EMBO reports

UNC13D inhibits STING signaling by attenuating its oligomerization on the endoplasmic reticulum

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

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

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

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Data availability

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

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

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Moreover, I have these editorial requests:

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

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Yours sincerely,

Achim Breiling
Senior Editor
EMBO Reports

Referee #1:

Song et al describe a novel role for the protein UNC13D in negatively regulating STING-specific signaling. The authors have provided sufficient evidence that UNC13D is a direct interactor with STING and shown that there are functional consequences of this interaction in attenuating STING signaling. Furthermore, they have laid the groundwork in showing physiological relevance and alluding to the possibility that hyperinflammation in FHL3 and HLH may be STING-dependent. The conceptual advance this work made is substantial. However, while the conclusions of the role of UNC13D are broadly sound, in many portions of the work, they are weakened by technical aspects of the experiment, including the inconsistent inclusions of control samples and measurements, as well as experiments that do not robustly exclude alternative interpretations. Thus, there is a need for more rigorous treatment of the data before publication.

First, can the authors show data from the yeast two-hybrid screen? How did the authors select UNC13D?

Figure 1: Although the blots broadly support the descriptions provided by the authors, the quality of the blots presented somewhat undermine the observations. For example, in 1C, pTBK1 and pIRF3 signals are visibly stronger in knockdown lanes compared to wild-type lanes at 24h. However, it is not apparent that overexpression of UNC13D dampens pIRF3 or pTBK1 appreciably (1A, 1B). The effects are also more apparent at 12 h than at 24 h. Also, the level of signal seems to vary greatly for what appears to be the same cell type e.g. UNC13D levels are similar for control cells in 1F and 1H, but residual signal for the KO cells are stronger in 1F.

If they wish to stake claims on effects of UNC13D on TBK1 and IRF3 phosphorylation, especially to make the point that UNC13D overexpression dampens STING signaling (1A, 1B) the authors should attempt to provide an average quantitation of the signals over their multiple repeats to determine the size of all effects on pTBK1/pIRF3.

Figure 2: Some claims appear overrepresented here. In 2D, phosphorylation of STING appears to be similar in wild-type compared to knockdown cells. In 2I, there is less total TBK1 present in WCL, slightly less in IP: HA-TBK1; could this explain why there is less STING pulled down as well?
In Figure 2K, it would be helpful to see a quantification of STING-ERGIC53 colocalization in all the different conditions. If the claim is that UNC13D KD promotes STING oligomerization and trafficking to ERGIC/Golgi, then this should be reflected in the cumulative microscopy data. From what is shown, the cGAMP-stimulated control cells and cGAMP-stimulated UNC13D KD cells look similar in terms of STING-ERGIC53 colocalization. An ER marker here could also be helpful to show that UNC13D overexpression promotes ER retention. STING also looks rather punctate in the UNC13D overexpression condition - any comments as to why?

Some other minor issues: What is the difference between lanes 2 vs 3 and 5 vs 6 in 2B? The H-151 treatment bar in 2G, con is colored incorrectly.

Figure 3E: This result is puzzling. The authors saw that cGAMP reduces affinity of UNC13D for STING, while BFA co-treatment restores this affinity, thus they concluded that retaining STING on the ER improves binding of UNC13D to STING. However, the co-IP is performed on cell lysates, where STING localization is presumably no longer relevant. Furthermore, the reported conditions for cell lysis and WCL blot for STING do not appear to support that the STING trafficked to the Golgi was excluded from the immunoprecipitation. The authors need to clarify how this experiment was performed in order to exclude their observations being possibly an artefact or a result of off-target effects of BFA. Otherwise, the confocal images presented in the same figure are sufficient evidence that STING and UNC13D interact on the ER.

Figure 3I: The total STING signal in the native PAGE seems to be overall higher in the knockdown lanes. It is thus difficult to conclude the effect of UNC13D on constitutive oligomerization of STING in the absence of stimuli. The authors should provide a denatured blot of STING similar to Figure 3J.

Figure 3K: The authors should provide further support that the Venus reporter designed here is a good readout of STING polymerization in live cells. STING is a constitutive dimer, and presumably co-expression of STING-N-Venus and STING-Venus-C will generate statistical proportions of mixed and homodimers. It is entirely possible that Venus dimerization in cis in mixed STING dimers causes the signal observed, especially since they see very robust fluorescence signal in the absence of any stimuli. Furthermore, consistent with the other data, UNC13D binding to STING dimer could merely prevent the proper reconstitution of the Venus protein. Since the authors are claiming that Venus fluorescence signals are representative of the degree of polymerization, they should perform similar native PAGE experiments on STING-Venus fusions to correlate to the number of green cells. Furthermore, they should provide evidence that cGAMP or pDNA stimuli cause increase in Venus signal, correlated with their native PAGE results, and that UNC13D knockdown enhances this signal (or UNC13D overexpression attenuates it). The authors should also clarify the language used to describe the constructs or provide a more precise description/figure: since they are clearly of different molecular weights it appears that STING is fused to two components of a split fluorescent protein, instead of simply being N and C-termini fusions to a single Venus entity.

Figure 4: The blots in Figure 4 are fine but hard to distinguish whether the signal is coming from FLAG-tag or HA-tag. Especially 4G where UNC13D-NN/UNC13D-MHD/FL-STING all run about the same molecular weight. Can these be probed on separate blots? If initial finding from 4B is that UNC13D mostly interacts with STING's TMD and CTT, why not try these IPs with STING-deltaCTT? Also, why is there more binding to STING with UNC13D-C2A compared to STING with FL-UNC13D? And why does

FL-STING have so many bands underneath - are these degradation products?

