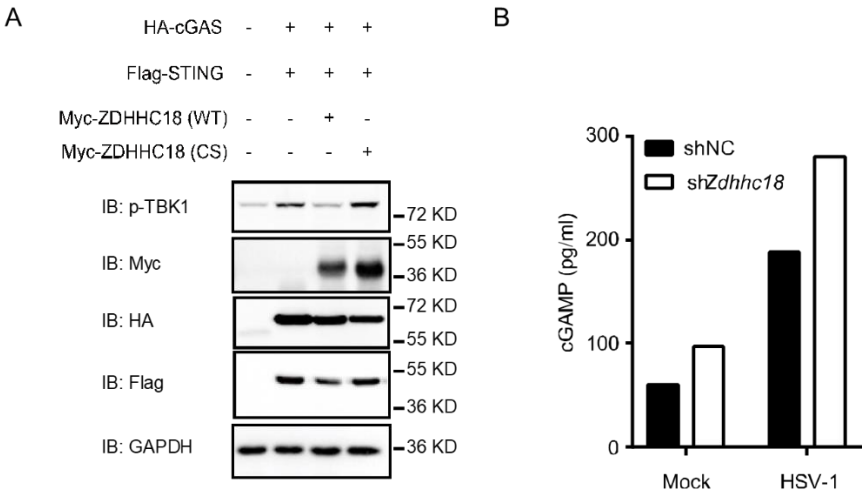
Response:

We thank the suggestion by the reviewer.

* We examined the phosphorylation level of TBK1 induced by overexpression of cGAS and STING in HEK293T cells. Overexpression of WT ZDHHC18 with cGAS and STING reduced the phosphorylation level of TBK1, whereas the enzymatically dead mutant ZDHHC18 (CS) rescued the phenotype of WT ZDHHC18 (Response fig. 7A; or Fig 5*B* in the MS).

* We measured the amount of cGAMP in ZDHHC18 KD L929 cells infected with HSV-1 to activate cGAS and found that KD of ZDHHC18 promoted the

production of cGAMP, which further confirmed the negative effect of ZDHHC18 in regulating cGAS activity (Response fig. 7B; or *Appendix*, Fig S5*C* in the MS).



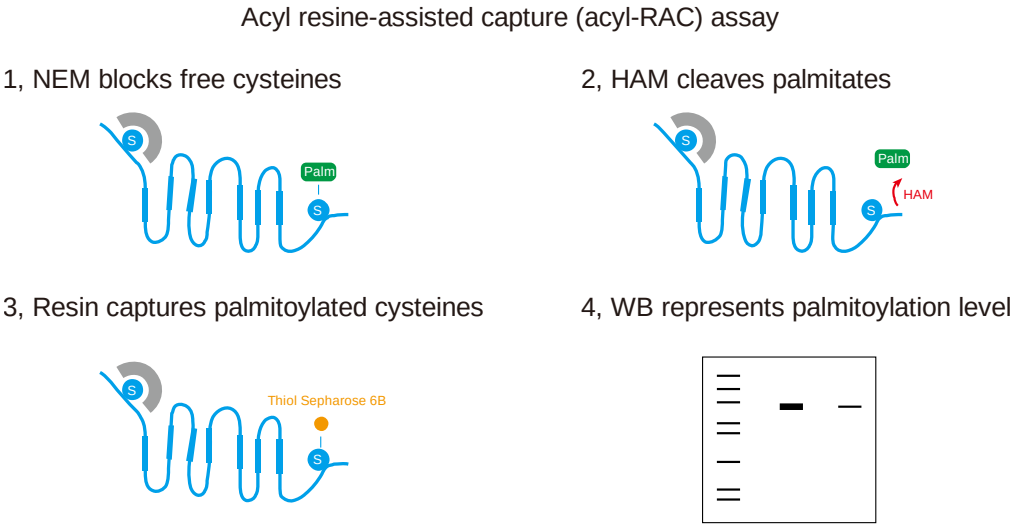
Response fig. 7 (A) HEK293T cells (1×10^6) were transfected with HA-cGAS (500 ng), Flag-STING (500 ng) and Myc-ZDHHC18 (500 ng) expression plasmids for 24 h. TBK1 phosphorylation was detected by immunoblotting. (B) L929 cells stably transfected with control shRNA or ZDHHC18 shRNA were infected with HSV-1 (MOI=5) for the indicated times, and the amount of cGAMP in the lysates was quantified by LC-MS/MS. LC-MS/MS: liquid chromatography-tandem mass spectrometry.

