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Nuclear RNA export factor variant initiates Piwi-piRNA-guided co-transcriptional silencing

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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 Correspondence (SB) 6th July 2019

We certainly appreciate the interest of the work and feel the scope and level is in principle appropriate for the journal. We are at this time focussing on outstanding technical issues raised by the referees. Unfortunately, we cannot at this time make a definitive decision for two reasons:

First of all, would you be able to also send us your point-by-point response to the second set of referee comments? From looking at the two other manuscripts that are accessible, we do think that it would be important to openly discuss the differences between the studies in the paper (as Bernd already noted, you should cite papers already published in E-life and posted on bioRxiv formally in the discussion and raise similarities and note, and where possible explain, discrepancies). However, we do not feel that these differences necessarily preclude publication (as suggested by ref 2), especially if they could possibly be the result of a slightly different experimental set-up (i.e. the time-points of analysis for the H3K9me3 occupancy or the readout of reporter repression as enzymatic activity vs. mRNA).

Secondly, we also noticed that the pre-print of the Brennecke lab (Batki et al. Biorxiv http://dx.doi.org/10.1101/609693) has a disclaimer added to the first page stating that there were issues with their own CLIP-seq data for Nxf2 in the engineered line. This manuscript now references the Nxf2 CLIP-seq data from your group:
"Consistent with this, Siomi and colleagues provide evidence based on CLIP experiments that Nxf2 directly interacts with piRNA complementary target RNAs in OSCs (Murano et al., 2019)". Could you please confirm that your CLIP-seq results were obtained using a different transgenic line than his?

Authors' correspondence 6th July 2019

First of all, we believe that many discrepancies between our study on Nxf2 and their (Drs Hannon, Brennecke and Yu) studies come from the facts that to analyze the molecular pathway of how Nxf2 functions in the piwi-mediated silencing, we mostly use a cultured cell line named OSC, which is derived from fly ovaries, and other groups mostly use transgenic and/or mutant flies. Using OSC, we can finely define the process of how Nxf2 contributes to the Piwi-mediated silencing by, for example, taking time course after Nxf2 expression and thus can observe both the early step and late phase of how the silencing is established. In contrast, other groups can only observe established or final stage of the silencing in transgenic and/or mutant flies but not the process of how the silencing is established.

> First of all, would you be able to also send us your point-by-point response to the second set of referee comments?

Please see our attached file for point-by-point response.

> we do think that it would be important to openly discuss the differences between the studies in the paper (as Bernd already noted, you should cite papers already published in E-life and posted on bioRxiv formally in the discussion and raise similarities and note, and where possible explain, discrepancies).

We added this in “Discussion” section, describing the difference in ours and the other groups’ studies (Revised manuscript page 22 line 9-).

> Could you please confirm that your CLIP-seq results were obtained using a different transgenic line than his?

We used the stable cell line of OSCs which we independently established in our laboratory. In addition to the stable cell line, we used the different protocol for CLIP library preparation, which includes the size selection of the associated RNA products (precise protocol is described within the manuscript). Other groups including Dr. Brennecke do not perform the size selection for CLIP experiments.

Our detailed point-by-point response to the referees’ comments is in attached pdf file (point-by-pointResponse.pdf).

Point-by-point response 1st round

Editor's comments:

the strongest concern expressed by the reviewers is that the molecular mechanism underlying the targeting of Nxf2 to transposon RNAs and its role in mediating silencing is too premature to justify publication of this study in Nature

In this revised study, we identified Nxf2, a nuclear RNA export factor (NXF) variant, as a protein that forms a complex with Panx and Piwi. Nxf2 further associates with p15 (Nxt1), a co-adaptor for nuclear RNA export. However, unlike Nxf1 that plays a major role in mRNA export, Nxf2–p15 instead transcriptionally regulates transposable elements (TEs) in the Piwi–piRNA pathway and also stabilises the protein level of Panx. Conversely, lack of Panx destabilizes Nxf2, showing that both Panx and Nxf2 are equally necessary in the silencing by stabilizing each other. Notably, ectopic targeting of Nxf2 could initiate co-transcriptional repression of the target reporter gene in a manner independent of H3K9me3 marks or H1 at an early time point. However, continuous silencing requires HP1a and H1. These results suggest that Nxf2 is required to enforce the association of Piwi–piRNA complexes with the nascent transcript of target TEs and trigger co-transcriptional repression, prior to heterochromatin formation in the nuclear silencing pathway (*two-step silencing model*). The amino-terminal domain of Nxf2 harbouring RNA binding activity is essential for recruitment of the Piwi–piRNA complex to target TEs. To get insights into the mechanistic details of how Nxf2 is recruited to TE transcripts, we performed CLIP experiments and were able to demonstrate *for the first time* that both Piwi and Nxf2 directly interact with target TE transcripts and that association of Nxf2 with target TE transcripts is Piwi-dependent. These findings suggest a model in which the Nxf2-Panx complex stabilizes the association of Piwi with target TE transcripts to trigger co-transcriptional repression and recruit chromatin factors for heterochromatin formation. Our results provide an unexpected link between an NXF variant and small RNA-mediated co-transcriptional silencing.

