

Requirements for multivalent Yb body assembly in transposon silencing in *Drosophila*

Shigeki Hirakata, Hirotsugu Ishizu, Aoi Fujita, Yumiko Tomoe, and Mikiko C. Siomi

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision

8th Feb 2019

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

As you will see, all referees think that your manuscript presents a well-designed and executed study. While referee #1 feels the novel insight the manuscript provides is limited, referees #2 and #3 point out the study should be published in EMBO reports, provided their concerns are addressed upon revision. As the reports are below, I will not detail them here. Nevertheless, I think that points 1, 2, 4 and most importantly 6 of referee #3 need to be addressed experimentally in a revised manuscript, which would also increase the novelty of the findings (in particular regarding the part on phase-separation).

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

REFeree REPORTS:

Referee #1:

This work sheds light on the assembly and function of the piRNA biogenesis granule called Yb body. Yb is a protein that characterizes this RNP granule and already heavily researched. The authors previously characterized helicase domain point mutants of Yb and studied Yb body formation and transposon silencing requirements (Murota et al., 2014). Now they make two additional deletion versions of Yb and examine Yb body formation, piRNA biogenesis and

transposon silencing.

In this study the requirements for Yb body formation by knock-down experiments. Study the interactions of the two deletion mutants. Complementation with deletion versions identify the domains required for piRNA biogenesis and complex formation. They identify two additional specific point mutations that can abrogate Yb body formation and affect piRNA biogenesis. Overall, a very detailed study that will be a resource to the piRNA community studying the Yb body. Well executed study. It adds to the current literature on Yb protein/Yb body, but does not bring new insights.

Comments:

1. One interesting finding is the distinct domain requirements of Yb protein for production of genic and flam (transposon-targeting piRNAs) (Figure 3C-D). Where does the specificity for sequences come from?
2. Summarize in a panel all the interactions/interaction domains they identify in a cartoon with lines connecting the domains. It is hard for even someone in the field to remember all this at the end of the text. There is some space at the corner of Fig.4.

Minor

1. Synopsis suggestions: "Transposon-targeting" or later in the text "non-transposon-targeting" Or "transposon-repressing or non-transposon-repressing".

Referee #2:

In their manuscript entitled "Requirements for multivalent Yb body assembly in transposon silencing in *Drosophila*", Hirakata and colleagues dissect the molecular function of Yb in piRNA biogenesis. Female sterile (1) Yb (Yb) is essential for primary piRNA biogenesis in *Drosophila* ovaries and ovarian somatic sheath cells (OSC). Yb defines cytoplasmic bodies that mark sites of piRNA production as they co-localize with additional piRNA biogenesis factors and reside adjacent to flam bodies, which are defined by the local enrichment of piRNA precursor RNA. Here, Hirakata and colleagues build upon previous work (by their own group and others) that characterized the genetic requirements of Yb and the hierarchical formation of Yb bodies. The authors characterize the differential requirement of individual protein domains of Yb for its interactions with RNA and protein partners. These analyses elucidate that all domains of Yb are required for formation of Yb bodies with differential requirements for protein-RNA and protein-protein interactions. Surprisingly, the production of mRNA-derived piRNAs, which constitute a minor fraction of total piRNAs (<10%), was not dependent on Yb in contrast to the major fraction of piRNAs that arise from and target transposon-derived sequences. Finally, Hirakata et al. provide evidence that Yb bodies like other RNA-protein granules assemble through liquid phase separation. This study is well designed and conducted, and the presented results further our understanding of Yb bodies and their function in piRNA biogenesis. I recommend publishing a revised version of this manuscript.

Specific comments:

Figure 3:

Fig. 3A.: Because the stability of Piwi protein and its nuclear localization depend on its interaction with piRNAs, it would be helpful to quantify Piwi protein levels and determine Piwi's subcellular localization in the different experimental conditions.

Fig. 3 C-E: Most piRNAs seem to be lost upon knock-down of Yb, and this defect is not restored with the dHel-C construct. To integrate information about this overall reduction in piRNAs into the differential analysis of genic and transposon-targeting piRNAs, the authors could normalize their read counts to the miRNA population rather than to total small RNAs (rpm). Normalization to the miRNA population could clarify, if genic piRNAs are increased in the mutant -as suggested by RPM normalization- or if the observed increase is only relative to a loss of transposon-targeting piRNAs (E). Surprisingly, this relative increase is not observed at the specific example of tj piRNAs. The authors should comment on this difference.