Technical critiques:

(1) In Figure 1, having the schematic for acyl-RAC assay would be helpful for general readers.

Response:

We thank the suggestion by the reviewer. We have drawn a schematic for acyl-RAC (*Appendix*, Fig S1*A* in the MS).



(2) All of the WB lack the molecular weight indicators. Some WB lack the appropriate legends (blotted with what antibody? such as Fig. 1G).

Response:

We thank the suggestion by the reviewer. We have modified the related content including the molecular weight indicators and legends of the WB.

Dear Hubert,

Thank you for submitting your revised manuscript to The EMBO Journal. This submission is a re-submission of MS 109272 that was rejected post review last year.

Given that you were able to address many of the key concerns raised, I was open to consider re-submission.

Your revision has now been re-reviewed by the two original referees. As you can see below, the referees appreciate the introduced changes and are supportive of publication here. The referees raise a few points that should be addressed for consideration here.

I) I agree with referee #1 that all data provided in the point-by-point response should be added to the manuscript. I also find your description regarding the new data and if it has been added to the manuscript a bit confusing in the point-by-point response.

2) Please check if cGAMP is needed for cGAS palmitoylation (referee #1).

3) Referee #2 still has some concerns about the ZDHHC18 localization and suggest making stable cell lines to better address this issue.

So almost there!

With best wishes

Karin

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

Instructions for preparing your revised manuscript:

I have attached a PDF with helpful tips on how to prepare the revised version

Further information is available in our Guide For Authors: https://www.embopress.org/page/journal/14602075/authorguide

Use the link below to submit your revision:

https://emboj.msubmit.net/cgi-bin/main.plex

Referee #1:

The authors have addressed most of the points I raised in the first round of review, and I generally find the work improved - regarding both mechanism and physiological relevance. However, some of the new data described in the point-by-point response are not included in the revised manuscript, which they should be and which also and makes it is difficult to evaluate the revised work.
In particular, the authors describe data on cGAS palmitoylation, being independent of STING and TBK1. This is very interesting and central for the mechanistic part of the work. Therefore, these data should therefore be shown in the paper. In addition, these data raise another important question, namely whether cGAMP (the enzymatic product of cGAS). This can easily be addressed by treatment with cGAMP or transfection with WT versus enzymatically dead cGAS. Such data would add significant impact and novelty to the work.

Referee #2:

The authors mostly addressed to my original concerns, except the one regarding the subcellualr localization part. This reviewer is still not convinced with the data presented in Figure 2K and Figure S2D.

In Figure S2D: the subcellular localizations of ZDHHC18 (FL) are significantly different. One in the top row suggest the Golgi localization, the one in the bottom row showed the punctate localization (dose not look like the Golgi). If the localization is prone

to be affected by the expression level of ZDHHC18, the stable cell lines should be establihsed and examined with/without HT-DNA. One cellular image should contain multiple cells.

Referee #1:

We appreciate this reviewer's comment. *"In this work Shi et al report that cGAS is palmitoylated by ZDHHC18 to exert negative regulation of its activity by impairing DNA binding. The data presented are generally strong, and appear convincing."*

He or she raised a number of concerns that can be fully addressed.

