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Siwi levels reversibly regulate secondary piRISC biogenesis by affecting Ago3 body morphology in Bombyx mori

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1st Editorial Decision 30th Apr 2020

Dear Mikiko,

Thank you for submitting your manuscript further defining the ping-pong cycle of piRNA biogenesis in silkworm for consideration by The EMBO Journal. We have now received three reports on your study, which are included below for your information.

As you will see, the referees appreciate the contribution the study makes towards the understanding of piRNA biogenesis in silkworm. However, they also raise several major issues that would need to be resolved in a revised version of the manuscript. In particular, it will be important to provide further support for the conclusions drawn from Northern blots using single piRNAs in Figure 1, including the binding of unloaded Siwi to Vret and Ago3 with VretL (ref#1- 2; ref#3- 2, 3; ref#2 - major point 1), at the least a quantification of multiple experiments should be provided. Furthermore, Ago3 bodies should be characterized and discussed in more detail (ref#1-4; ref2#; ref#3- 4- 11), as well as the role of Ago3 phosphorylation (ref#1-(5),6, ref#2- Fig3C,F and below; ref#3-9). In addition to these specific concerns, the referees note several instances where quantification or appropriate controls are missing, and these must be included in the revised manuscript. Moreover, the physiological context for the proposed regulatory pathway should be discussed in further detail
(see ref#2) and the text carefully revised to avoid any conclusions that are not fully supported by experimental data (ref#2's comment on liquid-liquid phase separation; ref#3- 9, 12). Finally, please also carefully respond to the other issues the reviewers raise, and potentially provide data when available.

REFEREE REPORTS

Referee #1:

This manuscript identifies the molecular function of a Tudor domain protein Vreteno in the Ping-pong cycle. The authors discover that phosphorylation of the Piwi protein Ago3 regulates its residence in the granules. I am happy to support it for EMBO J.

Minor comments.

1. Please cite the study on Vreteno in mosquitoes (Joosten, NAR, 2019). They show that Vreteno interacts with Ago3 to assemble the ping-pong complex to facilitate loading of its partner Piwi protein (like Siwi in the Bombyx system).
2. The data showing that Vreteno interacts with unloaded Siwi could be improved (Figure 1F). Northern blotting shows only one or two sequences. Deep sequencing or labelling of RNAs with radioactivity could have been used to give a complete picture. This is not essential, but would be good to have.
3. Dimerization of Vreteno is not very clear (Figure1H). Can the authors use Vret with two different tags to show that they at least self-associate into larger complexes. That should be easy to perform. Dimerziation claim will need more stringent experiments with purified proteins.
4. Is Ago3 body same as nuage? What other marker proteins can be found in this granule. Maybe they are all the same? If there is any information or suggestion, please discuss.
5. The phosphorylation of Ago3 is cool and novel. It would have been good to get a (quantified) sense of change in dot number/size etc for the 8SA and 8SE mutants. At least provide a few more pictures in the supplemental data. From the data presented it is not clear if there is a significant change in the dot size?
6. What does phosphorylation (or its absence) do to piRNA biogenesis? Please discuss.
7. The additional fragment seen in Ago3 is very similar to that previously reported in other contexts. The fact that they accumulate in the absence of Siwi, gives an idea of what it means for piRNA biogenesis. That is novel. For the sake of simplicity, indicate in a schematic/cartoon form of the piRNA biogenesis process.
8. Include a model in the main figures?

Referee #2:

In this manuscript, Sakakibara et al. introduce an intriguing novel quality control mechanism which ensures proper piRNA production in Bombyx mori (silk worm) germ cells. Moreover, this work establishes the TUDOR protein Vreteno (Vret) as an essential component in the ping-pong amplification pathway in Bombyx.
The authors show that Ago3 associates with two Vret-isoforms and that the long (L) isoform binds RNA as well as piRNA-loaded Ago3 and unloaded Siwi. The interaction between Vret-L and both PIWI proteins depends on binding of sDMA residues on the PIWI proteins and the first TUDOR domain of Vret-L. Vret colocalizes with piRNA-loaded Ago3 in so-called 'Ago3-bodies' in the perinuclear nuage and this granular localization is lost upon depletion of either of the interaction partners. To a minor extend, Siwi is also recruited to these Ago3-bodies. Siwi-depletion on the other

hand results in a significant increase in Ago3-body size, as well as phosphorylation and reduced solubility of Ago3.
Deep-sequencing of (small) RNA fragment associated with Ago3 in Siwi-depleted cells reveals the presence of long and short target RNA fragments, which largely originate from antisense piRNA precursor transcripts. These target RNAs are absent in Ago3-IP material from Vret-depleted cells, underscoring Vret's importance as an Ago3 cofactor. Further analyses show that the long target RNAs can be mapped sequentially to the genome, suggesting that they are phasing.
Altogether, this manuscript dissects a critical aspect of ping-pong amplification and uncovers an interesting mechanism in which loaded Ago3 together with its piRNA precursor target is sequestered in Ago3-bodies, when its reciprocal partner in ping-ping amplification, Siwi, is not present. Overall, the data are convincing and compelling.

Specific major concerns essential to be addressed to support the conclusions

Fig. 1D) In this panel, it is shown that production of the Siwi-associated R2Bm-piRNA is strongly affected by combined knockdown of Vret-S and -L, yet, unaffected by knockdown of Vret-L specifically. This would suggest that the effects in the combined Vret-S/-L knockdown are largely mediated by depletion of Vret-S and therefore, Vret-S is indispensable for the production of Siwi-associated piRNAs, while Vret-L is dispensable. However, the remainder of the manuscript focuses on Vret-L, while Vret-S is not analyzed further.
To strengthen these data, it is important to analyze the function of Vret-S in piRNA production, as well as its involvement in a protein complex involving Ago3 and Siwi. Judging from the supplemental table, there is a (short) stretch of sequence that is specific to Vret-S, which would make it feasible to specifically knock down Vret-S by siRNA-mediated knockdown to study its function.
Additionally, in this figure the authors base their conclusions on northern blotting analyses of a single piRNA sequence. It would be useful to provide a genome-wide view of the effects of Vret-S/-L knockdown on piRNA production through small RNA deep sequencing.