The proposed interaction mode is interesting - it would also be informative to perform the co-IP with STING delta 72-80 with only the F-NN domain to confirm the role of TM2-TM3 in NN domain interaction. Similarly, since the C-terminal region aa341-379 is unstructured, it would be instructive to express this 39-aa fragment (or use a synthetic HA-aa341-379 peptide) and perform co-IP directly with MHD and NN domains.

Figure 5. Why in Figure 5E does H-151 treatment show bump in IFNb mRNA?

Page 12: "UNC13D dysfunction may enhance STING oligomerization and facilitate subsequent downstream signaling events.." To support their model, can the authors also demonstrate definitively that the disease-relevant frameshift mutant A59T P147 FsX14 fails to inhibit STING oligomerization in response to pDNA/cGAMP stimuli?

Referee #2:

Song et al. reported the identification of UNC13D (Munc13-4), which is associated with familial hemophagocytic lymphohistiocytosis (FHL), as the novel homeostatic regulator of STING trafficking and signaling via direct interaction on ER. Deficiency of UNC13D enhanced ligand-induced STING activation through promoting STING oligomerization. Inhibition of STING by a small molecule inhibitor H-151 attenuated the expression of pro-inflammatory cytokines due to loss of UNC13D.

The reported work is of significance and general interest to the molecular biology field as it would advance the understanding of STING biology and the genetic disorder of FHL. The comprehensive biochemical analysis of STING signaling in UNC13D-deficient cells and mapping of the STING-UNC13D interaction interface are the major strengths of this report.

However, the overall quality and interpretation of the data/model, missing important controls, and the lack of convincing physiological importance need to be significantly improved prior to the publication in the current journal. Please find the specific comments below.

The overall model suggests that UNC13D acts as the cap for STING via direct interaction on ER and prevents the oligomerization of STING. The authors stably expressed FLAG-tagged UNC13D and showed that UNC13D was present on ER but not Golgi. The localization of ectopically expressed UNC13D is more than just ER as suggested by the authors as you can see by the overwhelmingly saturated green signal in Fig 3B and 3D, which might be artifacts from overexpression. Also, multiple prior publications showed that UNC13D (Munc13-4) is localized to the endosome or lysosome (PMID: 32582217, 15548590, and 21693760), which is contradictory to what the authors showed. It will be helpful if the authors could examine the localization of endogenous UNC13D in the cells used and modify the model to explain how endogenous lysosomal UNC13D would regulate STING oligomerization on ER.

The biochemical analysis suggesting UNC13D serves as a negative regulator of STING is comprehensive. However, the overall quality of the western blots/native pages could be improved for a clear and better interpretation of the data. The differences in key western blots between WT and UNC13D-KO/KD/overexpressed cells are fairly subtle including but not limited to p-IRF3 blots (Fig 1A-D, 1G), p-STING (Fig 1A, 2D, 3J, EV3A), p-TBK1 (Fig 1G) and native page of STING oligomerization (Fig 3I, 3J) which would undermine the interpretation of the data and conclusions made.

The authors could consider using non-reducing SDS-PAGE in addition to the native page to show the oligomerization of STING. P-STING and/or P-TBK1 blots are missing in many blots (Fig 1D-1H). It would be really important to include the full set of signaling pathway indicators (p-STING, p-TBK1, and p-IRF3) in these panels as the authors were trying to pinpoint where UNC13D plays a role in STING trafficking and signaling. It seems that the protein level of endogenous UNC13D in HeLa cells is decreasing after HSV-1 infection but not in other conditions (Fig 1E). Do the authors have any insights on whether UNC13D gets degraded post-infection or STING activation?

The authors showed reduced fluorescence of GFP-HSV in UNC13D KO cells, which seems contradictory to the comparable GFP level by western blots on the right side (Fig 1S). The reduced interaction of HA-TBK1 and FLAG-STING along with overexpression of FLAG-UNC13D in Fig 2J could be due to the reduced expression of TBK1 and STING in the whole cell lysate rather than the reduction of protein-protein interaction. The authors showed that overexpression of UNC13D inhibits the dimerization of STING using the split GFP assay in Fig 3K. However, the cells with reduced GFP signaling did not express UNC13D in red as well, which did not fully support the conclusion.

The conclusions will be more convincing as several critical controls were missing in the current report. IgG isotype controls should be included in the fluorescent images to ensure the accurate staining of the antibodies. Western blots showing the expression of indicated proteins should be included in the luciferase assays panels (Fig 3F, 4C) and rescue experiments (Fig 5B, 5D). The authors performed extensive IP experiments to identify the critical domains of STING-UNC13D interaction.

However, insufficient labeling and mixing of antibodies in the whole-cell lysates blots (like adding FLAG antibody and HA antibody together) makes it difficult to interpret the data and draw a conclusion. All western blots should include the size labels. It is not a fair comparison between WT and UNC31D KO-FHL3-mut in Fig 5D-5G. The authors should include WT, UNC13D KO, UNC13D KO-WT UNC13D, and UNC13D KO-FHL3-mut in the same panel.

Figure legends depicting different domains are missing in Fig 4F. The interaction of UNC13D-C2A and STING 139-340 was fairly weak (Fig 4I) to draw a "+" in Fig 4H. All bar graphs should be presented as a bar graph with a dot plot to show the distribution of individual data points. The proper statistical analysis should be performed using one-way ANOVA if there are more than 2 groups instead of the student t-test in Fig 1M-R, 2F-G, 3F, 5E, 5G, EV1, EV2, and EV4.