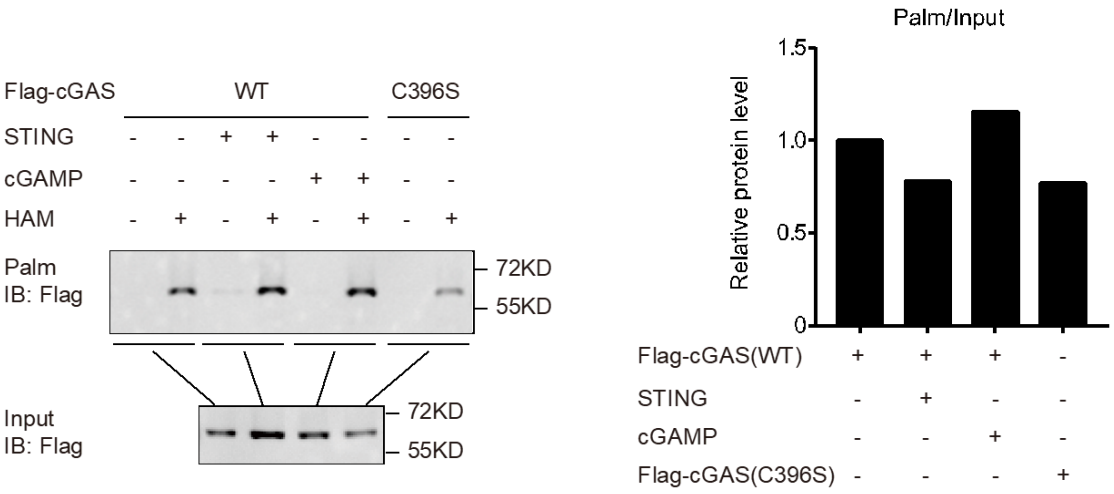
1. The authors have addressed most of the points I raised in the first round of review, and I generally find the work improved - regarding both mechanism and physiological relevance. However, some of the new data described in the point-by-point response are not included in the revised manuscript, which they should be and which also and makes it is difficult to evaluate the revised work. In particular, the authors describe data on cGAS palmitoylation, being independent of STING and TBK1. This is very interesting and central for the mechanistic part of the work. Therefore, these data should therefore be shown in the paper. In addition, these data raise another important question, namely whether cGAMP (the enzymatic product of cGAS). This can easily be addressed by treatment with cGAMP or transfection with WT versus enzymatically dead cGAS. Such data would add significant impact and novelty to the work.

Response:

We thank this reviewer's comment.

We have added all data provided in the point-by-point response into the manuscript.

Based on our data, we believe that the cGAS palmitoylation is not dependent on STING or TBK1. To further confirm this, we carried out additional works by overexpressing STING in the acyl-RAC assay. We found that overexpression of STING did not affect cGAS palmitoylation level. Then we focused on cGAMP by adding cGAMP into cells or using the C396S mutant, which failed to produce cGAMP, in acyl-RAC assays. The cGAS palmitoylation level showed little difference in these conditions, indicating that cGAS palmitoylation is not dependent on cGAMP or STING (Response Fig. 1; or *Appendix*, Fig S1, *B* and *C* in the MS).



Response fig. 1 Acyl-RAC assay of HEK293T cells transfected with Flag-cGAS and the indicated plasmids for 24 h or treated with cGAMP (1 μg/ml) for 1 h.

Referee #2:

This reviewer commented favorably on our manuscript. *"The authors demonstrated that cGAS undergoes palmitoylation, which restricts its activity. They identified the responsible palmitoyltransferase ZDHHC18, and further demonstrated the significance of this post-translational modification in KO mice. The work presented here is potentially interesting."*