Fig. 4E: It would be great, if the differential effect on flam compared to tj piRNAs as a surrogate for

transposon-targeting and genic piRNAs could be quantified by small RNA sequencing (as in Fig. 3.) Or supported by additional northern blots. The presented NB is rather weak and quantitative differences as stated in the text are not obvious.

Referee #3:

The manuscript by Hirakata et al. describes, in more detail than thus far, the molecular interactions that occur in, and are required for the Yb body in *Drosophila* somatic follicle cells of the ovary. This body is closely related to for instance P-granules in *C. elegans* and nuage in vertebrate germ cells. It is known to play an important role in the generation of piRNAs, above all those that come from the well-known flamenco locus. Interestingly, the authors demonstrate that the Yb body interactions are mostly needed to produce piRNA from the flamenco transcript, and not for generation of piRNAs in general. In absence of these bodies, genic piRNAs are produced, suggesting that these can be made fine in absence of Yb body factors such as Armi, Yb and Vret. Finally, the authors address the phase separated nature of the Yb body. This part, in my view is the weakest/least informative in the current form. However, I believe that with proper textual changes, most issues can be resolved. Overall, this manuscript would be a very good fit for EMBO Reports.

Major issues (in order as they come up in the manuscript)

Page 7 It cannot be concluded that Yb alone, without other proteins, initiates Yb body formation. Proteins that may help this process may be poorly retrieved in IP experiments. To make this conclusion, that Yb can do this on its own, purified Yb protein will need to be studied. This is not absolutely required for the manuscript, but the wording should be adjusted in case it is not done.

Page 8: RG motifs in Armi that may explain the interaction with eTud? In addition, the authors write: "eTud is also considered to be the domain that interacts with SoYb-Vret". Since the Yb-SoYb/Vret interaction is via Armi, this sentence is misleading. It suggests a direct interaction, where it most likely fully depends on Armi. Since this paper is really about resolving the details of interactions in these bodies, such a statement should not be made, unless a direct interaction between eTud and Vret/SoYb is experimentally demonstrated.

Page 9 To look at potential protein interactions of eTud (besides Armi) an IP experiment on eTud should be done in absence of Armi. The observed RNA interaction may well be via another protein. I ask this, since eTud domains are typically not thought to direct RNA interaction. At the very least, the authors should discuss how they think the eTud domain mediates interaction with RNA.

Page 9 The RNA found bound to Yb and the Hel-C deletion CLIP experiment should be sequenced. This could reveal that Flam RNA is indeed specifically bound, strengthening this aspect of the manuscript significantly. This should be a very simple experiment, since they have the CLIP running.

Page 10 The authors describe that loss of the HelC domain does not affect Tj piRNAs. Yet the authors also described before (Ishizu et al 2015) that Yb binds to Tj RNA and that this interaction would be required for Tj piRNA formation. Is this mediated by another part of Yb? The authors need to clarify this apparent discrepancy, as this seems to be directly contradicting the proposed model.

Page 11-12 The authors nicely show that Yb bodies have liquid-like properties. However, whether Yb really makes these, or is simply part of them remains unresolved. The introduced mutations could also affect RNA binding, and may not at all be related to IDR properties of Yb (if it has any). This relates to my previously mentioned concern (Page 7): the formation of Yb bodies in the cells may well depend on another protein that has not yet been identified. Proteins with extensive IDRs tend to come down poorly in IPs due to their tendency of self-aggregation. Perhaps repeating some of the IPs in presence of arginine in the buffer can improve such recovery, as it sometimes helps to solubilize such proteins, allowing identification through mass spectrometry. If the authors really want to claim that Yb induces phase separation, *in vitro* studies with purified Yb will need to be done. In absence of such *in vitro* experiments the authors have to tune down their conclusions on Yb

significantly, and make clear that is NOT clear whether Yb drives the phase separation or not. After all, Yb was simply just the first protein in these granules, and that provides zero information on its role in forming phase separated structures. Obviously, if Yb is used as a marker, by definition there will be no Yb bodies in a Yb mutant. But there may well be still the phase separated structure where Yb would go to if it were there.