The authors showed that inhibition of STING using small molecule H-151 reduced elevated IFN, IL-6, and CXCL10 mRNA expression in UNC13D KO cells and UNC13DKO-FHL3 mut cells as the physiological relevance. However, the effect is fairly small. Could the authors try using genetic means like siRNA knockdown or CRISPR KO to test whether transient or permeant removal of STING and or cGAS could rescue the elevated expression of cytokines in UNC13D KO and UNC13D KO-FHL3 mutant cells?

The authors proposed that UNC13D regulates STING in the unstimulated state. Do authors have any insights into whether cGAS plays a role in elevated IFN and ISGs in UNC13D-deficient cells? Does the basal STING activation require an endogenous ligand trigger like mtDNA or nuclear DNA damage due to the loss of UNC13D? It would be more convincing if the authors could use primary FHL patient-derived cells (PBMC or fibroblast) and/or FHL mouse models (cells or mice) to improve the physiological relevance. It will be important to test whether STING plays a role in these patient-derived cells or FHL mouse model-derived cells if they are used.

Referee #3:

Song et al. identify and characterize a novel interaction between UNC13D and STING, using a variety of cell biological, biochemical and molecular assays. They show that UNC13D and STING co-localize on the ER, and that the presence of UNC13D inhibits oligomerization of STING. The authors use cellular knockdown and knockout of UNC13D to demonstrate that its absence promotes proinflammatory signals, and that this effect can be diminished by an inhibitor of STING signaling. Song et al. furthermore map the domains involved in the UNC13D/STING interaction on both proteins and propose a model for the potential mechanism by which UNC13D may regulate the STING-mediated immune response.

Overall, this is a very interesting study with an abundance of data pointing in the same direction, the main novelty being a previously unknown cellular function of UNC13D. My main criticism is, that no quantification is provided for the immunoblot data and the cellular co-localization studies. The authors state that experiments were performed at least three times and data from individual experiments are shown. However, it is difficult to judge the magnitude of the reported effects, for instance the phosphorylation of TBK1, IRF3, p65 and STING, or the oligomerization of STING from a single blot or image shown for the respective experiments. I think the authors should provide quantification of their experimental replicates (normalized to their loading control) for the relevant immunoblot band intensities, as well as for the cellular co-localization analyses, maybe as part of the expanded view figures.

Major point:
1. I think quantification of experimental replicates (of the relevant bands, i.e. TBK1, IRF3, p65, STING) should be provided for the following figures:
Fig. 1 A-L,S
Fig. 2 B,C,D,E,H,I,J
Fig. 2 K,L (quantification of co-localization/aggregation)
Fig. 3 A,E,H,I,J
Fig. 3 B,C,D,G (quantification of co-localization)
Fig. EV5

Minor points:
2.
The authors state that "statistically significant differences between groups were determined using a two-tailed Student's t-test". I assume the authors compared KD#1 and KD#2, and in some cases #KD3 individually to the control, since a Student's t-test would not be an appropriate test for three or more groups. If this was the case, this should be stated clearly.

3.
Fig. 4 E, D Can the authors comment on why the interaction of STING delta-18-138 with UNC13D is stronger than the interaction of STING delta-18-68?

4.
The manuscript is well written, but I think the abstract could be improved.

Point by point responses:

Referee #1:

First, can the authors show data from the yeast two-hybrid screen? How did the authors select UNC13D?

Reply：

We have added the description of the yeast two-hybrid results (page 4). We screened a mixture of human fetal kidney and leukocyte cDNA libraries and obtained 66 independent positive clones. One of these clones encoded FHL3-associated protein UNC13D, dysfunction of which leads to hyperinflammatory responses. Its interaction with STING has not been reported, so we selected UNC13D as the subject of experiments to detect its role in innate immune signaling.

Figure 1: Although the blots broadly support the descriptions provided by the authors, the quality of the blots presented somewhat undermine the observations. For example, in 1C, pTBK1 and pIRF3 signals are visibly stronger in knockdown lanes compared to wild-type lanes at 24h. However, it is not apparent that overexpression of UNC13D dampens pIRF3 or pTBK1 appreciably ***(1A, 1B).*** *The effects are also more apparent at 12 h than at 24 h. Also, the level of signal seems to vary greatly for what appears to be the same cell type e.g. UNC13D levels are similar for control cells in* ***1F and 1H****, but residual signal for the KO cells are stronger in 1F. If they wish to stake claims on effects of UNC13D on TBK1 and IRF3 phosphorylation, especially to make the point that UNC13D overexpression dampens STING signaling (1A, 1B) the authors should attempt to provide an average quantitation of the signals over their multiple repeats to determine the size of all effects on pTBK1/pIRF3.*

Reply:

Following the reviewer's suggestion, we have provided quantitation of signals over multiple repeats in all western blot results, including Figure 1A and 1B (Fig EV1A and EV1B). The quantitation results showed that overexpression of UNC13D inhibits pTBK1, pIRF3 and pSTING induced by VACV and pDNA. We have also repeated experiments and replaced Fig 1F and 1H.

Figure 2: Some claims appear overrepresented here. In 2D, phosphorylation of STING appears to be similar in wild-type compared to knockdown cells. In 2I, there is less total TBK1 present in WCL, slightly less in IP: HA-TBK1; could this explain why there is less STING pulled down as well?