Our "two-step silencing model" is conceptually new. In addition, our findings also suggest that the silencing pathway may need an engaged-in system to distinguish "scanning Piwi" and "target-engaged Piwi" and repress only targets and avoid

unnecessary silencing of other cellular transcripts. Perhaps, Nxf2 may play a role in supporting association of Piwi that has found its *bona fide* targets. Panx preferentially associates with Piwi, which loads piRNAs targeting TEs (Sienski et al., 2015 *Genes Dev*), suggesting that there may be a system for the Panx-Nxf2 to form the Piwi-Panx-Nxf2-p15 complex, specifically with Piwi that is engaged to its target TEs. Our CLIP analysis shows that Nxf2 cannot associate with target TEs without the activity of Piwi, indicating that the Panx-Nxf2-p15 complex itself does not recognize target TEs, but rather recognizes target-engaged Piwi. This is the key finding to describe the recognition mechanism of piRNA-target nascent RNA by Piwi-piRNA silencing machinery, which has not been proven for years since the model arise.

Referee #1:

The mechanistic details of how Nxf2 is recruited to transposon RNAs is sadly less well developed, and direct proof that Panx-Piwi are recruited to transposable elements via Nxf2 or vica versa (e.g. via RNA immunoprecipitation or CLIP assays) are missing.

As described above, now we have CLIP data to demonstrate that both Piwi and Nxf2 directly interact with target TE transcripts and that association of Nxf2 with target TE transcripts is Piwi-dependent (revised Fig. 6).

Nxf2 and p15 are important for transposon silencing (refs 16-18). Work in the human system has shown that both Nxf1 and Nxf2 bind p15 via the NTF2L domain (Herold et al., Mol. Cell. Biol. 20:8996). Therefore, the data showing the association of Nxf2 with p15 is incremental since it is merely confirming what was known in the human system.

TEs have been previously shown to co-opt mRNA export machiner**y.** For example, Dr. Bill Theurkauf and colleagues previously demonstrated that UAP56 couples piRNA clusters to the perinuclear transposon silencing machinery (Zhang et al., 2012 *Cell*). Recently Dr. Julius Brennecke has posted his new paper on the involvement of Nxf3, another ovary specific NXF variant, in piRNA production

(bioRxiv #596171; doi:10.1101/596171). However, findings described in all these papers suggest that UAP56 and Nxf3 function as part of a piRNA precursor-processing/export machinery and not part of co-transcriptional repression mediated by the Piwi-piRNA complex. Our findings described in this paper instead point to the active role of Nxf2 in the Piwi-mediated transcriptional silencing, unexpectedly as a co-transcriptional repressor rather than RNA export factor. This is an entirely new finding which will open a new field of TE silencing and epigenetic process of gene silencing in general.

Specific comments: 1. The manuscript fails to explain how Nxf2 is recruited to transposable elements to elicit silencing. This could have been demonstrated by iCLIP, RNA-IP, or some other means in cells where other components of the piRNA pathway were depleted. The suggestion is that piRNA-PIWI complexes might direct NXF2 to TE RNAs, but this needs to be established. For Nxf1, the TREX complex plays a key role in recruiting Nxf1 to target mRNAs. Moreover both Nxf1 and Nxf2 in humans use the N-terminal region to bind Alyref (Herold et al., Mol. Cell. Biol. 20:8996) and this is key for recruitment of Nxf1 to mRNA and subsequent stable binding. Does it play a similar role with Nxf2 or is transposable element targeting carried out by piRNA pathway components exclusively? As the manuscript stands, far too much of the model relies on speculation based on indirect assumptions.

As described above, now we have CLIP data to demonstrate that both Piwi and Nxf2 directly interact with target TE transcripts and that association of Nxf2 with target TE transcripts is Piwi-dependent (revised Fig. 6). As suggested by the referee, we have performed knock down of Alyref, and found that Alyref is not involved in silencing of TEs (revised Extended Data Fig. 5e).

2. Whilst the authors show that depletion of Panx weakens the silencing mediated by Nxf2, the authors do not go on to show that Panx is recruited to its target TEs via Nxf2, as suggested by their model and tethering data. Direct proof of this could have been obtained in vivo using an assay such as RNA immunoprecipitation and using a reconstituted system in vitro.

As described above, now we have CLIP data to demonstrate that both Piwi and

Nxf2 directly interact with target TE transcripts and that association of Nxf2 with target TE transcripts is Piwi-dependent (revised Fig. 6). Also, since Panx-Nxf2 interaction is necessary for their protein stability (revised Fig. 2 and 5), we believe that Panx is recruited to target TEs together with Nxf2. This point is now clearly indicated in the revised manuscript (p16 line9~).

1. *The mass spectrometry analysis of Panx shows cherry-picked interactions. Instead, the full datasets of mass spectrometry hits should be published as a Supplementary Table. Cherry picking which data to present is unacceptable.*
2. *The ΔLRR NXf2 mutant is poorly described. In fact, the N-terminus of Dm Nxf2 has RRM domains embedded between the LRR repeats. Whilst it is clear from the study that the N-terminal region of Nxf2 is important for TE silencing, its precise role is unclear. Importantly this region of Nxf1 allows interaction with export adaptors as well as RNA. The role of this N-terminal region of Nxf2 in TE silencing needs to be better defined. Is its loss preventing RNA binding or association with some other factor critical for recruitment of Nxf2 or both?*

Now we show the list of proteins obtained from mass spectrometry analysis (revised Extended Data table 1, and 2).

As described above, now we have CLIP data to demonstrate that both Piwi and Nxf2 directly interact with target TE transcripts and that association of Nxf2 with target TE transcripts is Piwi-dependent (revised Fig. 6). We also performed KD of Alyref (see our response to 1).