Figure 4D: The authors show mutant versions of Yb that are not in a discrete body. It would be great to know whether the SoYb/vret/Armi interactions would be affected by this. This would address whether Yb needs to be in a phase separated body to do what the authors describe, or not. If not done, the authors should mention that this question remains unresolved for now.

Minor issues:

Page 12 The FlamBG data is all from others and published. While it is cited correctly, this belongs in the discussion, not the results section. Placing it in the results section may create the wrong impression that this is primary data from this paper itself.

-Armi shows up as a double band in the Western blot. Please comment/explain.

Minor text issues:

'Transposon-repressible piRNA' is not a correct phrase I believe. It suggests that piRNAs are repressed by transposons. I would suggest the following wording:
 Transposon-repressing instead of transposon-repressible
 non-transposon-repressing instead of transposon-irrepressible

Page 4 In the context of Co-transcriptional repression panoramix/silencio should also be mentioned.

Page 5 'conprehensive' (typo)

Page 6 'propensities' should be 'properties. Or use a different phrase like 'propensity to phase separate'.

1st Revision - authors' response

25th Mar 2019

Referee #1:

Comment #1: One interesting finding is the distinct domain requirements of Yb protein for production of genic and flam (transposon-targeting piRNAs) (Figure 3C-D). Where does the specificity for sequences come from?

This is a very important question to answer. To address this, we attempted several experiments, but the answer still remains unknown. We predict that the length of piRNA precursors and/or the density of the Yb binding sites in them are the key here. This was largely based on our CLIP-sequencing data (Ishizu et al. *Cell Reports* 2015), which showed that the *flam* RNA transcripts contain numerous Yb-binding sites throughout them, while the genic piRNA sources (mRNAs) have far fewer Yb-binding sites and these sites are almost exclusively in their 3' UTRs. The *flam* piRNA cluster is ~180 kb long and it has only one promoter. Thus, the primary transcripts are estimated to be ~180 kb long, but a previous study indicated that they undergo splicing (Goriaux et al. *EMBO Reports* 2014). However, that study did not involve a comprehensive investigation. They analyzed only *flam* exon 1-3 regions and at present the actual size of mature *flam* RNA splicing variants is unknown. Furthermore, no comprehensive determination of the exons/intron boundaries in *flam* has been performed. We first need to resolve these issues prior to addressing the reviewer's question. Future analyses to resolve these points are anticipated.

Comment #2: Summarize in a panel all the interactions/interaction domains they identify in a cartoon with lines connecting the domains. It is hard for even someone in the field to remember all this at the end of the text. There is some space at the corner of Fig.4.

We have now included a panel summarizing the functionalities of each domain of Yb including protein-protein interactions (revised Fig. 4F).

Minor comment: Synopsis suggestions: "Transposon-targeting" or later in the text "non-transposon-targeting" Or "transposon-repressing or non-transposon-repressing".

We thank the reviewer for this kind suggestion. We now use the phrases “*transposon-repressing*” and “*non-transposon-repressing*” in the revised manuscript.

Referee #2:

Surprisingly, the production of mRNA-derived piRNAs, which constitute a minor fraction of total piRNAs (<10%), was not dependent on Yb in contrast to the major fraction of piRNAs that arise from and target transposon-derived sequences. Finally, Hirakata et al. provide evidence that Yb bodies like other RNA-protein granules assemble through liquid phase separation. This study is well designed and conducted, and the presented results further our understanding of Yb bodies and their function in piRNA biogenesis. I recommend publishing a revised version of this manuscript.

We thank the reviewer for this positive comment.

Specific comments:

Fig. 3A: Because the stability of Piwi protein and its nuclear localization depend on its interaction with piRNAs, it would be helpful to quantify Piwi protein levels and determine Piwi's subcellular localization in the different experimental conditions.