Fig. EV4D) This model suggests that the target-L RNA fragments are the pre-piRNAs that will be maturated into Siwi-bound piRNAs. If this model in correct, 5' ends of Siwi-associated piRNAs should display a strong overlap with target-L fragments. Please provide these analyses to strengthen the model that is proposed here.

Minor concerns that should be addressed

The authors propose a regulatory mechanism in which Ago3-piRISC rapidly and reversibly stalls in response to experimental modification of Siwi expression (knockdown). I suggest that the authors speculate under what natural conditions this regulatory mechanism becomes important. Are there cellular conditions in Siwi expressed at low levels? Is Siwi expression dynamically regulated? In this regard, it would also be interesting to comment on the presence of phosphorylated Ago3 in control conditions in Fig 3C (at least, I seem to notice that there is slight signal on the western)

Fig. 1A) It is not entirely clear to me how Ago3-IP/MS was performed. Was Ago3-IP material loaded on SDS-PAGE gel followed by resection of specific bands which were sent for MS; or was the entire immunoprecipitate processed for analyses by MS. Regardless, it might be useful for the community to provide information on other interacting proteins (if any) and/or to make the Mass Spec data available.

Fig. 1E) It is stated that the experiment was repeated three times. Please provide the data for those replicates (e.g. in expanded data) or show the quantification of the fold-enrichment of Ago3-

associated piRNAs in Vret-IP. Additionally, for the Ago3-IP, it is stated that IP 'was conducted under harsh conditions and so no other proteins co-purified with Ago3'. Please provide the data that support this statement (for instance by silver staining of the Ago3-IP material).

Fig. 1G) To correlate the effects of Ago3- and Vret-depletion on Siwi-associated piRNA levels, the authors stratify piRNA levels based on the effect of Ago3-depletion (decreased, unchanged, increased).
Instead of stratifying these data to three groups, it would be more informative to provide the data in a scatter plot showing this correlation (i.e. plotting Ago3-KD/WT ratios against Vret-KD/WT ratios).

Fig. 1H) Based on the data shown in this panel, the authors claim that Ago3 and Siwi interaction with Vret is mediated through the N-terminal TUDOR domain. While the association is greatly reduced by mutations in this first TUDOR domain, it is not fully abolished. Moreover, Ago3-association with Vret is also reduced slightly upon mutation of the C-terminal TUDOR domain, suggesting that there may be cooperative activity of both TUDOR domains involved in binding Ago3. It would be helpful to add a Vret mutant in which both TUDOR domains are mutated simultaneously, to establish whether both TUDOR domains may be involved in cooperative PIWI-binding.

Fig. 2H) Could the authors clarify how it is made sure that the endogenous, but not overexpressed, Ago3 is targeted by the knockdown; especially as the siRNA appear to target in the coding sequence and not the UTR (likewise for Fig. 5A-C). Also, although the authors show in Figure 2E that Ago3 knockdown is efficient, it would be nice to see an additional control to verify the efficiency of the knockdown in this particular experiment (e.g. Ago3 RNAi without overexpression of an Ago3 construct).

Fig. 3A-B) Instead of stratifying the Ago3-body size into 2 groups ('small' vs 'large'), it is more informative to show the actual granule size that was measured, to get a better idea about the spread of the data. I suggest providing this data as an expanded view figure.

Fig. 4B-C, F-K) The text states that only reads mapping to transposons were used for the analyses. Please provide information on the number of reads that are not mapping to transposons, and therefore disregarded in these analyses.

Fig. 5B) It would be nice to show whether ectopic expression of Siwi (and mutants) restored the activity of the ping-pong cycle and therefore piRNA production.

Fig. 5C) This figure shows that ectopically expressing a mutant Siwi that is unable to bind piRNAs (Siwi-KA) reduces Ago3 phosphorylation to WT-levels and restores Ago3-bodies to their normal size. Could the authors speculate on the mechanism that may be responsible for this normalization, as ping-pong amplification is still not restored to its normal activity, and there still is a functional depletion of Siwi?

-In the materials and methods, I could not find the procedure for cell fractionation (fig 3C). Please provide it.
Fig. 4C, G, H, I, J, K) The data do not seem to add up to 100%. Please double-check.

Fig. 5C) Please provide the quantification (as in Fig 5A).

Please define n.i. as used in some of the figures.

Fig. EV3A) It seems that the labelling of this figure is not correct, as there seems to be lysate in all lanes. Please correct.

Additional non-essential suggestions for improving the study (which will be at the author's/editor's discretion)

Fig. 1C) Is RNA binding by Vret-L direct, e.g. mediated by the TUDOR protein, or could it be indirect through its interaction with Ago3/Siwi? It would be interesting to repeat the CLIP-experiment, using the TUDOR-domain mutant shown in 1H, in which interaction with PIWI-proteins is greatly reduced.

Fig. 3C) Later in the manuscript (Fig 3F), it is suggested that only loaded Ago3 loaded is phosphorylated. In figure 3C, the authors show a beautiful fractionation where they separate phosphorylated from non-phosphorylated Ago3. It should be interesting to evaluate the amount of piRNA loading in Ago3 in these two fractions, to further substantiate the claim that only loaded Ago3 is phosphorylated.

Fig. 3F) There appears to be an increase in phosphorylation for the DDH-mutant. Could the authors speculate on what might be the cause of this?

- Throughout the manuscript, the authors use apo-PIWI to refer to unloaded PIWI-proteins. It would be helpful to define this term in the introduction; or alternatively, to replace with term 'unloaded PIWI-protein', which may be more intuitive.

- Figure legends. The legend for Figure 2 could be condensed as there is repetition in the description of separate panels. Also, I noticed several of concluding statements in the legends (for several of the figures). I suggest using non-conclusive figure legends instead.

- It might help readers to spend a few words clarifying why the 8SE mutant mimics phosphorylation.

- It would be interesting to analyze the presence of classical stress granule components in Ago3 bodies, to evaluate to what extent these granules differ from each other, especially given their role in reversible store RNA.

-In the discussion, the authors suggest that Ago3 bodies are generated by liquid-liquid phase separation. While this may be true, I do not think that there is direct evidence for this in the study. I suggest commenting on this.