Reply:

We have provided quantitation of western blot signals over multiple repeats in Fig 2D (Fig EV2J). The results showed that phosphorylation of STING increased in knockdown cells compared to control cells upon cGAMP stimulation. We repeated the experiment and replaced Fig 2I, in which the amounts of TBK1 in WCL and IP were nearly equivalent.

In Figure 2K, it would be helpful to see a quantification of STING-ERGIC53 colocalization in all the different conditions. If the claim is that UNC13D KD promotes STING oligomerization and trafficking to ERGIC/Golgi, then this should be reflected in the cumulative microscopy data. From

what is shown, the cGAMP-stimulated control cells and cGAMP-stimulated UNC13D KD cells look similar in terms of STING-ERGIC53 colocalization. An ER marker here could also be helpful to show that UNC13D overexpression promotes ER retention. STING also looks rather punctate in the UNC13D overexpression condition - any comments as to why?

Reply:

Following the reviewer's suggestion, we have provided the quantification of STING-ERGIC53 colocalization (Fig 2J), and performed co-staining of ER marker calnexin and STING under different conditions (Fig EV2O and EV2P). The results showed that in comparison with control cells, there was more STING accumulation on the ERGIC and less STING distribution on the ER following cGAMP stimulation in UNC13D KO cells, while UNC13D overexpression promoted ER retention of STING upon cGAMP stimulation. Overexpression of UNC13D only partially inhibited STING aggregation and activation, as shown in Figure A and B; therefore, some STING puncta were present in the results obtained under the UNC13D overexpression condition.

Some other minor issues: What is the difference between lanes 2 vs 3 and 5 vs 6 in 2B? The H-151 treatment bar in 2G, con is colored incorrectly.

Reply:

In Fig 2B, lane 2/3 and Lane 5/6 are two different stable cells. We have relabeled Fig 2B. The results of Fig 2G have been merged to Fig 5F, and Fig 2G have been deleted.

Figure 3E: This result is puzzling. The authors saw that cGAMP reduces affinity of UNC13D for STING, while BFA co-treatment restores this affinity, thus they concluded that retaining STING on the ER improves binding of UNC13D to STING. However, the co-IP is performed on cell lysates, where STING localization is presumably no longer relevant. Furthermore, the reported conditions for cell lysis and WCL blot for STING do not appear to support that the STING trafficked to the Golgi was excluded from the immunoprecipitation. The authors need to clarify how this experiment was performed in order to exclude their observations being possibly an artefact or a result of off-target effects of BFA. Otherwise, the confocal images presented in the same figure are sufficient evidence that STING and UNC13D interact on the ER.

Reply:

We agree with the reviewer that the co-IP lysates did not exclude STING trafficked to the Golgi, but we clearly observed that the interaction between STING and UNC13D was weakened upon stimulation, and BFA treatment restored the interaction. The reason for this phenomenon may be the different modification states of STING on the ER or Golgi, such as ubiquitination, palmitoylation et al.; if these modifications were retained in the lysate buffer, they could lead to a change in the affinity of UNC13D for STING, which was shown in the IP results.

Figure 3I: The total STING signal in the native PAGE seems to be overall higher in the knockdown lanes. It is thus difficult to conclude the effect of UNC13D on constitutive oligomerization of STING in the absence of stimuli. The authors should provide a denatured blot of STING similar to Figure 3J.

Reply:

We have replaced Fig 3I and provided the denatured blot of STING in SDS-PAGE in Fig 3I and Fig 3J. We agree with reviewer that the effect of UNC13D on oligomerization of STING in the

absence of stimulation is not sufficiently obvious in the native PAGE experiment (Fig 3I and 3J, Fig EV3J and 3K), and we have deleted the relevant description in the text (page 8 and 15).

Figure 3K: The authors should provide further support that the Venus reporter designed here is a good readout of STING polymerization in live cells. STING is a constitutive dimer, and presumably co-expression of STING-N-Venus and STING-Venus-C will generate statistical proportions of mixed and homodimers. It is entirely possible that Venus dimerization in cis in mixed STING dimers causes the signal observed, especially since they see very robust fluorescence signal in the absence of any stimuli. Furthermore, consistent with the other data, UNC13D binding to STING dimer could merely prevent the proper reconstitution of the Venus protein. Since the authors are claiming that Venus fluorescence signals are representative of the degree of polymerization, they should perform similar native PAGE experiments on STING-Venus fusions to correlate to the number of green cells. Furthermore, they should provide evidence that cGAMP or pDNA stimuli cause increase in Venus signal, correlated with their native PAGE results, and that UNC13D knockdown enhances this signal (or UNC13D overexpression attenuates it). The authors should also clarify the language used to describe the constructs or provide a more precise description/figure: since they are clearly of different molecular weights it appears that STING is fused to two components of a split fluorescent protein, instead of simply being N and C-termini fusions to a single Venus entity.

Reply:

In the Venus reporter experiment, we separately fused the two components of the fluorescent protein Venus (VN or VC) to the N terminal of STING to construct VN-STING and VC-STING. We have corrected the description of the constructs in the text (page 9) and deleted the cartoon to avoid misunderstanding (Fig 3K). We agree with the reviewer that co-expression of VN-STING and VC-STING would generate a heterodimer or homodimer. We believe that the fluorescence signal was mainly caused by polymerization of these heterodimers or homodimers. Following the reviewer's suggestion, we performed native PAGE on STING-Venus fusion proteins (Fig EV3M). We found prominent polymers when VN-STING and VC-STING were co-expressed, and the abundance of polymers was reduced by UNC13D overexpression. These results were found to be correlated with the fluorescence results (Fig 3K). We also found an enhanced Venus fluorescence signal and VN/VC-STING polymerization in native-PAGE upon stimulation with cGAMP, and UNC13D overexpression attenuated this polymerization (Fig 3K and L, Fig EV3M). We have added relevant information in the results section of the main text (page 9).