The IF results of endogenous Piwi have already been presented in original Appendix Fig S2 (Fig EV3 in the revised manuscript). In the absence of Yb, the level of endogenous Piwi was low, as has been reported previously (Szakmary et al. *J Cell Biol* 2009). (This control value was missing in the original figure, so we added it to the revised figure.) The Piwi level returned to normal upon the ectopic expression of DHel-C, but not upon the ectopic expression of DeTud. Thus, the correlation between Piwi stability and piRNA expression (and Piwi loading) is clear.

Fig. 3 C-E: Most piRNAs seem to be lost upon knock-down of Yb, and this defect is not restored with the dHel-C construct. To integrate information about this overall reduction in piRNAs into the differential analysis of genic and transposon-targeting piRNAs, the authors could normalize their read counts to the miRNA population rather than to total small RNAs (rpm). Normalization to the miRNA population could clarify, if genic piRNAs are increased in the mutant -as suggested by RPM normalization- or if the observed increase is only relative to a loss of transposon-targeting piRNAs (E). Surprisingly, this relative increase is not observed at the specific example of tj piRNAs. The authors should comment on this difference.

We immunoprecipitated the Piwi-piRNA complexes from OSCs prior to the piRNA library construction. Therefore, the libraries did not contain miRNAs and so it was impossible to normalize the read counts to the miRNA population.

The loss of the Hel-C domain attenuated the Yb-*flam* interaction (Fig 3F). This led the DHel-C mutant to bind genic piRNA sources (including *tj* mRNAs) more strongly and so the level of genic piRNAs produced in the cells was increased (Fig 3E). We now discussed this more clearly in the revised text (page 10).

The relative increase was not observed for *tj*-piRNAs. This was likely due to the relatively high abundance of *tj*-piRNAs among genic piRNAs in normal OSCs. In that case, the discrepancy in the presence and absence of the Hel-C domain may not be very obvious. This comment now appears in the revised text (page 10).

Fig. 4E: It would be great, if the differential effect on flam compared to tj piRNAs as a surrogate for transposon-targeting and genic piRNAs could be quantified by small RNA sequencing (as in Fig. 3.) Or supported by additional northern blots. The presented NB is rather weak and quantitative differences as stated in the text are not obvious.

We repeated the experiments and replaced the data with new ones (revised Fig 4D). We hope that this replacement will satisfy the reviewer's concern.

Referee #3:

I believe that with proper textual changes, most issues can be resolved. Overall, this manuscript would be a very good fit for EMBO Reports.

We thank the reviewer for this positive comment.

Major issues:

Page 7: It cannot be concluded that Yb alone, without other proteins, initiates Yb body formation. Proteins that may help this process may be poorly retrieved in IP experiments. To make this conclusion, that Yb can do this on its own, purified Yb protein will need to be studied. This is not absolutely required for the manuscript, but the wording should be adjusted in case it is not done.

We purified recombinant Yb and examined whether this protein was able to form Yb body-like structures *in vitro* in the presence and absence of RNAs (2-3 kb long). In neither case did such structures form. We suspected that much longer RNAs like *flam* RNAs (the gene is ~180 kb long) should be employed in the experiments, but technically speaking it is very difficult to prepare such long RNAs. We modified the corresponding text to tone it down, which now reads: “*The hierarchical manner of Yb body assembly is suggested: Yb triggers Yb body formation in a manner independent of Armi, SoYb, and Vret*” (page 7 in the revised text). We also added the following text in the section: “*Yb may or may not require other factors to initiate the process of Yb body formation. Further analysis to address this is anticipated.*”

Page 8: RG motifs in Armi that may explain the interaction with eTud? In addition, the authors write: "eTud is also considered to be the domain that interacts with SoYb-Vret". Since the Yb-SoYb/Vret interaction is via Armi, this sentence is misleading. It suggests a direct interaction, where it most likely fully depends on Armi. Since this paper is really about resolving the details of interactions in these bodies, such a statement should not be made, unless a direct interaction between eTud and Vret/SoYb is experimentally demonstrated.

We checked the peptide sequence of Armi and found no RG motifs (GRG, ARG, or GRA) in it. We also found that the amino acids involved in the formation of the aromatic cage in Tud domain are not conserved in eTud. Thus, RG motifs might not explain the interaction between Armi and eTud. Pandey et al. previously showed that Tudor domain containing-protein BmTDRD12 bound Siwi sDMA-independently (PNAS 2013).