-I would be interested in the authors' ideas about the mechanism responsible for sensing Siwi levels and the mechanism to discriminate loaded and unloaded Ago, leading to the phosphorylation of the former. Perhaps the authors could speculate on this in the discussion.

Referee #3:

In this manuscript Sakakibara et al. provide a further analysis of piRNA biogenesis in Bombyx. The group recently published a model of piRNA biogenesis in Bombyx describing the function of Papi (Tudor domain protein) and Zucchini (endonuclease) at the mitochondrial membrane for primary piRNA production (Nishida et al. Nature 2018). Here, they analyze another part of piRNA biogenesis,

the ping-pong cycle. They identify the role of Vreteno, another Tudor domain protein, together with the PIWI protein Ago3, in Ago3 RNA granules (referred to as Ago3 bodies). Ago3 bodies are part of the nuage, an RNA granule structure that localizes around germ cell nuclei and known as the place of the ping-pong cycle in other species. This shuttling between primary piRNA biogenesis at the mitochondrial membrane and ping-pong cycle in the nuage is well described in other species.
The study is of interest to further understand piRNA biogenesis in silkworm. However, in several instances the conclusions are overstated. Particularly for experiments regarding Ago3 bodies, the presented data do not strongly support the conclusions.

Major concerns:
1) piRNA biogenesis in Bombyx is rather different than in other species, therefore the title should indicate the name of the species.

2) An important point of the study is that Vret in the nuage is in complex with loaded Ago3 and unloaded Siwi. The authors conclude from data in Fig. 1E that Vret is preferentially associated with loaded Ago3, as compared with unloaded Ago3. To confirm this point the quantification of the three northern blots mentioned in the text should be provided. Here only one northern blot is shown and no quantification.
In addition, could this difference in piRNA levels between Ago3-IP and Vret-IP arise from the stringent conditions used in Ago3-IP, which might result in some level of dissociation between Ago3 and piRNAs?

3) Fig. 1G: The authors conclude that "Vret functions in Ago3-dependent secondary Siwi-piRISC production but is unnecessary for Ago3-independent primary Siwi-piRISC production". This conclusion is based on the fact that piRNAs bound to Siwi vary in the same direction in Ago3 KD and Vret KD. Analysis of piRNAs from these different samples to determine whether they are primary or secondary (with ping-pong signatures) should be performed to confirm this conclusion.

4) A key point is that Vret is required for the formation of granules that contain loaded Ago3 and unloaded Siwi: the Ago3 bodies. Overlap between Vret and Ago3 is 80%. However, overlap between Vret and Siwi is only 20% (Fig. 2A, B). Figure 2 should show and quantify the colocalization Ago3/Siwi to see if it is compatible with their model.

In Vret KD, some Siwi foci remain (Fig. 2D). The authors propose that these Siwi-positive particles are the place for Siwi-dependent Ago3-piRISC production (supposed before to be at the mitochondrial membrane). In that case, Ago3 should colocalize with Siwi in these particles. However, no Ago3 foci were formed in Vret KD.

5) In Ago3 KD (Fig. 2E, F) a low number of Vret foci and Siwi foci remain. Vret-Siwi interaction is reduced in Ago3 KD, therefore, the remaining Vret and Siwi foci are expected not to colocalize. This point should be verified.

6) In Fig. 2G, H colocalization of Ago3 with Vret would be useful to conclude about Ago3 bodies that are defined in this paper to contain Ago3 and Vret.

7) The information that Ago3 KA and DDH do interact with Ago3 WT is lacking in Fig. EV2F.

8) The point of Fig. 3 A, B is to show that in Siwi KD, Ago3 bodies become larger. Since Ago3 bodies are defined as containing Ago3 and Vret, and their function relies on both proteins, quantification of Ago3/Vret colocalization in large granules in Siwi KD is mandatory.

9) Immunostaining experiments in Fig. 3H and EV3E are lacking a control with normal cells without Ago3 overexpression. Particularly because the effect of Siwi KD on granule size (Fig. EV3E) is much lower than its effect recorded in Fig. 3A.
This part is not convincing. The conclusion "Once the Siwi level relative to the Ago3 level becomes lower by any means, the germ cells sense the situation and induce Ago3 phosphorylation and insolubilization resulting in Ago3-body enlargement" is overstated.
In addition, data obtained with unphosphorylated Ago3 (Fig. 3I) seem contradictory to this conclusion.

10) In Fig. 5A, the number of large Ago3 bodies in Siwi KD is lower than that obtained in Fig. 3A, although the staining appears quite similar in both figures. Is there an explanation for this difference?

11) The quantification is lacking in Fig. 5C. The staining of Flag-Siwi KA appears different to that of Flag-Siwi in Fig. 5A. Quantification of Ago3/Siwi colocalization in both conditions would help to determine whether Ago3 bodies are similar in both conditions.

12) The conclusion "These findings suggest that cells lacking Siwi cause Ago3 bodies to granulize to store and protect the piRNA intermediates from RNA degradation" is again very strong and not supported by experiments.
A possible way to substantiate this conclusion might be to look whether piRNA intermediates are lacking (possibly degraded) in Ago3 IP in the double Siwi KD-Vret KD when Ago3 bodies cannot form.

Referee #1:

This manuscript identifies the molecular function of a Tudor domain protein Vreteno in the Ping-pong cycle. The authors discover that phosphorylation of the Piwi protein Ago3 regulates its residence in the granules. I am happy to support it for EMBO J.

We thank referee #1 for his/her positive comment.

Minor comments

1) *Please cite the study on Vreteno in mosquitoes (Joosten, NAR, 2019). They show that Vreteno interacts with Ago3 to assemble the ping-pong complex to facilitate loading of its partner Piwi protein (like Siwi in the Bombyx system).*

The paper by Joosten *et al.* describes Veneno, which is not a homologue of Vreteno, although both proteins are Tudor proteins functioning in the piRNA pathway and indeed the names are similar.

2) *The data showing that Vreteno interacts with unloaded Siwi could be improved (Figure 1F). Northern blotting shows only one or two sequences. Deep sequencing or labelling of RNAs with radioactivity could have been used to give a complete picture. This is not essential, but would be good to have.*

We thank the suggestion raised by the referee. We isolated Siwi from the Vret complex by tandem immunoprecipitation, extracted piRNAs from the immunoprecipitate and labeled them with ^{32}P, which clearly indicated that Vret interacts with unloaded Siwi as we claimed originally. The data are included as revised Fig EV1G.