Figure 4: The blots in Figure 4 are fine but hard to distinguish whether the signal is coming from FLAG-tag or HA-tag. Especially 4G where UNC13D-NN/UNC13D-MHD/FL-STING all run about the same molecular weight. Can these be probed on separate blots? If initial finding from 4B is that UNC13D mostly interacts with STING's TMD and CTT, why not try these IPs with STING-deltaCTT? Also, why is there more binding to STING with UNC13D-C2A compared to STING with FL-UNC13D? And why does FL-STING have so many bands underneath - are these degradation products?

Reply:

Fig 4G was renumbered to Fig 4F. We have repeated and replaced Fig 4F (summarized in Fig 4G) with the results of experiments in which FLAG-tag or HA-tag was probed on separate blots to

distinguish UNC13D-NN/UNC13D-MHD/FL-STING. Our goal was to map the UNC13D region(s) that interacted with full-length STING, and the binding of STING1-340 and UNC13D was weak (Fig 4A and 4B), so we performed IPs with STING-FL, but not with STING-delta CTT. With regard to the greater level of binding between STING and UNC13D-C2A in comparison with the binding between STING and UNC13D-FL, we reasoned that UNC13D-C2A may have formed a structure that was able to bind more easily to STING. The lowest bands in the IP results are the degradation products of STING.

The proposed interaction mode is interesting - it would also be informative to perform the co-IP with STING delta 72-80 with only the F-NN domain to confirm the role of TM2-TM3 in NN domain interaction. Similarly, since the C-terminal region aa341-379 is unstructured, it would be instructive to express this 39-aa fragment (or use a synthetic HA-aa341-379 peptide) and perform co-IP directly with MHD and NN domains.

Reply:

Following the reviewer's suggestion, we have performed co-IP between the HA-NN domain of UNC13D and the TM domain deletion mutants of STING. Similar to full-length UNC13D, the interaction between the NN domain and the STING mutant lacking the first short helix of the TM2/TM3 linker (aa 72-80) was weaker than that of the mutant lacking the second short helix (aa 82-90) (Fig EV4B). These results also suggest that the NN domain of UNC13D associates with the STING TM2-TM3 linker region that is exposed to the cytoplasm. We have added relevant information in the results section in the main text (page 10 and 11).

To address the direct interaction between the STING CTT and the MHD/NN domains of UNC13D, we constructed a plasmid containing the STING CTT (340-379) and performed co-IP. The WB results showed that the binding of the STING CTT with the FL and NN of UNC13D was obvious, whereas binding with the MHD of UNC13D was weak, and there was no detected binding with the C2A or C2B of UNC13D. These results confirmed the interaction between the STING CTT and the NN/MHD domains of UNC13D (Fig EV4C and D). Based on these results, we modified the model as follows: UNC13D associates with STING mainly via interactions between the C2A domain of UNC13D and the STING LBD, as well as between the NN domain of UNC13D and the STING TMD/CTT (Fig 4K).

Figure 5. Why in Figure 5E does H-151 treatment show bump in IFNb mRNA?

Reply:

We have repeated these experiments, and similar phenomena were observed. In WT cells, H-151 treatment enhanced mRNA basal levels of IFN-β, but not IL6 or CXCL10, in the absence of stimulation (Fig 5E). We thought it possible that H-151 has a previously unknown weak effect on IFN-β expression. H-151 effectively blocked elevated expression of cytokines, including IFN-β, in cells with UNC13D dysfunction under stimulated conditions (Fig 5F).

Page 12: "UNC13D dysfunction may enhance STING oligomerization and facilitate subsequent downstream signaling events." To support their model, can the authors also demonstrate definitively that the disease-relevant frameshift mutant A59T P147 FsX14 fails to inhibit STING oligomerization in response to pDNA/cGAMP stimuli?

Reply:

Following the reviewer's suggestion, we performed native PAGE with the disease-relevant mutant A59T P147 FsX14. In comparison with control cells, cells with mutant UNC13D or lacking UNC13D showed enhanced STING oligomerization upon stimulation with cGAMP (Fig 5D). These results demonstrated that the disease-relevant mutant of UNC13D fails to inhibit STING oligomerization in response to stimuli. We have added the results and related information in Fig 5 and the main text (page 12).

Referee #2:

The overall model suggests that UNC13D acts as the cap for STING via direct interaction on ER and prevents the oligomerization of STING. The authors stably expressed FLAG-tagged UNC13D and showed that UNC13D was present on ER but not Golgi. The localization of ectopically expressed UNC13D is more than just ER as suggested by the authors as you can see by the overwhelmingly saturated green signal in Fig 3B and 3D, which might be artifacts from overexpression. Also, multiple prior publications showed that UNC13D (Munc13-4) is localized to the endosome or lysosome (PMID: 32582217, 15548590, and 21693760), which is contradictory to what the authors showed. It will be helpful if the authors could examine the localization of endogenous UNC13D in the cells used and modify the model to explain how endogenous lysosomal UNC13D would regulate STING oligomerization on ER.