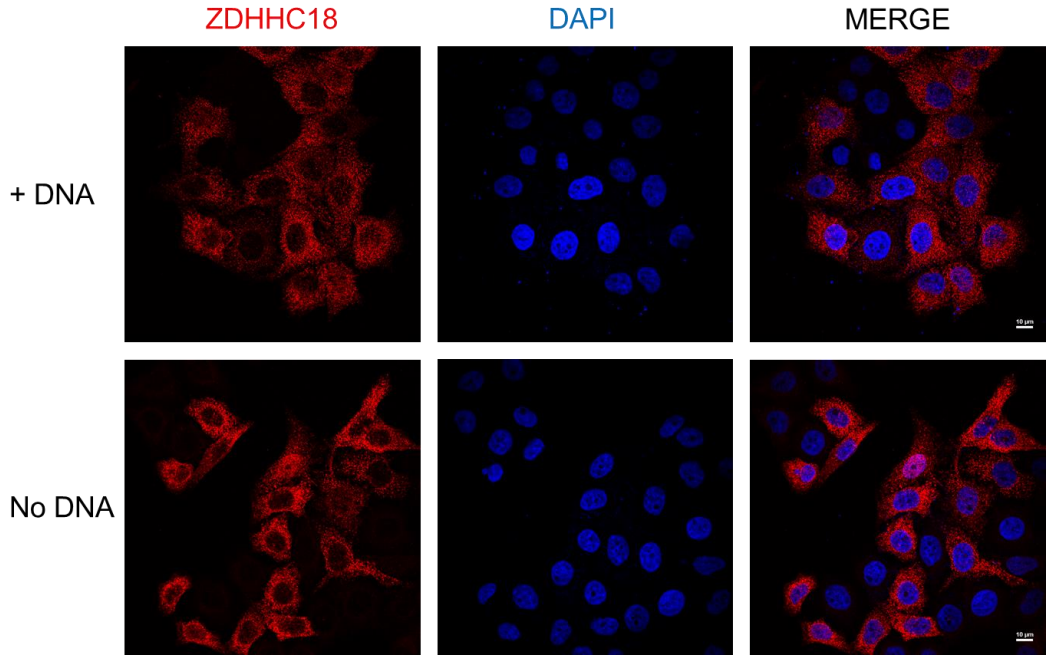
He or she raised a number of specific points that we can fully address with our new data.

The authors mostly addressed to my original concerns, except the one regarding the subcellular localization part. This reviewer is still not convinced with the data presented in Figure 2K and Figure S2D.

In Figure S2D: the subcellular localizations of ZDHHC18 (FL) are significantly different. One in the top row suggest the Golgi localization, the one in the bottom row showed the punctate localization (dose not look like the Golgi). If the localization is prone to be affected by the expression level of ZDHHC18, the stable cell lines should be established and examined with/without HT-DNA. One cellular image should contain multiple cells.

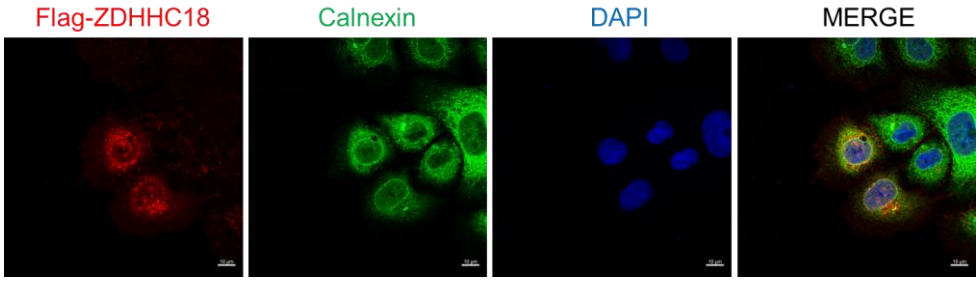
Response:

We agreed to dissect the subcellular localization of ZDHHC18. To do so, we firstly performed cell staining assay of endogenous ZDHHC18 in HeLa cells. Endogenous ZDHHC18 showed a dispersed pattern in HeLa cells with or without DNA stimulation (Response fig. 2), which seemed like ER or Golgi. However, limited by the conflict between antibody species, we could not confirm the colocalization of endogenous ZDHHC18 with ER or Golgi.