3) *Dimerization of Vreteno is not very clear (Figure1H). Can the authors use Vret with two different tags to show that they at least self-associate into larger complexes. That should be easy to perform. Dimerziation claim will need more stringent experiments with purified proteins.*

We performed the suggested experiments using two different tags. Data are provided as revised Fig EV1K. New data were also provided in revised Fig 1H.

4) *Is Ago3 body same as nuage? What other marker proteins can be found in this granule. Maybe they are all the same? If there is any information or suggestion, please discuss.*

PIWI-positive perinuclear granules in germ cells are considered to be nuage. In this regard, Ago3 bodies are nuage in BmN4 cells. However, as shown in the original manuscript, all nauge is not identical such that Ago3 bodies disappeared upon Vret

depletion but Siwi-positive nuage partly remained in the cells (original Fig 2D). Our earlier study showed that Spn-E and Vasa reside in different subsets of nuage in BmN4 cells (Nishida *et al,* 2015). Currently we do not know what (marker) proteins other than Ago3, Vret and Siwi are found in Ago3 bodies. Further analysis will answer this question.

5) *The phosphorylation of Ago3 is cool and novel. It would have been good to get a (quantified) sense of change in dot number/size etc for the 8SA and 8SE mutants. At least provide a few more pictures in the supplemental data. From the data presented it is not clear if there is a significant change in the dot size.*

Another set of cell images of the 8SA and 8SE mutants are provided as revised Fig EV 3I. We hope that this satisfied the concern of the referee.

6) *What does phosphorylation (or its absence) do to piRNA biogenesis? Please discuss.*

We are currently engaged in addressing this question and would like to report the outcome in a future paper.

7) *The additional fragment seen in Ago3 is very similar to that previously reported in other contexts. The fact that they accumulate in the absence of Siwi, gives an idea of what it means for piRNA biogenesis. That is novel. For the sake of simplicity, indicate in a schematic/cartoon form of the piRNA biogenesis process.*

Unfortunately, we do not understand the comment raised by the referee: "*The additional fragment seen in Ago3 is very similar to that previously reported in other contexts."*

8) *Include a model in the main figures?*

The model is now shown as revised Fig 6.

Referee #2:

Altogether, this manuscript dissects a critical aspect of ping-pong amplification and uncovers an interesting mechanism in which loaded Ago3 together with its piRNA precursor target is sequestered in Ago3-bodies, when its reciprocal partner in ping-ping amplification, Siwi, is not present. Overall, the data are convincing and compelling.

We thank this reviewer for his/her positive comment.

Major concerns

1) *Fig. 1D: In this panel, it is shown that production of the Siwi-associated R2Bm-piRNA is strongly affected by combined knockdown of Vret-S and -L, yet, unaffected by knockdown of Vret-L specifically. This would suggest that the effects in the combined Vret-S/-L knockdown are largely mediated by depletion of Vret-S and therefore, Vret-S is indispensable for the production of Siwi-associated piRNAs, while Vret-L is dispensable. However, the remainder of the manuscript focuses on Vret-L, while Vret-S is not analyzed further. To strengthen these data, it is important to analyze the function of Vret-S in piRNA production, as well as its involvement in a protein complex involving Ago3 and Siwi. Judging from the supplemental table, there is a (short) stretch of sequence that is specific to Vret-S, which would make it feasible to specifically knock down Vret-S by siRNA-mediated knockdown to study its function. Additionally, in this figure the authors base their conclusions on northern blotting analyses of a single piRNA sequence. It would be useful to provide a genome-wide view of the effects of Vret-S/-L knockdown on piRNA production through small RNA deep sequencing.*

We have depleted Vret-S and Vret-L individually and found that R2Bm-piRNA was still produced nicely under the conditions used (revised Fig 1D). However, when both Vret isoforms were depleted simultaneously, R2Bm-piRNA was hardly produced. Northern blotting was also performed for RT3-piRNA, Bmmar6-piRNA and R1Bm-piRNA. The results from the northern blotting were fundamentally identical to that of R2Bm-piRNA (revised Fig 1D). These results support the concept that Vret expression, irrespective of its isoform, ensures that piRNA biogenesis occurs properly.

Because Ago3 preferably bound with Vret-L (original Figs 1A and 1B), we think that it is reasonable to stay with Vret-L in Fig 1E and other figures. We have already provided data showing the effect of Vret knockdown on genome-wide piRNA sequences in the original Fig 1G.

2) *Fig. EV4D: This model suggests that the target-L RNA fragments are the pre-piRNAs that will be maturated into Siwi-bound piRNAs. If this model in correct, 5' ends of Siwi-associated piRNAs should display a strong overlap with target-L fragments. Please provide these analyses to strengthen the model that is proposed here.*

We have examined this and found that 5'-ends of Siwi-associated piRNAs displayed strong overlap with the 5'-ends of Target-L fragments. Data are provided in revised Fig EV4D.

Minor concerns

1) *The authors propose a regulatory mechanism in which Ago3-piRISC rapidly and reversibly stalls in response to experimental modification of Siwi expression (knockdown). I suggest that the authors speculate under what natural conditions this regulatory mechanism becomes important. Are there cellular conditions in Siwi expressed at low levels? Is Siwi expression dynamically regulated? In this regard, it would also be interesting to comment on the presence of phosphorylated Ago3 in control conditions in Fig 3C (at least, I seem to notice that there is slight signal on the western).*

We hardly detected a western band corresponding to phosphorylated Ago3 under normal conditions (*e.g.,* see 'Control' in original Fig 3C) as we originally noted this in the text (page 17). We do not know if at some point through gonadal development, for example, Siwi is naturally depleted. This is described in the revised Discussion section (page 27).

2) *Fig. 1A: It is not entirely clear to me how Ago3-IP/MS was performed. Was Ago3-IP material loaded on SDS-PAGE gel followed by resection of specific bands which were sent for MS; or was the entire immunoprecipitate processed for analyses by MS. Regardless, it might be useful for the community to provide information on other interacting proteins (if any) and/or to make the Mass Spec data available.*

We excised P150 and P130 bands from the gel and performed MS using these samples. This information is provided in the revised Materials and Methods section (page 37). Currently, we have no information as to what other proteins co-immunoprecipitated with Ago3. Further analysis is required to answer this question.