Referee #2:

1. Abstract, line 10: "Nxf2 further associates with p15 (Nxt1), a co-adaptor for nuclear RNA export." The way it is described in the abstract, it appears that this study identifies for the first time interaction between Nxf2 and Nxt1. This interaction is known for almost 20 years. Since other introductory sentences are properly referenced, please add references here also to avoid misrepresentation.

Thank you. We added a relevant citation for this in Abstract.

2. Fig. 1a: It is not clear to this reviewer which band on the gel corresponds to which protein. There are so many bands. Did the authors find Gtsf1? A Western blot for Gtsf1 and Mael should be included. Is Panx complex with Piwi and Nxf2 distinct from other Piwi complexes that have Gtsf1, etc?

We performed western blots with anti-Gtsf1 and anti-Mael antibodies and were able to show Mael is present in the complex. These results are now shown in extended data (revised Extended Data Fig. 1c).

3. Fig. 2b: The absence of reporter repression with Piwi tethering is in conflict with their own observed interaction data (Fig. 1a,b). Is the Piwi that is tethered loaded with piRNAs and nuclear? They see interaction between Panx, Piwi, and Nxf2, so why can tethered Piwi not recruit Panx? They explain that tethering of Panx recruits Nxf2 and vice versa, so why not recruit Piwi? This is inconsistent.

lN-HA-myc-tagged Piwi localized at the nucleus (revised Extended Data Fig. 4b), indicating that lN-HA-myc-tagged Piwi harbored piRNA in OSCs (Saito et al., 2010 *Genes Dev*). To address the question why tethered Piwi cannot silence reporters, we introduced revised reporter constructs with fragments of the Flamenco locus, the major piRNA cluster. Using this system, we could show that Piwi which is guided to the target reporter via loaded piRNA, could represses its targets (revised Extended Data Fig3), indicating that it is likely that only piRNA-guided Piwi is able to recruit silencing machinery to the target (revised Extended Data Fig. 7i). Introduction of revised reporters has led us to propose that the silencing pathway may need an engaged-in system to distinguish “scanning Piwi” and “target-engaged Piwi” and repress only targets and avoid unnecessary silencing of other cellular transcripts. Perhaps, Nxf2 may play a role in supporting association of Piwi that has found its *bona fide* targets. Panx preferentially associates with Piwi, which loads piRNAs targeting TEs (Sienski et al., 2015 *Genes Dev*), suggesting that there may be a system for the Panx-Nxf2 to form the Piwi-Panx-Nxf2-p15 (PPNP) complex, specifically with Piwi that is engaged to its target TEs. Our CLIP analysis shows that Nxf2 cannot associate with target TEs without the activity of Piwi, indicating that the Panx-Nxf2-p15 complex itself does not recognize target TEs, but rather recognizes target-engaged Piwi (revised Fig. 6). This is the key finding to describe the recognition mechanism of piRNA-target nascent RNA by Piwi-piRNA silencing machinery.

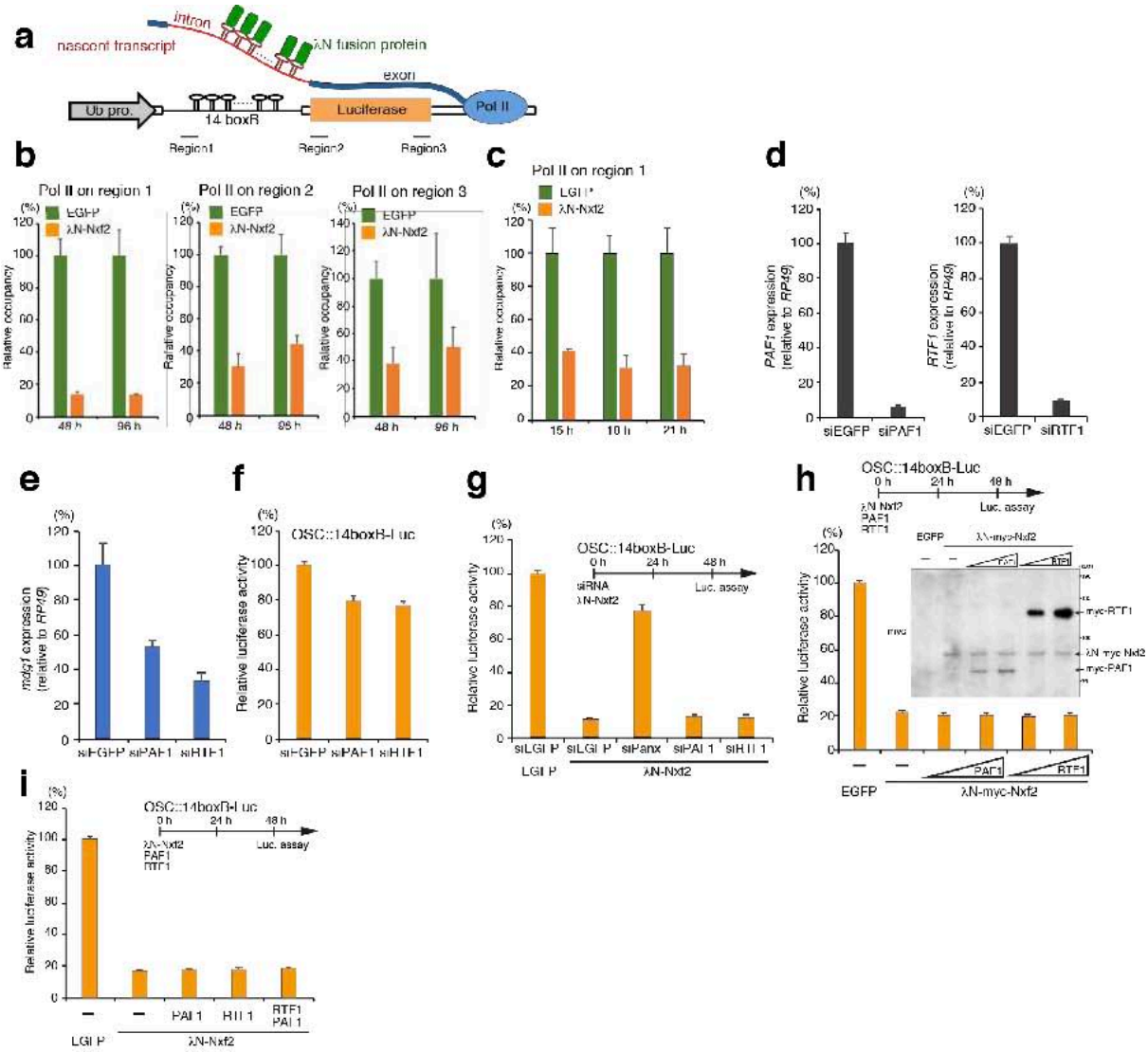
4. Fig. 3: Strong conclusions about the time-course of repression is better obtained by genome-integrated inducible expression of the tethered proteins (like the genome-integrated reporter they use). The transient expression strategy used by the authors may hide effects due to differences in plasmid transfection, etc.