We modified the text in the revised manuscript accordingly (page 8), which now reads: “*eTud is also considered to be the domain for the recruitment of SoYb-Vret heterodimer to the Yb complex.*”

Page 9: To look at potential protein interactions of eTud (besides Armi) an IP experiment on eTud should be done in absence of Armi. The observed RNA interaction may well be via another protein. I ask this, since eTud domains are typically not thought to direct RNA interaction. At the very least, the authors should discuss how they think the eTud domain mediates interaction with RNA.

Despite our efforts, we failed to express eTud alone in OSCs. This domain is composed of a Tudor domain and a nuclease-like domain. This latter domain may contribute to the activity. We now discuss this issue in the revised text (page 9).

Page 9: The RNA found bound to Yb and the Hel-C deletion CLIP experiment should be sequenced. This could reveal that Flam RNA is indeed specifically bound, strengthening this aspect of the manuscript significantly. This should be a very simple experiment, since they have the CLIP running.

Despite our extensive efforts, we failed to produce CLIP-seq libraries for the DHel-C mutant. This was most likely because of the low Myc-Yb immunoprecipitation efficiency using anti-Myc antibodies. To respond to the reviewer’s comment, however, we carried out CLIP-qPCR analysis for genic piRNA sources other than *tj* (revised Fig 3F). These provide evidence supporting our idea that Yb-*flam* binding specifically diminishes in the absence of Hel-C.

Page 10: The authors describe that loss of the HelC domain does not affect Tj piRNAs. Yet the authors also described before (Ishizu et al 2015) that Yb binds to Tj RNA and that this interaction would be required for Tj piRNA formation. Is this mediated by another part of Yb? The authors need to clarify this apparent discrepancy, as this seems to be directly contradicting the proposed model.

In this study, we found that the Hel-C domain is required for Yb-Yb (*i.e.*, protein-protein) interaction and that the interaction between Yb and genic piRNA sources (mRNAs) does not require the Hel-C domain. However, the Yb-mRNA interaction requires the RNA helicase and eTud domains of Yb. To make this clearer, we summarize the functionalities of each domain of Yb in revised Fig 4F.

Page 11-12: The authors nicely show that Yb bodies have liquid-like properties. However, whether Yb really makes these, or is simply part of them remains unresolved. The introduced mutations could also affect RNA binding, and may not at all be related to IDR properties of Yb (if it has any). This relates to my previously mentioned concern (Page 7): the formation of Yb bodies in the cells may

well depend on another protein that has not yet been identified. Proteins with extensive IDRs tend to come down poorly in IPs due to their tendency of self-aggregation. Perhaps repeating some of the IPs in presence of arginine in the buffer can improve such recovery, as it sometimes helps to solubilize such proteins, allowing identification through mass spectrometry. If the authors really want to claim that Yb induces phase separation, in vitro studies with purified Yb will need to be done. In absence of such in vitro experiments the authors have to tune down their conclusions on Yb significantly, and make clear that is NOT clear whether Yb drives the phase separation or not. After all, Yb was simply just the first protein in these granules, and that provides zero information on its role in forming phase separated structures. Obviously, if Yb is used as a marker, by definition there will be no Yb bodies in a Yb mutant. But there may well be still the phase separated structure where Yb would go to if it were there.

In accordance with the reviewer's suggestion, we carried out Yb immunoprecipitation in the presence of arginine. However, under such conditions, we found that Yb immunoprecipitation efficiency was greatly reduced. We also attempted *in vitro* assays using purified recombinant Yb in the presence and absence of RNAs (2-3 kb long). In neither case did Yb body-like structures form. We suspected that much longer RNAs like *flam* RNAs (the gene is ~180 kb long) should be employed in the experiments, but technically speaking, at present, it is very difficult to prepare such long RNAs. To tone down our conclusions, we modified the corresponding text, which now reads: "However, their interactions with *Armi*, *SoYb*, and *Vret* were maintained. These findings strongly suggest the importance of Yb-Yb interaction via the *Hel-C* domain in the phase separation of Yb bodies, transposon-repressing *flam*-piRNA biogenesis, and transposon silencing" (pages 12-13 in the revised text).