3) *Fig. 1E: It is stated that the experiment was repeated three times. Please provide the data for those replicates (e.g. in expanded data) or show the quantification of the fold-enrichment of Ago3-associated piRNAs in Vret-IP. Additionally, for the Ago3-IP, it is stated that IP 'was conducted under harsh conditions and so no other proteins co-purified with Ago3'. Please provide the data that support this statement (for instance by silver staining of the Ago3-IP material).*

We quantified the data of all three experiments and found that they were nearly identical to each other (*i.e.,* Ago3-IP:Flag-Vret-IP was 0.27:1). The fold-change was noted as 2.1 in the original text but is 3.7. This correction was made in the revised text (page 10). The purity of Ago3 immunoisolated in the experiment is shown in revised Fig EV1F.

4) *Fig. 1G: To correlate the effects of Ago3- and Vret-depletion on Siwi-associated piRNA levels, the authors stratify piRNA levels based on the effect of Ago3-depletion (decreased, unchanged, increased). Instead of stratifying these data to three groups, it would be more informative to provide the data in a scatter plot showing this correlation (i.e. plotting Ago3-KD/WT ratios against Vret-KD/WT ratios).*

A scatter plot is provided in revised Fig EV1I.

5) *Fig. 1H: Based on the data shown in this panel, the authors claim that Ago3 and Siwi interaction with Vret is mediated through the N-terminal TUDOR domain. While the association is greatly reduced by mutations in this first TUDOR domain, it is not fully abolished. Moreover, Ago3-association with Vret is also reduced slightly upon mutation of the C-terminal TUDOR domain, suggesting that there may be cooperative activity of both TUDOR domains involved in binding Ago3. It would be helpful to add a Vret mutant in which both TUDOR domains are mutated simultaneously, to establish whether both TUDOR domains may be involved in cooperative PIWI-binding.*

We found that a Vret mutant with mutations in both TUDOR domains was unstable and thus no conclusions could be drawn. We have repeated the experiments and provide new data in revised Fig 1H. Tud1mut weakly associated with Siwi and Ago3, whereas Tud2mut bound both to a similar extent when compared with that of WT. If the interaction were 'cooperative' as this referee suggested, the Tud2mut interaction would also be weakened; however, this was not the case. Thus, we have not changed our original statement, *"These results suggest that Vret interacts with Siwi and Ago3 through Tudor1."*

6) *Fig. 2H: Could the authors clarify how it is made sure that the endogenous, but not overexpressed, Ago3 is targeted by the knockdown; especially as the siRNA appear to target in the coding sequence and not the UTR (likewise for Fig. 5A-C). Also, although the authors show in Figure 2E that Ago3 knockdown is efficient, it would be nice to see an additional control to verify the efficiency of the knockdown in this particular experiment (e.g. Ago3 RNAi without overexpression of an Ago3 construct).*

Exogenous Ago3 proteins used in this study were all RNAi-resistant. We have added text stating in the revised Materials and Methods (page 29). The signal of endogenous Ago3 upon Ago3 depletion was negligible, as indicated in revised Fig EV2G.

7) *Fig. 3A-B: Instead of stratifying the Ago3-body size into 2 groups ('small' vs 'large'), it is more informative to show the actual granule size that was measured, to get a better idea about the spread of the data. I suggest providing this data as an expanded view figure.*

A boxplot is provided as revised Fig EV3A.

8) *Fig. 4B-C, F-K: The text states that only reads mapping to transposons were used for the analyses. Please provide information on the number of reads that are not mapping to transposons, and therefore disregarded in these analyses.*

The information was provided in the revised Materials and Methods (page 35).

9) *Fig. 5B: It would be nice to show whether ectopic expression of Siwi (and mutants) restored the activity of the ping-pong cycle and therefore piRNA production.*

The data were provided as revised Fig EV5C.

10) *Fig. 5C: This figure shows that ectopically expressing a mutant Siwi that is unable to bind piRNAs (Siwi-KA) reduces Ago3 phosphorylation to WT-levels and restores Ago3-bodies to their normal size. Could the authors speculate on the mechanism that may be responsible for this normalization, as ping-pong amplification is still not restored to its normal activity, and there still is a functional depletion of Siwi?*

The findings suggest that the aberrancy caused by Siwi loss is restored without Ago3-piRISC supply. This was noted in the original Abstract and original text (page 21).

11) *-In the materials and methods, I could not find the procedure for cell fractionation (fig 3C). Please provide it.*

The procedure was provided in the revised Materials and Methods (page 31).

12) *Fig. 4C, G, H, I, J, K: The data do not seem to add up to 100%. Please double-check.*

The original figures showed the main parts of the data. Full data are now provided as Table EV2 and EV3.

13) *Fig. 5C: Please provide the quantification (as in Fig 5A).*

We have provided the quantification in revised Fig 5C.

14) Please define n.i. as used in some of the figures.

We have defined n.i. (*i.e.,* non-immune IgG) in the revised figure legends.

15) Fig. EV3A: It seems that the labelling of this figure is not correct, as there seems to be lysate in all lanes. Please correct.

We thank the referee for raising this point. We have fixed the problem in revised Fig EV3C (original Fig EV3A).

Additional non-essential suggestions

1) *Fig. 1C: Is RNA binding by Vret-L direct, e.g. mediated by the TUDOR protein, or could it be indirect through its interaction with Ago3/Siwi? It would be interesting to repeat the CLIP-experiment, using the TUDOR-domain mutant shown in 1H, in which interaction with PIWI-proteins is greatly reduced.*

Considering the size of the protein band shown in Fig 1C and the nature of CLIP experiments, we state that Vret directly interacts with RNA.

2) *Fig. 3C: Later in the manuscript (Fig 3F), it is suggested that only loaded Ago3 loaded is phosphorylated. In figure 3C, the authors show a beautiful fractionation where they separate phosphorylated from non-phosphorylated Ago3. It should be interesting to evaluate the amount of piRNA loading in Ago3 in these two fractions, to further substantiate the claim that only loaded Ago3 is phosphorylated.*

We thank the referee for this suggestion. We will pursue these experiments in future efforts. The Ago3 KA mutant was not phosphorylated. This suggests that only loaded Ago3 is phosphorylated.