We would not agree with this, because genome-integrated inducible expression system may alter genome structure and/or mutate genes dependent upon integration sites and numbers, thereby changing transcriptome. We can get consistent and reproducible results using our transient expression strategy, which supports well our claim.

5. Page 9, line 20: "In other words, the silencing mode in the Piwi–piRISC pathway may switch from Nxf2–Panx-dependent to heterochromatin-dependent". This sentence is unsubstantiated and not true. The reporter they use to study silencing is NOT repressed by Piwi or targeted by piRNAs bound to Piwi. Also, what is Nxf2-Panx-dependent repression due to?

Thank you. We have rewritten relevant sentences. Also, please see our response to 3. We have performed series of experiments to analyze what Nxf2-Panx-dependent repression is due to. We obtained the results indicated below: In *C. elegans*, NRDE-2 (nuclear RNAi defective-2) associates with the Argonaute protein NRDE-3 in the nucleus and is recruited by the NRDE-3/siRNA complex to nascent transcripts. This complex inhibits Pol II during the elongation, and directs the accumulation of Pol II at genomic loci targeted by RNAi. We investigated whether lN-Nxf2 directs the accumulation of Pol II at boxB sites in the co-transcriptional silencing (Response Fig. a-c). Although the time course of Pol II occupancy upon lN-Nxf2 was examined by ChIP-qPCR at the boxB sites, we could not observe any accumulation of Pol II along the reporter gene. Recently, it was reported that Pol II-associated proteins, PAF1 and RTF1, antagonize Piwi-directed silencing, which are factors involved in the modulation of promoter release, elongation, and termination of Pol II. The Panx-Nxf2-p15 complex might interfere with Pol II *via* the PAF1 complex, considering that fission yeast PAF1 represses AGO1/siRNA-directed silencing. Although we examined the effect of PAF1 and RTF1 on the co-transcriptional silencing, PAF1 and RTF1 do not appear to be involved in the co-transcriptional silencing mediated by lN-Nxf2 (Response Fig. d-i). These results suggest that Pol II regulation by the PPNP complex differs from that of the small RNA-mediated Pol II regulation model proposed previously. It may be necessary to identify key factors involved in this regulation, possibly by performing proteome analysis of the unknown factors tethered to our

reporter recruitment system. We found a decrease in the active histone H3K4me3 marks on the reporter gene even at an earlier time point (48 hpt) at which the level of H3K9me3 marks remained low (revised Extended Data Fig. 7a, Fig. 4c). Two reports about H3K4 methylation in Piwi-mediated silencing have been published. The level of H3K4 methylation on TEs is increased upon Piwi-KD. The expression of artificial piRNAs that target a reporter locus induced transcriptional silencing associated with a decrease in the active H3K4-methylation marks. In addition, previous report has shown that the depletion of LSD1, which removes H3K4-methylation marks from promoters, had significant effects on the ability of Panx to repress the reporter gene (Yu et al., 2015 *Science*). Therefore, we performed LSD1-KD and examined the effect of LSD1 on co-transcriptional silencing by the enforced tethering of Nxf2. However, the depletion of LSD1 had a limited impact on the silencing, whereas the level of *mdg1* was increased 27-fold upon LSD1-KD (revised Extended Data Fig. 7b-g). These findings together suggest that the decrease observed in H3K4me3 levels at 48 hpt in the tethering assay may not have been due to LSD1.



Response Figure.

(a) The scheme shows regions targeted by qPCR, following ChIP experiments.

(b) ChIP-qPCR analysis of RNA polymerase II (Pol II) occupancy on the reporter gene upon λN-Nxf2 tethering. Bar graph shows the occupancy relative to that of EGFP. Error bars indicate SD (n=3). OSCs were harvested at 48 or 96 hpt.

(c) Time course of Pol II occupancy on region 1, immediate upstream of 14 boxB sites, upon λN-Nxf2 tethering. OSCs were harvested at the indicated time points. Error bars indicate SD (n=3). Contrary to our expectations, Pol II accumulation by the enforced tethering of Nxf2 at 14 boxB sites was not observed.