Reply:

Since antibodies against UNC13D could not effectively recognize endogenous UNC13D in immunofluorescence experiments, we constructed HeLa cells that stably expressed FLAG-UNC13D. Compared with the ER marker, no obvious co-localization of FLAG-UNC13D with the Golgi marker was found in the immunofluorescence experiments conducted before or after stimulation (Fig 3B and 3D, Fig EV3B and EV3D). Therefore, we believe that the co-localization of FLAG-UNC13D with the ER was not artifact of overexpression, and we suggest that UNC13D interacts with STING on the ER. Previous studies of the localization and function of UNC13D have mainly focused on hematopoietic cells, such as cytotoxic CD8+ T cells or mast cell line RBL-2H3 (PMID 32582217, 15548590, and 21693760). In these cells, exogenous UNC13D is present on secretory lysosomes, which differ from classic lysosomes. Thus far, the localization and function of UNC13D in many nonhematopoietic cells remain unknown. We do not rule out the possibility that UNC13D may also be present on lysosomes in the HeLa cells we used in our experiments, but we have not yet found any lysosome-related functions of UNC13D, such as regulating the protein level of STING.

The biochemical analysis suggesting UNC13D serves as a negative regulator of STING is comprehensive. However, the overall quality of the western blots/native pages could be improved for a clear and better interpretation of the data. The differences in key western blots between WT and UNC13D-KO/KD/overexpressed cells are fairly subtle including but not limited to p-IRF3 blots (Fig 1A-D, 1G), p-STING (Fig 1A, 2D, 3J, EV3A), p-TBK1 (Fig 1G) and native page of STING oligomerization (Fig 3I, 3J) which would undermine the interpretation of the data and conclusions made.

Reply:

Following the reviewer's suggestion, we have replaced Fig 1C-1H, and provided quantitation of western blot signals over multiple repeats for the p-IRF3, p-TBK1, p-STING blots in Figure 1, Figure 2 and Figure3 (Fig EV1A-H, Fig EV2H-K, Fig EV3H-I), and native page of STING oligomerization in Figure 3I, 3J.

The authors could consider using non-reducing SDS-PAGE in addition to the native page to show the oligomerization of STING.

Reply:

Non-reducing SDS-PAGE can depolymerize the polymer and preserve the dimer of STING (PMID 31230712, 35613581). Following the reviewer's suggestion, we have performed non-reducing SDS-PAGE to detect STING oligomerization. In comparison with their control cells, UNC13D-knockout cells showed significantly more STING dimers, indicating that more polymers were formed upon cGAMP stimulation (Fig EV3L). This result is in line with the native PAGE results. Relevant information has been added to the results section (page 8).

P-STING and/or P-TBK1 blots are missing in many blots (Fig 1D-1H). It would be really important to include the full set of signaling pathway indicators (p-STING, p-TBK1, and p-IRF3) in these panels as the authors were trying to pinpoint where UNC13D plays a role in STING trafficking and signaling.

Reply:

Following the reviewer's suggestion, we repeated experiments and replaced Fig 1C-1H (including the p-STING and p-TBK1 blots). We also provided quantitation of western blot signals over multiple repeats in Fig 1 (Fig EV1A-L).

It seems that the protein level of endogenous UNC13D in HeLa cells is decreasing after HSV-1 infection but not in other conditions (Fig 1E). Do the authors have any insights on whether UNC13D gets degraded post-infection or STING activation?

Reply:

We detected the protein level of UNC13D upon pDNA, VACV and cGAMP stimulation, and we did not find significant changes (Fig 1C, D, G, H, I, and J, Fig 2D and E). We believe that infection or STING activation does not generally lead to the degradation of UNC13D. However, HSV infection did induce an obvious decrease in the protein level of endogenous UNC13D, and we think that this change may have been caused by stagnation of cell proliferation induced by HSV; as shown in Fig 1E and 1F, the abundance of tubulin also decreased significantly.

The authors showed reduced fluorescence of GFP-HSV in UNC13D KO cells, which seems contradictory to the comparable GFP level by western blots on the right side (Fig 1S).

Reply:

We have provided quantitation of the western blot signals in Fig 1S (relabeled as Fig EV1P). The result showed reduced abundance of GFP-HSV in UNC13D KO cells.

The reduced interaction of HA-TBK1 and FLAG-STING along with overexpression of FLAG-UNC13D in Fig 2J could be due to the reduced expression of TBK1 and STING in the whole cell lysate rather than the reduction of protein-protein interaction.

Reply:
We have replaced Fig 2I (we think that the reviewer may have meant 2I instead of 2J), and Fig 2I was renumbered to Fig 2G. The expression levels of TBK1 and STING in the whole cell lysate were nearly equivalent (Fig 2G).

The authors showed that overexpression of UNC13D inhibits the dimerization of STING using the split GFP assay in Fig 3K. However, the cells with reduced GFP signaling did not express UNC13D in red as well, which did not fully support the conclusion.

Reply:
We have replaced Fig 3K. The red fluorescence signal of UNC13D has been clearly identified.

The conclusions will be more convincing as several critical controls were missing in the current report. IgG isotype controls should be included in the fluorescent images to ensure the accurate staining of the antibodies.

Reply:
We have included the IgG control in the immunofluorescence results to ensure accurate antibody staining (Fig 2I, 2K, Fig EV2O, Fig 3B, C, D, G).

Western blots showing the expression of indicated proteins should be included in the luciferase assays panels (Fig 3F, 4C) and rescue experiments (Fig 5B, 5D).