3) *Fig. 3F) There appears to be an increase in phosphorylation for the DDH-mutant. Could the authors speculate on what might be the cause of this?*

We postulate that the Ago3 kinase may be more accessible, structure wise, to the DDH mutant.

4) *Throughout the manuscript, the authors use apo-PIWI to refer to unloaded PIWI-proteins. It would be helpful to define this term in the introduction; or alternatively, to replace with term 'unloaded PIWI-protein', which may be more intuitive.*

We replaced apo-Siwi and apo-Ago3 with unloaded Siwi and unloaded Ago3, respectively, throughout the revised version of the manuscript.

5) *Figure legends. The legend for Figure 2 could be condensed as there is repetition in the description of separate panels. Also, I noticed several of concluding statements in the legends (for several of the figures). I suggest using non-conclusive figure legends instead.*

We have reduced the length of the figure legends as much as possible in the revised text by eliminating repetitive phrases and conclusive statements.

6) *It might help readers to spend a few words clarifying why the 8SE mutant mimics phosphorylation.*

We have explained the phosphorylation state of the 8SE mutant in the revised text (page 18).

7) *It would be interesting to analyze the presence of classical stress granule components in Ago3 bodies, to evaluate to what extent these granules differ from each other, especially given their role in reversible store RNA.*

We thank the referee for this suggestion. We will analyze stress granules in future work to be conducted soon as a new project.

8) *In the discussion, the authors suggest that Ago3 bodies are generated by liquid-liquid phase separation. While this may be true, I do not think that there is direct evidence for this in the study. I suggest commenting on this.*

We recently found that Ago3 bodies disappear upon 1,6-hexanediol treatment, supporting the notion that Ago3 bodies are generated through liquid-liquid phase separation. We are currently preparing another manuscript to present this finding.

9) *I would be interested in the authors' ideas about the mechanism responsible for sensing Siwi levels and the mechanism to discriminate loaded and unloaded Ago, leading to the phosphorylation of the former. Perhaps the authors could speculate on this in the discussion.*

Currently, we have no speculation for this. Identification of new Ago3 binders would help us to understand the mechanism. We are currently engaged in such experiments our laboratory.

Referee #3:

Major concerns

1) *piRNA biogenesis in Bombyx is rather different than in other species, therefore the title should indicate the name of the species.*

We wanted to add "*Bombyx"* to the title but the letter count of the title should not be more than 100 in total. Siwi is one of the PIWI proteins expressed in silkworm and so its appearance informs readers that this study is about piRNA biogenesis in *Bombyx*. Because of the letter count limitation, we have revised the title to read, "*Reversible regulation of secondary Siwi-piRISC biogenesis in response to Siwi levels in germ cells*."

2) *An important point of the study is that Vret in the nuage is in complex with loaded Ago3 and unloaded Siwi. The authors conclude from data in Fig. 1E that Vret is preferentially associated with loaded Ago3, as compared with unloaded Ago3. To confirm this point the quantification of the three northern blots mentioned in the text should be provided. Here only one northern blot is shown and no quantification. In addition, could this difference in piRNA levels between Ago3-IP and Vret-IP arise from the stringent conditions used in Ago3-IP, which might result in some level of dissociation between Ago3 and piRNAs?*

We quantified the data of all three experiments and found that they were nearly identical to each other (*i.e.,* Ago3-IP:Flag-Vret-IP was 0.27:1). The PIWI–piRNA interaction is so strong that it is hardly disturbed by the stringent (Empigen) conditions used. We also used these conditions in our previous study (Nishida *et al,* Nature 2018).

3) *Fig. 1G: The authors conclude that "Vret functions in Ago3-dependent secondary Siwi-piRISC production but is unnecessary for Ago3-independent primary Siwi-piRISC production". This conclusion is based on the fact that piRNAs bound to Siwi vary in the same direction in Ago3 KD and Vret KD. Analysis of piRNAs from these different samples to determine whether they are primary or secondary (with ping-pong signatures) should be performed to confirm this conclusion.*

The ping-pong cycle amplifies Siwi-loaded piRNAs, meaning that primary and secondary pools are not identical but overlap. This fact complicates analysis of piRNAs based on their sequences. Therefore, we used a strategy where we sorted piRNAs into decreased, unchanged, and increased groups, and compared results between Ago3 and Vret knockdowns.

4) *A key point is that Vret is required for the formation of granules that contain loaded Ago3 and unloaded Siwi: the Ago3 bodies. Overlap between Vret and Ago3 is 80%.*

However, overlap between Vret and Siwi is only 20% (Fig. 2A, B). Figure 2 should show and quantify the colocalization Ago3/Siwi to see if it is compatible with their model. In Vret KD, some Siwi foci remain (Fig. 2D). The authors propose that these Siwi-positive particles are the place for Siwi-dependent Ago3-piRISC production (supposed before to be at the mitochondrial membrane). In that case, Ago3 should colocalize with Siwi in these particles. However, no Ago3 foci were formed in Vret KD.

We performed double immunostaining of Siwi and Ago3. The data are provided in Fig EV2C. Data are basically compatible with our original model. Ago3 was hardly detected in Siwi-positive nuage in Vret-depleted cells. This suggests that Ago3 quickly comes in and out of the nuage during processing. This observation is in agreement with our earlier work that Siwi-piRISC association with unloaded Ago3 is very weak but strengthened tremendously when an ATP-hydrolysis-defective Vasa mutant is used for the assays (Nishida *et al,* Cell Reports 2015).

5) *In Ago3 KD (Fig. 2E, F) a low number of Vret foci and Siwi foci remain. Vret-Siwi interaction is reduced in Ago3 KD, therefore, the remaining Vret and Siwi foci are expected not to colocalize. This point should be verified.*

The remaining Vret and Siwi foci in Figs 2E and 2F were minor and weak and thus examining co-localization is difficult. Furthermore, the experiments were performed in Ago3-depleted cells but not Ago3-knockout cells. Thus, Siwi and Vret may co-localize but it does not mean that Vret and Siwi can interact in the absence of Ago3.