(d) RNA levels of *PAF1* and *RTF1* were quantified by qRT-PCR upon treatment of siEGFP (control), siPAF1, or siRTF1. Error bars represent SD (n=3).

(e) RNA level of *mdg1* was quantified by qRT-PCR upon treatment of siEGFP (control), siPAF1, or siRTF1. Error bars represent SD (n=3). Depletion of PAF1 or RTF1 enhanced the silencing of *mdg1* in OSCs.

(f) Depletion of PAF1 and RTF1 slightly weakened the luciferase activities from the reporter gene with 14 boxB sites integrated in the OSC genome. Error bars represent SD (n=4).

(g) Effects of knockdown of PAF1 and RTF1 on boxB reporter activity upon λN-Nxf2 expression. Bar graph shows luciferase activity relative to that of the sample co-transfected with myc-EGFP and siEGFP (control). Error bars indicate SD (n=4). Transfection schedule of siRNA and protein expression plasmids is shown at the top of the figures. Depletion of PAF1 or RTF1 had no impact on the silencing by the enforced tethering of Nxf2 on a nascent transcript.

(h and i) Effects of exogenous expression of PAF1 and RTF1 on boxB reporter activity upon λN-Nxf2 expression. Bar graph shows luciferase activity relative to that of the sample transfected with myc-EGFP (control). Error bars indicate SD (n=4). Transfection schedule of plasmids is shown at the top of the figures. Exogenous expression of PAF1 or RTF1 did not cancel the silencing by the enforced tethering of Nxf2 on a nascent transcript.

6. *Page 9, line 23: "To reveal the role of the Nxf2-Panx complex…". It should be called as Nxf2-Panx-Piwi complex as shown in Fig. 1a-b. Rephrase.*

Thank you. We correct the sentence.

7. Ext. Data Fig. 6b: Move this interaction with Piwi to the main figure. This is critical to identify this Panx-Nxf2 complex as being linked to Piwi pathway. Provide a cartoon of Panx and Nxf2 and mark functional areas of interaction. It is too much to remember all this as the reader proceeds down the text.

Thank you. We have moved the results to the main figure accordingly, and provided a cartoon of Panx and Nxf2 with their functional areas (revised Fig. 5)

8. Page 10, lines 11-12: Mention the N-term of Panx as the interaction domain for Piwi. Looking at Ext. Data Fig. 6b, only an infinitesimal amount of Piwi is associated with Panx-Nxf2.

We have rewritten the text accordingly. The low amount of Piwi associated with Panx-Nxf2 may be because not all of the Panx-Nxf2 complexes are associated with Piwi (see also revised Extended Data Fig. 7h).

9. Page 11, line 13: Speculation about the Piwi-piRISC complex recruited to nascent RNA by base-pairing and stabilization by Nxf2 is not demonstrated in this study. The authors should use piRNA-targeted reporters to show that in these situations, depletion of Nxf2 has an impact (and can be rescued by Nxf2 full-length, but not ΔLRR).

As described above, now we have CLIP data to demonstrate that both Piwi and Nxf2 directly interact with target TE transcripts and that association of Nxf2 with target TE transcripts is Piwi-dependent (revised Fig. 6). We also showed the impact of Nxf2 depletion using piRNA-targeted reporters (revised Extended Data Fig. 5d).

10. Ext. Data Fig. 7a,b: Did they find Piwi in the IPs? A Western blot should be

shown for Piwi and include the peptide counts for Piwi in the table (if it is there).

We found Piwi in the mass spec analysis and added this result in extended figure (revised Extended Data Fig. 2b and Extended Data Table 2).
Additionally, IP of myc-Nxf2 followed by Western blot could detect Piwi (revised Extended Data Fig. 6c).

11. Page 11, line 20: The authors may want to clearly acknowledge that p15/Nxt1 is already described as associating with Nxf2 (ref. #13), at this point in the paragraph. They mention this later in p. 12, line 2. Nxt1/p15 being a co-factor of Nxf2 was not in doubt.

Thank you. We have changed writing accordingly.

12. Did the anti-Panx purification in Fig. 1a have Nxt1?

We add the results in extended figure. We performed Panx-IP using the same condition as Fig 1a, and were able to detect Nxt1 (revised Extended Data Fig. 2c).

13. Fig. 3c and final model: The role of Nxf2 in piRNA-dependent silencing is not demonstrated. How the proposed "initial phase of repression" works is not clear and beyond the proposed RNA-binding to the nascent RNA from the repressed locus, it is not clear how Nxf2 works. If it is all a question of RNA-binding, why use a Nuclear Export Factor variant? Is the use of an NXF and its co-factor Nxt1 mean anything more?

As described above, now we have CLIP data to demonstrate that both Piwi and Nxf2 directly interact with target TE transcripts and that association of Nxf2 with target TE transcripts is Piwi-dependent. Therefore, these findings suggest that the Piwi-piRNA complex has co-opted the RNA-binding activity of nuclear export variant for the regulation. We further discussed this in the manuscript. Also, please see our response to 5 regarding "initial phase of repression".

1st Editorial Decision(BP) 7th Jul 19

We believe all issues can be dealt with textually and I list key points below.