Reply:
We have shown the expression levels of the indicated proteins in the luciferase assays (Fig 3F and Fig EV4A), and the expression levels of the proteins in the rescue experiments are shown in Fig EV5C.

The authors performed extensive IP experiments to identify the critical domains of STING-UNC13D interaction. However, insufficient labeling and mixing of antibodies in the whole-cell lysates blots (like adding FLAG antibody and HA antibody together) makes it difficult to interpret the data and draw a conclusion. All western blots should include the size labels.

Reply:
We have repeated experiments and replaced Fig 4F. The FLAG tag and HA tag were each probed on separate blots of whole cell lysates (WCL) to distinguish UNC13D-NN/UNC13D-MHD/FL-STING. We also included other IP results in Figure 4. Size labels were added to all western blots.

It is not a fair comparison between WT and UNC31D KO-FHL3-mut in Fig 5D-5G. The authors should include WT, UNC13D KO, UNC13D KO-WT UNC13D, and UNC13D KO-FHL3-mut in the same panel.

Reply:
We have replaced Fig 5D-5G with Fig 5B-5C, in which WT, UNC13D KO, UNC13D KO-WT UNC13D RE, and UNC13D KO-FHL3 mut results are included in the same panel. Statistically significant differences were labeled.

Figure legends depicting different domains are missing in Fig 4F.

Reply:
Fig 4F was renumbered to Fig 4E. We have added relevant information to depict different domains in the figure and figure legend of Fig 4E.

The interaction of UNC13D-C2A and STING 139-340 was fairly weak (Fig 4I) to draw a "+" in Fig 4H.

Reply:
We created a new version of Fig 4H in which the interaction of UNC13D-C2A and STING 139-340 is clearly shown.

All bar graphs should be presented as a bar graph with a dot plot to show the distribution of individual data points. The proper statistical analysis should be performed using one-way ANOVA if there are more than 2 groups instead of the student t-test in Fig 1M-R, 2F-G, 3F, 5E, 5G, EV1, EV2, and EV4.

Reply:
The results of Fig 2F-G have been merged to Fig 5E-F, and Fig 2F-G have been deleted. The statistical analyses of more than 2 groups were performed using ANOVA for Fig 1M-R, 3F, 5E-F, 5G, EV1, EV2, and EV4, and the all bar graphs were replaced as a bar graph with a dot plot to show the distribution of individual data points.

The authors showed that inhibition of STING using small molecule H-151 reduced elevated IFN, IL-6, and CXCL10 mRNA expression in UNC13D KO cells and UNC13DKO-FHL3 mut cells as the physiological relevance. However, the effect is fairly small. Could the authors try using genetic means like siRNA knockdown or CRISPR KO to test whether transient or permeant removal of STING and or cGAS could rescue the elevated expression of cytokines in UNC13D KO and UNC13D KO-FHL3 mutant cells?

Reply:
Following the reviewer's suggestion, we knocked out STING in UNC13D KO cells, and we found that permanent removal of STING clearly rescued the elevated expression of cytokines due to the loss of UNC13D (Fig 5B, 5C and Fig EV5C), which confirmed the effect of H-151. Relevant information has been added to the results section (page 12).

The authors proposed that UNC13D regulates STING in the unstimulated state. Do authors have any insights into whether cGAS plays a role in elevated IFN and ISGs in UNC13D-deficient cells? Does the basal STING activation require an endogenous ligand trigger like mtDNA or nuclear DNA damage due to the loss of UNC13D?

Reply:
We have also attempted to knock out cGAS in UNC13D KO cells, but we only obtained cGAS knockdown cells (Fig EV5C). While we found that knockdown of cGAS significantly decreased the elevated expression levels of cytokines (Fig 5B and 5C), indicating that cGAS plays a role in elevated levels of IFN and ISGs in UNC13D-deficient cells. We predicted that basal STING activation requires an endogenous ligand trigger like mtDNA or nuclear DNA damage, which is recognized by cGAS. Relevant information has been added to the results section (page 12).

It would be more convincing if the authors could use primary FHL patient-derived cells (PBMC or fibroblast) and/or FHL mouse models (cells or mice) to improve the physiological relevance. It will be important to test whether STING plays a role in these patient-derived cells or FHL mouse model-derived cells if they are used.

Reply:

We fully agree with the reviewer's opinion, but we are not able to obtain patient-derived cells or FHL mouse model-derived cells in the short term. We will try to conduct such experiments in the future, and we hope that readers of the article could also help to verify these results. We have added this information to the discussion section (page 16).

Referee #3:

Major point:

1. I think quantification of experimental replicates (of the relevant bands, i.e. TBK1, IRF3, p65, STING) should be provided for the following figures:

Fig. 1 A-L,S

Fig. 2 B,C,D,E,H,I,J

Fig. 2 K,L (quantification of co-localization/aggregation)

Fig. 3 A,E,H,I,J

Fig. 3 B,C,D,G (quantification of co-localization)

Fig. EV5

Reply:

Following the reviewer's suggestion, we have provided quantitation of the relevant bands (p-TBK1, p-IRF3, p-p65, p-STING…) in the figures mentioned above over multiple repeats (Fig EV1A-L, Fig EV1P, Fig 1S) (Fig EV2H-N) (Fig EV3A and E, Fig EV3G-I) (Fig EV5F, G and L) , as well as quantification of co-localization/aggregation (Fig 2J, Fig 2L, Fig EV2P, Fig 3L, Fig EV3B-D, Fig EV3F, Fig EV5H).