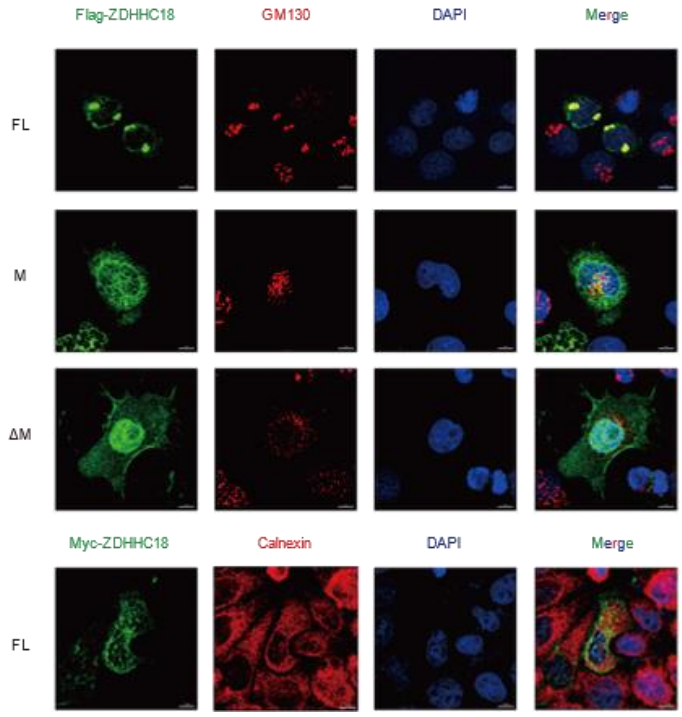


Response fig. 2 cell staining of endogenous ZDHHC18 in HT-DNA-stimulated (or not) HeLa cells. Scale bars: 10 μm.

Then we detected the subcellular localization of transiently overexpressed Flag-tagged ZDHHC18 in HeLa cells. We found that transiently overexpressed Flag-tagged ZDHHC18 showed both dispersed and punctate pattern in different cells (Response fig. 3). Consistently, it is worthy to point out that the images in top row of *Figure S2D* (here referred Response fig. 4) showed that most fraction of ZDHHC18, which is transiently overexpressed, localized at Golgi, whereas some fraction of ZDHHC18 showed a dispersed pattern (Response fig. 4, top row).



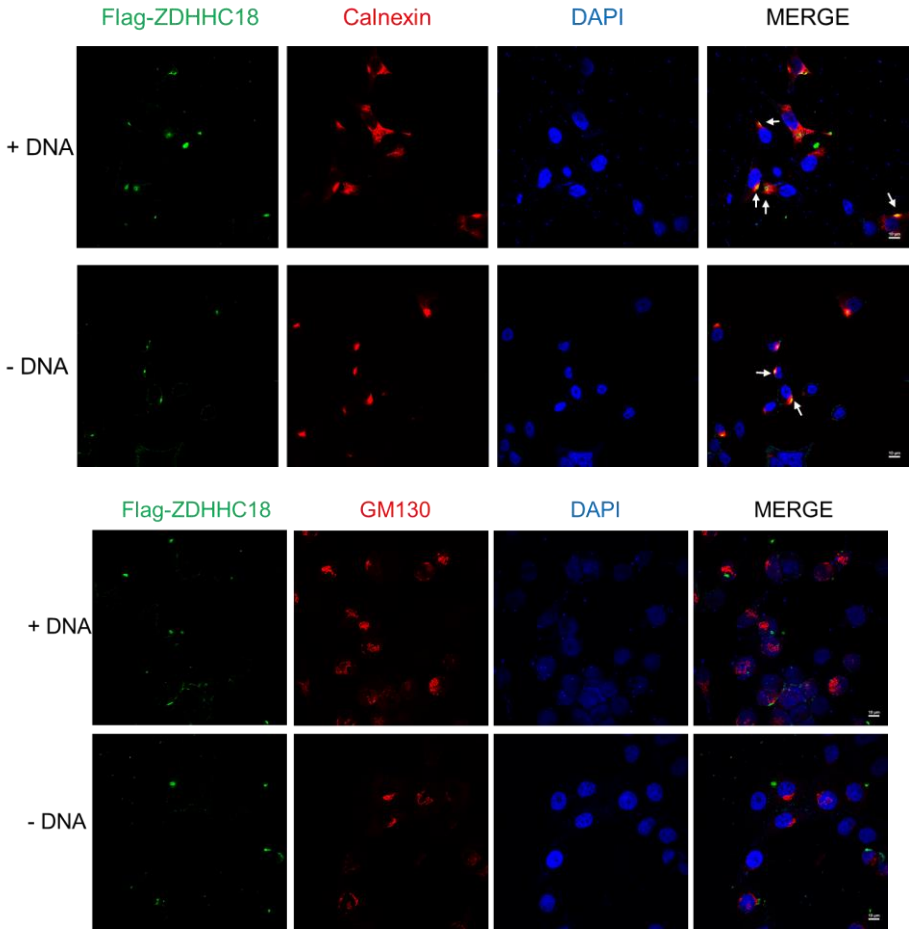
Response fig. 3 cell staining of transiently overexpressed Flag-ZDHHC18 (1 μg) in HeLa cells. Scale bars: 10 μm.