[2] Key points to be addressed textually in the revised manuscript:

Referee #2 raises a few discrepancies between you work and the other two (maybe three) manuscripts:
(i) You see an interaction of Piwi with the complex while the others do not.
→ We note that the other studies do report a weak interaction by co-IP of whole cell lysates (but don't see a Mass Spec signal), while you use nuclear fractions. This should be clearly stated in the manuscript.
(ii) The requirement and order of heterochromatin formation for repression. Eggless knockout, H3K9me3 ChIP at reporter genes differs. You propose a 2 step mechanism were transcriptional repression occurs first and then H3K9me3 deposition, while the other studies directly detect H3K9me3 (likely at later time points)
→ We note that the differences might be due to the experimental system and your study looks at fairly early time-points. This should be clearly discussed in the manuscript.
(iii) Mael - inconsistency between papers. In our view this is a discussion point and requires toning down of the relevant 2 sentences in your paper.
(iv) We also agree with your statement that the studies agree on many issues and this should also be noted (citing the published paper and preprint where appropriate):
- new complex of Nxf2-p15-Panx and (possibly) weakly Piwi
- Nxf2 function in piRNA mediated silencing not nuclear export
- Mostly on the domains of Nxf2 needed for the Panx interaction and silencing (please discuss discrepancies in detail in the paper; we understand that the Zhao et al. preprint appears not to have described the deletions in sufficient detail to be sure; since this is a preprint, we leave it to you how best to describe this issue; we agree with you that this is not necessarily an inconsistency between the studies).
(v) your studies has unique aspects that should be highlighted explicitly relative to the other published studies:
- Your study finds that the RNA-binding domain of Nxf2 is required for its role in piRNA silencing
- Your study is apparently the only one of the 4 that proposes a 2-step model, however how the initial repression occurs remains unclear. This latter point could be articulated maybe more clearly in the discussion as ref #2 seems to have missed this.
(vi) you stated in response to the referee: 'It is not clear why we should present transcriptome-wide analysis of our CLIP data in this study.' We note: please provide access to the full data-set, although a further discussion beyond the current scope of the paper will not be necessary
(vii) we agree that point 8 of ref #2 can be ignored entirely

1st Revision - authors' response 8th Jul 2019

[2] Key points to be addressed textually in the revised manuscript:

Referee #2 raises a few discrepancies between you work and the other two (maybe three) manuscripts:

(i) You see an interaction of Piwi with the complex while the others do not.
→ We note that the other studies do report a weak interaction by co-IP of whole cell lysates (but don't see a Mass Spec signal), while you use nuclear fractions. This should be clearly stated in the manuscript.

Other groups did also observe the interaction of Nxf2 with Piwi both in their original studies (Yu et

al. Science 2015; Sienski et al., Genes Dev 2015) and new studies (Fabry et al. eLIFE 2019; Batki et al., bioRxiv 2019) using IP-Western blot analysis. Although this time Hannon and colleagues state in their new eLife paper that "co-immunoprecipitation experiments detected weak but reproducible interaction between Piwi and Panx, Nxf2, and Nxt1, but not with a negative control" (page 5, line 137), we cannot simply compare band intensities between our experiments and theirs, and thus, we cannot tell what they observed in their gels is weaker than those observed in our gels. We believe that the difference is very likely due to the antibodies and IP/Western conditions-----we and other groups used different antibodies and different conditions for IP/Western blot experiments. Brennecke and Hannon and their colleagues did not observe significant levels of Piwi peptides in their shotgun MS spec analyses. We did not perform shotgun MS spec analysis, and therefore we cannot directly compare this result. Again importantly, Brennecke and Hannon too clearly observed the interaction between Piwi and Nxf2/Panx by IP-Western blot analysis. Therefore, we all agree that Nxf2/Panx interact with Piwi as long as the IP-Western blot analysis is concerned, and the final models in ours and their paper show the interaction between Piwi and Nxf2-Panx-p15 complex. Taking all these into account, we added the sentence in Discussion that reads that "We all have identified a new complex of Panx-Nxf2-p15, which associates with Piwi." (page 23, line 12).

(ii) *The requirement and order of heterochromatin formation for repression. Eggless knockout, H3K9me3 ChIP at reporter genes differs. You propose a 2 step mechanism were transcriptional repression occurs first and then H3K9me3 deposition, while the other studies directly detect H3K9me3 (likely at later time points)*

→ We note that the differences might be due experimental system and *your study looks at fairly early time-points. This should be clearly discussed in the manuscript.*

We added the sentences to clearly discuss this point (page 23, lines 15-22). Also. please see our response to *(v)*.

(iii) *Mael -inconsistency between papers. In our view this is a discussion point and requires toning down of the relevant 2 sentences in your paper.*

We have rewritten the corresponding sentence now to read "Thus, Nxf2 acts with Panx most likely downstream from or in parallel to Piwi, Gtsf1, and Mael" (page 15, line 12-13).

(iv) *We also agree with your statement that the studies agree on many issues and this should also be noted (citing the published paper and preprint where appropriate): -new complex of Nxf2-p15-Panx and (possibly) weakly Piwi -Nxf2 function in piRNA mediated silencing not nuclear export -Mostly on the domains of Nxf2 needed for the Panx interaction and silencing (please discuss discrepancies in detail in the paper; we understand that the Zhao*

et al. preprint appears not to have described the deletions in sufficient detail to be sure; since this is a preprint, we leave it to you how best to describe this issue; we agree with you that this is not necessarily an inconsistency between the studies).

We have added several sentences and citations in Discussion to read ”Four groups including ours have obtained the similar findings regarding the essential role of Nxf2 in the Piwi-piRNA pathway rather than in nuclear RNA export (Batki, Schnabl et al., 2019, Fabry, Ciabrelli et al., 2019, Zhao, Cheng et al., 2019). We all have identified a new complex of Panx-Nxf2-p15, which associates with Piwi. The UBA domain is necessary for the interaction with Panx, and the first LRR domain is needed for the regulation of TEs (Batki et al., 2019, Fabry et al., 2019).” (page 23, line 9-15). Please note that since it is difficult to refer to Zhao et al., due to the lack of information about their constructs, we focused on referring to the other papers for domain structure analysis of Nxf2.