Minor points:

2.The authors state that "statistically significant differences between groups were determined using a two-tailed Student's t-test". I assume the authors compared KD#1 and KD#2, and in some cases #KD3 individually to the control, since a Student's t-test would not be an appropriate test for three or more groups. If this was the case, this should be stated clearly.

Reply:

We have performed ANOVA instead of Student's t-test to identify statistically significant differences between three or more groups. We also replaced all bar graphs with a bar graph/dot plot to show the distribution of individual data points.

3.Fig. 4 E, D Can the authors comment on why the interaction of STING delta-18-138 with UNC13D is stronger than the interaction of STING delta-18-68?

Reply:

TM2 (residues 45–69) is the longest transmembrane helix in STING. The structure-based

mutational analyses showed that E68 in TM2 is an important mediator of the interaction between the transmembrane and cytoplasmic regions of STING, allowing it to form a dimeric assembly (PMID: 30842659). We suspected that STING delta-18-68 might not be able to form a stable structure to interact with UNC13D. STING delta-18-138 has an intact CTD, which has been reported to form a stable homodimer structure (PMID: 22579474 and 22728659), and this difference may be one of the reasons that the interaction of STING delta-18-138 with UNC13D is stronger than that of STING delta-18-68.

4.The manuscript is well written, but I think the abstract could be improved.

Reply:

We have modified the abstract as follows:

Stimulator of interferon genes (STING) is an essential signaling protein that is located on the endoplasmic reticulum (ER) and triggers the production of type I interferons (IFN) and proinflammatory cytokines in response to pathogenic DNA. Aberrant activation of STING is linked to autoimmune diseases. The mechanisms underlying homeostatic regulation of STING are unclear. Here, we report that UNC13D, which is associated with familial hemophagocytic lymphohistiocytosis (FHL3), is a negative regulator of the STING-mediated innate immune response. UNC13D co-localized with STING on the ER and inhibited STING oligomerization. Cellular knockdown and knockout of UNC13D promoted the production of interferon-β (IFN-β) induced by a DNA virus, but not an RNA virus. Furthermore, UNC13D deficiency increased the basal level of proinflammatory cytokines. and this effect was diminished by an inhibitor of STING signaling. Furthermore, the domains involved in the UNC13D/STING interaction on both proteins were mapped. Our findings provide an insight into the regulatory mechanism of STING, the previously unknown cellular function of UNC13D and the potential pathogenesis of FHL3.

1st Revision - Editorial Decision **30th Aug 2022**

Dear Dr. Chen,

Thank you for the submission of your revised manuscript to our editorial offices. I have now received the reports from the two of the three referees that I asked to re-evaluate your study, you will find below. Referee #1 remained completely unresponsive to my invitations to re-assess the manuscript. However, going through your point-by-point response, I consider his/her points as adequately addressed.

As you will see, the other two referees now support the publication of your study. Before proceeding with formal acceptance, I have these editorial requests I also you to address in a final revised manuscript:

- Please have your final manuscript file carefully proofread. There are still some typos and grammatical errors present (see also the report of referee #2).

- Please provide the abstract written in present tense throughout.

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

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- Please make sure that the number "n" for how many independent experiments were performed, their nature (biological versus technical replicates), the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values is indicated in the respective figure legends (main, EV and Appendix figures), and that statistical testing has been done where applicable. Please avoid phrases like 'independent experiment', but clearly state if these were biological or technical replicates. Please add complete statistical testing to all diagrams (main, EV and Appendix figures). Please also indicate (e.g. with n.s.) if testing was performed, but the differences are not significant. In case n=2, please show the data as separate datapoints without error bars and statistics.

- As they are significantly cropped, please provide the source data for the Western blots shown in the manuscript (including the EV figures). The source data will be published in separate source data files online along with the accepted manuscript and will be linked to the relevant figures. Please submit scans of entire gels or blots together with the revised manuscript. Please include size markers for scans of entire gels, label the scans with figure and panel number, and send one PDF file per figure (main and EV).

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Best,

Achim Breiling
Senior Editor

EMBO Reports

Referee #2:

I sincerely thank the authors for addressing all of my concerns and questions. I firmly believe the current version of the manuscript should be accepted at EMBO reports.

Referee #3:

The authors now provide quantitative analyses of their biochemical and cell biological assays. The majority of replicates used for quantification are biological replicates. I think that the revised version of the manuscript convincingly supports their claim that UNC13D negatively regulates the STING-mediated immune response by inhibiting STING oligomerization.

One minor detail in the abstract should be corrected:

"Furthermore, UNC13D deficiency increased the basal level of proinflammatory cytokines. and this effect was diminished by an inhibitor of STING signaling."

The period after "cytokines" should be deleted. Alternatively, "and" should be deleted and "This" capitalized.

The authors have addressed all minor editorial requests.

Dr. Dan-Ying Chen
Peking University
School of Life Sciences
School of Life Sciences
Peking University
Beijing 100871
China

Dear Dr. Chen,

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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.

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

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If your work benefited from core facilities, was their service mentioned in the acknowledgments section?	Yes	acknowledgments

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

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

Ethics

Ethics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Studies involving **human participants**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval.	Not Applicable	
Studies involving **human participants**: Include a statement confirming that **informed consent** was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	Not Applicable	
Studies involving **human participants:** For publication of **patient photos**, include a statement confirming that consent to publish was obtained.	Not Applicable	
Studies involving experimental **animals**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval. Include a statement of compliance with ethical regulations.	Not Applicable	
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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	