*Figure 4D: The authors show mutant versions of Yb that are not in a discrete body. It would be great to know whether the *SoYb/vret/Armi* interactions would be affected by this. This would address whether Yb needs to be in a phase separated body to do what the authors describe, or not. If not done, the authors should mention that this question remains unresolved for now.*

We performed co-immunoprecipitation experiments and confirmed that the Yb mutants interact with *Armi*, *SoYb* and *Vret* as efficiently as Yb WT does. The results are shown in revised Fig EV4D.

*Minor issues: Page 12: The *FlamBG* data is all from others and published. While it is cited correctly, this belongs in the discussion, not the results section. Placing it in the results section may create the wrong impression that this is primary data from this paper itself.*

Thank you for the kind suggestion. However, the original study (Malone et al. *Cell* 2009) focused only on *flam* piRNAs but did not analyze genic piRNAs. Thus, the data shown in Fig 4E are new (although the analysis was carried out using their sequence data) and in our opinion worthy if inclusion in the Result section.

**Armi* shows up as a double band in the Western blot. Please comment/explain.*

The *Armi* doublet on western blots corresponds to two *Armi* isoforms. This was previously reported by Olivieri et al. (*EMBO J* 2010). We added this information in the revised text (page 7).

Minor text issues: 'Transposon-repressible piRNA' is not a correct phrase I believe. It suggests that piRNAs are repressed by transposons. I would suggest the following wording:

*Transposon-repressing instead of transposon-repressible
non-transposon-repressing instead of transposon-irrepressible*

We now use the phrases "transposon-repressing" and "non-transposon-repressing" throughout the revised manuscript.

*Page 4: In the context of Co-transcriptional repression *panoramix/silencio* should also be mentioned.*

Panoramix/Silencio and other co-factors now appear in the revised text (page 4).

Page 5 'comprehensive' (typo)

Page 6 'propensities' should be 'properties. Or use a different phrase like 'propensity to phase separate'.

We have corrected these in the revised text (pages 5 and 6).

2nd Editorial Decision

10th Apr 2019

Thank you for the submission of your revised manuscript to our editorial offices. We have now received the reports from the two referees that were asked to re-evaluate your study (you will find below). As you will see, the both referees now support the publication of your manuscript in EMBO reports.

Before we can proceed with formal acceptance, I have these editorial requests, which I ask you to also address in a final revised version of the manuscript:

REFeree REPORTS:

Referee #2:

The authors have addressed all comments. The improved manuscript contains an important body of work and should be published without further revision.

Referee #3:

The authors have addressed the issues that were raised in a satisfying manner. I support publication of the current manuscript in EMBO Reports.

2nd Revision - authors' response

14th Apr 2019

The authors performed all requested editorial changes.

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Mikiko C. Siomi

Journal Submitted to: EMBO Reports

Manuscript Number: EMBOR-2019-47708V1

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- 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.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs 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 and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship 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 χ^2 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.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Sample size was chosen based on previous studies in the field. No statistical method was used to predetermine the sample size.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	N/A
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	N/A
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	No.
For animal studies, include a statement about randomization even if no randomization was used.	N/A
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	No.
4.b. For animal studies, include a statement about blinding even if no blinding was done	N/A
5. For every figure, are statistical tests justified as appropriate?	Materials and Methods section, page 25.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Assumption that the Ct values follow normal distribution is based on previous studies.
Is there an estimate of variation within each group of data?	Yes.
Is the variance similar between the groups that are being statistically compared?	Yes.

C- Reagents**USEFUL LINKS FOR COMPLETING THIS FORM**

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http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
<http://www.selectagents.gov/>

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	Materials and Methods section, pages 18-20.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Materials and Methods section, page 16.

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	N/A
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	N/A
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	N/A

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	N/A
12. 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.	N/A
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	N/A
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	N/A
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	N/A
16. 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.	N/A
17. 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.	N/A

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	Materials and Methods section, page 25.
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	Deposited to Gene Expression Omnibus.
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	N/A
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	N/A

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	N/A
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