(v) your studies has unique aspects that should be highlighted explicitly relative to the other published studies: -Your study finds that the RNA-binding domain of Nxf2 is required for its role in piRNA silencing -Your study is apparently the only one of the 4 that proposes a 2-step model, however how the initial repression occurs remains unclear. This latter point could be articulated maybe more clearly in the discussion as ref #2 seems to have missed this.

Although Brennecke group does not have CLIP data, they also suggest that RNA-binding domain of Nxf2 is required for piRNA silencing (et al., bioRxiv 2019), so we focused on the 2-step model as our unique aspect, and added some sentences to highlight this which read “Among these groups, we were the only one which was able to observe the initial step of the silencing. This is because we performed our analysis using cultured cell line OSCs, and took different timepoints after expression of Nxf2 (Fig 4). No other group has examined the regulation at the time point as early as 48 hours, and the regulation observed in 96 hours after transfection (later stages of TE silencing) was consistent with the observation by the other group (Batki et al., 2019). An important issue to be clarified next is the mechanism by which the PPNP complex initiates the silencing of TEs.” (page 23, line 15-22)

(vi) *you stated in response to the referee: ‘It is not clear why we should present transcriptome-wide analysis of our CLIP data in this study.’ We note: please provide access to the full data-set, although a further discussion beyond he current scope of the paper will not be necessary.*

We provided the accession number of GEO for full data-set access within the manuscript under “Data availability” section (page 40, line 31-) (GSE131950, it will be open to public once the manuscript is accepted).

(vii) we agree that point 8 of ref can be ignored entirely.

Thank you.

2nd Editorial Decision (BP) 9th Jul 2019

I am pleased to inform you that your manuscript is now in principle accepted for publication in the EMBO Journal, pending a number of additional changes as outlined below:

Issues related to the specific points raised previously:
(i) We appreciate that you have added on p. 23 'We all have identified a new complex of Panx-Nxf2-p15, which associates with Piwi.' but would recommend a little more detail e.g. include 'in IP/Western conditions using different antibodies, although [Brennecke ref.] and [Hannon ref.] did not observe Piwi peptides in their shotgun MS spec analyses'
(ii) OK
(iii) The apparent inconsistency between the studies regarding Mael pointed out by ref 2 was not really described on p15. We encourage a brief mention in the paper.
(iv) OK
(v) Please add to the discussion 'Although Brennecke group does not have CLIP data, they also suggest that RNA- binding domain of Nxf2 is required for piRNA silencing'
We suggest some edits to the new section:
"Among these groups, we were the only one which was able to observe the initial steps of the silencing. This is because we performed our analysis using cultured cell line OSCs, and took different timepoints after expression of Nxf2 (Fig 4). The present study is the only one to consider time points as early as 48 hours. The regulation observed at 96 hours after transfection, representing later stages of TE silencing, was consistent with the observation by Batki et al., 2019. An important issue to be clarified is the mechanism by which the PPNP complex initiates the silencing of TEs."
(vi) Ok. Please use present tense in p. 40, line 32 'All ....is deposited in the...' Please ensure the deposit is set life before publication.
For clarity, we suggest to include in the manuscript a note 'transcriptome-wide analysis of the CLIP data is available at NCBI-GEO, accession number GSE131950'

New issue raised in relation to previous reviewing process:
(i) in your response to the initial round of review dated May 24th, on p. 6/7, you had added a whole figure of data to address the effect on polII ('Response fig b-i). You conclude that 'PolII regulation by the PPNP complex differs from that of the small RNA-mediated polII regulation model proposed previously'. This data is relevant to the mechanistic insight presented by this paper - yet it appears not to be included in the current manuscript. We would like to encourage inclusion of this data or a subset of the data to address mechanistic insight. Please contact us separately to discuss this issue further prior to resubmission if this presents a challenge for you.

3rd Revision - authors' response 10th Jul 2019

Issues related to the specific points raised previously:

(i) We appreciate that you have added on p. 23 'We all have identified a new complex of Panx-Nxf2-p15, which associates with Piwi.' But would recommend a little more detail e.g. include 'in IP/Western conditions using different antibodies, although [Brennecke ref.] and [Hannon ref.] did not observe Piwi peptides in their shotgun MS spec analyses'

We have added a new sentence that reads ”Although Batki et al. and Fabry et al. did not observe significant amount of Piwi peptides in their shotgun MS analysis of Panx immunoprecipitants, they showed the interaction between Panx and Piwi in immunoprecipitation followed by western blot

analysis and their former studies (Batki et al., 2019, Fabry et al., 2019, Sienski et al., 2015, Yu et al., 2015). The inconsistency could be due to the immunoprecipitation conditions and/or antibodies used."

(ii) OK

Thank you.

(iii) The apparent inconsistency between the studies regarding Mael pointed out by ref 2 was not really described on p15. We encourage a brief mention in the paper.

We have added a new sentence that reads "Deficiency of Mael, however, does not result in decrease of H3K9me3 marks (Sienski et al., 2012), and thus Mael may act in parallel to the silencing mediated by the Piwi-Panx-Nxf2 complex; knockdown of each component of the complex all results in depletion of H3K9me3 marks."

(iv) OK

Thank you.