Response fig. 4 (*Figure S2E* in manuscript) cell staining of transiently overexpressed ZDHHC18 (1 μg) in HeLa cells. Scale bars: 7 μm.

Moreover, we established a HeLa cell line stably expressing Flag-tagged ZDHHC18 as the reviewer requested. We found that stably overexpressed Flag-ZDHHC18 showed significantly punctate pattern and colocalized with ER

(indicated by Calnexin) with or without DNA (Response fig. 5). In this Flag-ZDHHC18-stably-overexpressing HeLa cells, we did not detect the colocalization of ZDHHC18 with Golgi apparatus (indicated by GM130) with or without DNA stimulation (Response fig. 5).



Response fig. 5 (*Figure S2G* in manuscript) cell staining of stably overexpressed Flag-ZDHHC18 in HeLa cells with HT-DNA-stimulation (or not). Scale bars: 10 μm.

Therefore, the subcellular localization of ZDHHC18 was diverse. Endogenous ZDHHC18 showed a dispersed pattern. Transiently overexpressed ZDHHC18 showed both punctate and dispersed pattern and colocalized with Golgi. Stably overexpressed ZDHHC18 showed punctate pattern which colocalized with ER but not Golgi. We modified the statements accordingly in our manuscript.

3rd Revision - Editorial Decision **15th Mar 2022**

Dear Hubert,

Thank you for submitting the revised version. I have now had a chance to take a look at the revised version and I appreciate the introduced revisions. I am therefore very pleased to accept the manuscript for publication here. Before sending you the formal acceptance letter there are just a few formatting issues to resolve.

- Please relabel COI to Disclosure Statement & Competing Interests

- For the data availability section if your study does not include datasets, please insert the following statement: This study includes no data deposited in external repositories.

- The reference format also needs to be corrected

- Please upload individual high resolution figure files

- Callouts to Fig 6I callout and Appendix Table S3 is missing.

- The nomenclature for the appendix figures need to be corrected to "Appendix Figure S#" "Appendix Table S#".

- We include a synopsis of the paper (see http://emboj.embopress.org/). Please provide me with a general summary statement and 3-5 bullet points that capture the key findings of the paper.

- We also need a summary figure for the synopsis. The size should be 550 wide by [200-400] high (pixels). You can also use something from the figures if that is easier.

- Our publisher has also done their pre-publication check on your manuscript. When you log into the manuscript submission system you will see the file "Data Edited Manuscript file". Please look at the word file and the comments regarding the figure legends and respond to the issues.

- We generally discourage displaying statistic when N=2 (Figure 1).