6) *In Fig. 2G, H colocalization of Ago3 with Vret would be useful to conclude about Ago3 bodies that are defined in this paper to contain Ago3 and Vret.*

We have performed the experiments and data are provided as Figs EV2G and EV2H.

7) *The information that Ago3 KA and DDH do interact with Ago3 WT is lacking in Fig. EV2F.*

We apologize upfront, but we do not understand the relevance of examining the interaction between Ago3 WT and its mutants.

8) *The point of Fig. 3 A, B is to show that in Siwi KD, Ago3 bodies become larger. Since Ago3 bodies are defined as containing Ago3 and Vret, and their function relies on both proteins, quantification of Ago3/Vret colocalization in large granules in Siwi KD is mandatory.*

We have performed immunofluorescence to show co-localization of Ago3 and Vret in Siwi-depleted cells. Data are provided as Fig EV3B.

9) *Immunostaining experiments in Fig. 3H and EV3E are lacking a control with normal cells without Ago3 overexpression. Particularly because the effect of Siwi KD on granule size (Fig. EV3E) is much lower than its effect recorded in Fig. 3A. This part is not convincing. The conclusion "Once the Siwi level relative to the Ago3 level becomes lower by any means, the germ cells sense the situation and induce Ago3 phosphorylation and insolubilization resulting in Ago3-body enlargement" is overstated. In addition, data obtained with unphosphorylated Ago3 (Fig. 3I) seem contradictory to this conclusion.*

The effect of Siwi depletion on granule size (original Fig EV3E) was lower than its effect shown in Fig 3A. This was because overexpression of Ago3 decreases the Siwi level relative to the Ago3 level. We have already noted this in the original text (page 17): *"Once the Siwi level relative to the Ago3 level becomes lower by any means, the germ cells sense the situation and induce Ago3 phosphorylation and insolubilization, resulting in Ago3-body enlargement."* Nonetheless, to avoid overstating this point, we have amended the sentence to read (page 18): *"We postulate that once the Siwi level relative to the Ago3 level becomes lower by any means, the germ cells sense the situation and induce Ago3 phosphorylation and insolubilization, resulting in Ago3-body enlargement."* Based on the data obtained with the 8SA mutant (Fig 3I), we originally noted (page 17): *"This indicates that Ago3 phosphorylation and Ago3-body enlargement are separable from each other."* We think this claim is appropriate.

10) *In Fig. 5A, the number of large Ago3 bodies in Siwi KD is lower than that obtained in Fig. 3A, although the staining appears quite similar in both figures. Is there an explanation for this difference?*

We found no such obvious difference between them. However, for clarification, we have provided other cell images in revised Fig EV5A.

11) *The quantification is lacking in Fig. 5C. The staining of Flag-Siwi KA appears different to that of Flag-Siwi in Fig. 5A. Quantification of Ago3/Siwi colocalization in both conditions would help to determine whether Ago3 bodies are similar in both conditions.*

We have quantified and provided data in revised Fig 5C.

12) The conclusion "These findings suggest that cells lacking Siwi cause Ago3 bodies to granulize to store and protect the piRNA intermediates from RNA degradation" is again very strong and not supported by experiments. A possible way to substantiate this conclusion might be to look whether piRNA intermediates are lacking (possibly degraded) in Ago3 IP in the double Siwi KD-Vret KD when Ago3 bodies cannot form.

The notion was a “suggestion” but not a “firm conclusion”. Thus, we have not made changes to the original statement in the revised manuscript. The suggested experiment is complicated and difficult to perform and even though we find piRNA intermediates absent in Ago3 IP in the Siwi/Vret double KD, we are unsure what causes the outcome and this this remains inconclusive.

2nd Editorial Decision 29th Jul 2020

Dear Mikiko,

Thank you for submitting your revised manuscript. Please apologize the delay in communicating this decision to you, which was due to delayed referee reports as well as the high number of new submission we had been receiving. We now have the reports from the original referees (see comments below) and I am pleased to say that the referees overall support publication. However, referee # 2 and, in particular referee #3, have some remaining issues that should be addressed by textual edits in a final revised version. In this version, I would also ask you to take care of a number of editorial issues that are listed in detail below. Please make any changes to the manuscript text in the attached document only using the "track changes" option. Once these remaining issues are resolved, we will be happy to formally accept the manuscript for publication.

REFEREE REPORTS

Referee #1:

The authors have addressed all my concerns in the revised manuscript. It is now ready for publication.

Referee #2:

Dear editor,

I reviewed the original manuscript by Nishida et al. The authors have successfully addressed all my comments and I am happy to support his work for EMBO J.

There is one minor point that is still unclear to me and may be addressed in the final version of this manuscript:

In response to one of my earlier comments, the authors now state in their methods section that 'Exogenous Ago3 proteins used in this study were all RNAi-resistant'. It is still not fully clear to me how this RNAi-resistance was achieved. Was the coding sequence altered in such a way that it is no longer targetable by the siRNA used for knockdown of endogenous Ago3? If so, it would be helpful if the authors comment on this briefly in the methods section.

Kind regards

Referee #3:

In this revised version, the authors have addressed most of my concerns. A number of points remain to be addressed though.

Remaining concerns.
4) The authors have performed the Ago3/Siwi colocalization as requested. They describe this results as follows (p. 13)."We also examined the overlapping status of Siwi and Ago3 (Fig EV2C), which was comparable with the data shown in Figs 2A and 2B." Vret/Ago3 overlap is 80.7% (Fig. 2A); Vret/Siwi overlap is 19.7% (Fig. 2B); Ago3/Siwi overlap is 38% (Fig. EV2C). Therefore the overlaps are different and cannot be considered as comparable. It would be useful to acknowledge this point and provide a possible interpretation.

7) On p. 15 the authors indicate "Both the KA and DDH mutants interacted with Vret and Ago3 WT (Fig EV2I)" hence my remark.