(v) Please add to the discussion 'Although Brennecke group does not have CLIP data, they also suggest that RNA-binding domain of Nxf2 is required for piRNA silencing'

We suggest some edits to the new section: "Among these groups, we were the only one which was able to observe the initial steps of the silencing. This is because we performed our analysis using cultured cell line OSCs, and took different timepoints after expression of Nxf2 (Fig 4). The present study is the only one to consider time points as early as 48 hours. The regulation observed at 96 hours after transfection, representing later stages of TE silencing, was consistent with the observation by Batki et al., 2019. An important issue to be clarified is the mechanism by which the PPNP complex initiates the silencing of TEs."
Thank you. We have added the edits you suggested to Discussion (page 22).

(vi) Ok. Please use present tense in p. 40, line 32 'All ....is deposited in the...' Please ensure the deposit is set life before publication. For clarity, we suggest to include in the manuscript a note 'transcriptome-wide analysis of the CLIP data is available at NCBI-GEO, accession number GSE131950'

As you suggested, we have used present tense in p. 40, line 32 'All ....is deposited in the...' Deposit is

set life now.

We also added the sentence “Transcriptome-wide CLIP data is available at NCBIGEO, accession number GSE131950.” at the end of CLIP-seq data analysis section of Methods (page 44).

New issue raised in relation to previous reviewing process:
(i) in your response to the initial round of review dated May 24th, on p. 6/7, you had added a whole figure of data to address the effect on polII ('Response fig b-i). You conclude that 'PolII regulation by the PPNP complex differs from that of the small RNA-mediated polII regulation model proposed previously'. This data is relevant to the mechanistic insight presented by this paper -yet it appears not to be included in the current manuscript. We would like to encourage inclusion of this data or a subset of the data to address mechanistic insight. Please contact us separately to discuss this issue further prior to resubmission if this presents a challenge for you.

We have added two paragraphs regarding the points you suggested in Discussion. (page 23 line 7-)

Additional Issues:
(i) ORCID ID: please include missing IDs minimally for Iwasaki and Siomi (see below)

We already did this on July 9th. Could you check your record?

(ii) Please include the Materials & Methods section after the Discussion.

We did this.

(iii) Scale bars are missing in Appendix Fig S1G, Fig S 2H, Fig 3B,G.

We added scale bars to Fig EV1G, Fig EV2H, and Fig EV4B&D.

(iv) We noticed that you solely use 'Appendix' figures. We encourage the use of collapsible 'expanded view' figures -please see details in our author guidelines at https://www.embopress.org/page/journal/14602075/authorguide#expandedvi ew

We moved Appendix fig S1-6 to Expanded view figures.

(v) As per our long-standing recommendation, we suggest inclusion of SOURCE DATA (that is minimally processed data underlying the figure panels) for key data

We provided source data for our key data (Fig 1B: Panx-IP Piwi WB and Fig 2C & Fig 2G:

Destabilization of Panx/Nxf2 upon Nxf2/Panx/p15-KD). Additionally, we have provided source data for CLIP experiments in the supplementary figure (EV Fig 6D).

(vi) Thank you for the author checklist, which will be included as a supplementary file. We suggest inclusion of the cell line information (#7) in the main paper.

We cited the original papers that described how the cell line was established in the materials and methods section. Which reads "Ovarian somatic cells (OSCs) was established in the previous studies (Niki et al., 2006, Saito et al., 2009)."

(vii) Please check the references carefully: journal names are capitalized ('Nature Communications'); no 'New York' after Science; only first authors are listed for multiauthor papers in the main body of the text (see our guide to authors, references at https://www.embopress.org/page/journal/14602075/authorguide#referencesf ormat); please use EMBO's special reference style for preprint citations in bioRxiv on p.41, line 8 (see guide to authors at https://www.embopress.org/page/journal/14602075/authorguide#preprintserv ers)

We did this.

(viii) Please supply the synopsis as a separate file

We did this.

(ix) Please include a data availability section. Please consider the following instructions: Before submitting your revision, primary datasets (and computer code, where appropriate) produced in this study need to be deposited in an appropriate public database (see http://msb.embopress.org/authorguide#dataavailability). <optional: Specifically, we would kindly ask you to provide public access to the the following datasets: -Dataset ... -Dataset ... >

We did this.

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PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Haruhiko Siomi and Yuka W. Iwasaki
Journal Submitted to: EMBO J
Manuscript Number: EMBOJ-2019-102870

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 χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

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?	Each experiment was repeated at least three times.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	NA
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	NA
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.	NA
For animal studies, include a statement about randomization even if no randomization was used.	NA
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.	NA
4.b. For animal studies, include a statement about blinding even if no blinding was done	NA
5. For every figure, are statistical tests justified as appropriate?	Yes
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Yes
Is there an estimate of variation within each group of data?	Yes, error bars are shown
Is the variance similar between the groups that are being statistically compared?	Yes

C- Reagents

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repo

http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title

http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tun

http://datadryad.org

http://figshare.com

http://www.ncbi.nlm.nih.gov/gap

http://www.ebi.ac.uk/ega

http://biomodels.net/

http://biomodels.net/miriam/
http://jjj.biochem.sun.ac.za
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).	Yes
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	OSC was obtained in the former studies (Niki et al., PNAS 2006; Saito et al., Nature 2009). myc-Nxf2 stable line of OSC was generated in this study.

* 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.	Reported in the methods section.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	NA
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.	NA

E- Human Subjects

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

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	Yes. The accession codes for data generated in this study are listed in "Data Availability" section.
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).	NA
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).	NA
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.	NA

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.	NA