That should be all - let me know if there is anything further to discuss

Best

Karin

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

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We realize that it is difficult to revise to a specific deadline. In the interest of protecting the conceptual advance provided by the work, we recommend a revision within 3 months (13th Jun 2022). Please discuss the revision progress ahead of this time with the editor if you require more time to complete the revisions. Use the link below to submit your revision:

https://emboj.msubmit.net/cgi-bin/main.plex

4th Revision - Editorial Decision **24th Mar 2022**

Dear Hubert,

Thank you for submitting your revised manuscript to The EMBO Journal. I have now had the opportunity to take a careful look at everything and all looks good!

I am therefore very pleased to accept the manuscript for publication here.

Congratulations on a nice study

With best wishes

Karin

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

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EMBO Press Author Checklist

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USEFUL LINKS FOR COMPLETING THIS FORM

The EMBO Journal - Author Guidelines
EMBO Reports - Author Guidelines
Molecular Systems Biology - Author Guidelines
EMBO Molecular Medicine - Author Guidelines

Reporting Checklist for Life Science Articles (updated January 2022)

This checklist is adapted from Materials Design Analysis Reporting (MDAR) Checklist for Authors. MDAR establishes a minimum set of requirements in transparent reporting in the life sciences (see Statement of Task: 10.31222/osf.io/9sm4x). Please follow the journal's guidelines in preparing your manuscript.

Please note that a copy of this checklist will be published alongside your article.

Abridged guidelines for figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- ideally, figure panels should include only measurements that are directly comparable to each other and obtained with the same assay.
- plots include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n<5, the individual data points from each experiment should be plotted. Any statistical test employed should be justified.
- Source Data should be included to report the data underlying figures according to the guidelines set out in the authorship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements.
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.
- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- 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.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Please complete ALL of the questions below.
Select "Not Applicable" only when the requested information is not relevant for your study.

Materials

Newly Created Materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
New materials and reagents need to be available; do any restrictions apply?	Not Applicable	

Antibodies	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
For **antibodies** provide the following information: - Commercial antibodies: RRID (if possible) or supplier name, catalogue number and or/clone number - Non-commercial: RRID or citation	Yes	Reagents and Tools Table

DNA and RNA sequences	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Short novel DNA or RNA including primers, probes: provide the sequences.	Yes	Reagents and Tools Table

Cell materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Cell lines: Provide species information, strain. Provide accession number in repository **OR** supplier name, catalog number, clone number, and/**OR** RRID.	Yes	Materials and methods
Primary cultures: Provide species, strain, sex of origin, genetic modification status.	Not Applicable	
Report if the cell lines were recently **authenticated** (e.g., by STR profiling) and tested for mycoplasma contamination.	Yes	Materials and methods

Experimental animals	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Laboratory animals or Model organisms: Provide species, strain, sex, age, genetic modification status. Provide accession number in repository **OR** supplier name, catalog number, clone number, **OR** RRID.	Yes	Materials and methods
Animal observed in or captured from the field: Provide species, sex, and age where possible.	Not Applicable	
Please detail **housing and husbandry conditions**.	Yes	Materials and methods

Plants and microbes	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Plants: provide species and strain, ecotype and cultivar where relevant, unique accession number if available, and source (including location for collected wild specimens).	Not Applicable	
Microbes: provide species and strain, unique accession number if available, and source.	Yes	Materials and methods

Human research participants	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If collected and within the bounds of privacy constraints report on age, sex and gender or ethnicity for all study participants.	Not Applicable	

Core facilities	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If your work benefited from core facilities, was their service mentioned in the acknowledgments section?	Yes	Materials and methods

Design

Study protocol	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If study protocol has been **pre-registered, provide DOI in the manuscript**. For clinical trials, provide the trial registration number **OR** cite DOI.	Not Applicable	
Report the **clinical trial registration number** (at ClinicalTrials.gov or equivalent), where applicable.	Not Applicable	

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.	Not Applicable	
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?	Not Applicable	

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.

Adherence to community standards	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
State if relevant guidelines or checklists (e.g., **ICMJE, MIBBI, ARRIVE, PRISMA**) have been followed or provided.	Not Applicable	
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	