9) The part relating to Ago3 bodies in the presence of Ago3 overexpression (p. 17) remains obscure. Ago3 overexpression alone has no effect on the size of Ago3 bodies. Or it may have a slight effect, but in that case it should be quantified with the right control (without Ago3 overexpression). For Ago3 overexpression with Siwi KD, if the authors mean to show that Ago3 body enlargement is weaker in this condition than in Siwi KD alone (as I understand from their response in the letter), this should be quantified with both conditions in the same experiment. But this is not what is described on p. 17.
Finally, I don't see how data in Fig. 3I showing that unphosphorylated Ago3-8SA forms large Ago3 bodies, agree with their model "once the Siwi level relative to the Ago3 level becomes lower by any means, the germ cells sense the situation and induce Ago3 phosphorylation and insolubilization, resulting in Ago3-body enlargement."
The rationale for these experiments should be clarified and their results/conclusions better explained.

10) and 11) Siwi KD produces 35%, 17% and 8% of large granules in Figs. 3A, 5A and 5C, respectively. This indicates a large variability between experiments. Is the rescue with Flag-Siwi and Flag-Siwi KA statistically significant?

Referee #2:

In response to one of my earlier comments, the authors now state in their methods section that 'Exogenous Ago3 proteins used in this study were all RNAi-resistant'. It is still not fully clear to me how this RNAi-resistance was achieved. Was the coding sequence altered in such a way that it is no longer targetable by the siRNA used for knockdown of endogenous Ago3? If so, it would be helpful if the authors comment on this briefly in the methods section.

"*RNAi-resistant*" means that the cDNAs encoding exogenous Ago3 proteins, both WT and mutants, were mutated to avoid Ago3 siRNA targeting, which was, however, effective for repressing endogenous Ago3. Therefore, as intended, exogenous Ago3 proteins were expressed in BmN4 cells. To make this facet clearer, we have added a sentence in the revised Materials and Methods section (page 29) that reads "*For this, the cDNAs were mutated to be insensitive to Ago3 siRNA. The sequences of the oligos used for the mutagenesis are presented in Table EV4.*"

Referee #3:

Remaining concerns.

4) The authors have performed the Ago3/Siwi colocalization as requested. They describe this results as follows (p. 13)."We also examined the overlapping status of Siwi and Ago3 (Fig EV2C), which was comparable with the data shown in Figs 2A and 2B." Vret/Ago3 overlap is 80.7% (Fig. 2A); Vret/Siwi overlap is 19.7% (Fig. 2B); Ago3/Siwi overlap is 38% (Fig. EV2C). Therefore the overlaps are different and cannot be considered as comparable. It would be useful to acknowledge this point and provide a possible interpretation.

Nuage is non-membranous organelles formed through liquid-liquid phase separation, famous for its fluidity and dynamicity *in vivo*. Furthermore, the numbers, sizes, and spatial relationships of nuage are not uniform in each cell. We have also realized that nuage in BmN4 cells can be divided into, at least, three types by their residences and functions (Namba and Siomi *et al.* in preparation). In each type of nuage, multiple reactions for producing piRNAs take place and over time their components change because of their continuous movement '*in and out of the organelles*'. Because of these unique characteristics of nuage and the nature of the experiments, where we had to set certain parameters to count the nuage signals numerically regardless of the natures of the organelles, the overlap in percentages of Vret/Ago3, Vret/Siwi and Ago3/Siwi may not be represented by a simple '*addition and subtraction*' formula. However, to respond to the concern raised by the referee, we have modified the original sentence to read (page 13), "*which was <u>reasonably</u> comparable to the data shown in Figs 2A and 2B."* We hope that our interpretation above is reasonable and that the amendment we made satisfies the concern of the referee.

7) On p. 15 the authors indicate "Both the KA and DDH mutants interacted with Vret and Ago3 WT (Fig EV2I)" hence my remark.

We are very sorry for our misunderstanding. We actually meant "*Both the KA and DDH mutants interacted with Vret <u>as well as</u> Ago3 WT (Fig EV2I)."* We have corrected this error in the newly revised text (page 15).

9) The part relating to Ago3 bodies in the presence of Ago3 overexpression (p. 17) remains obscure. Ago3 overexpression alone has no effect on the size of Ago3 bodies. Or it may have a slight effect, but in that case it should be quantified with the right control (without Ago3 overexpression). For Ago3 overexpression with Siwi KD, if the authors mean to show that Ago3 body enlargement is weaker in this condition than in Siwi KD alone (as I

understand from their response in the letter), this should be quantified with both conditions in the same experiment. But this is not what is described on p. 17.

The degree of phosphorylation of Flag-Ago3 WT in the control cells was very subtle (Control in Fig 3F) such that the enlargement of the bodies was not obvious (upper cell images in Fig 3H). In Fig 3A, we used the anti-Ago3 antibody to show body enlargement. In Fig 3H (lower cell images), we used the anti-Flag antibody to show body enlargement. Thus, it is difficult to compare them in the same experiments.

Finally, I don't see how data in Fig. 3I showing that unphosphorylated Ago3-8SA forms large Ago3 bodies, agree with their model "once the Siwi level relative to the Ago3 level becomes lower by any means, the germ cells sense the situation and induce Ago3 phosphorylation and insolubilization, resulting in Ago3-body enlargement."
The rationale for these experiments should be clarified and their results/conclusions better explained.

We postulated "*once the Siwi level relative to the Ago3 level becomes lower by any means, the germ cells sense the situation and induce Ago3 phosphorylation and insolubilization, resulting in Ago3-body enlargement*" based on the results shown before Fig 3I. We then obtained the data shown in Figs 3I, EV3H and EV3I and based on these, we newly claimed that Ago3 phosphorylation and Ago3-body enlargement are separable from each other. This aspect of our work was already written clearly in the revised text (page 18).

10) and 11) Siwi KD produces 35%, 17% and 8% of large granules in Figs. 3A, 5A and 5C, respectively. This indicates a large variability between experiments. Is the rescue with Flag-Siwi and Flag-Siwi KA statistically significant?

We admit that there are slight variabilities in the percentages. However, we believe that it is reasonable because the granule enlargement happens inconsistently in each cell: if higher numbers of small granules happen to merge and become a large granule, it consequently reduces the ratio of small granules to large granules. We applied the Fisher's exact test, which indicated that rescue with Flag-Siwi and Flag-Siwi KA was statistically significant. We added a sentence in both figure legends that reads "*A significant difference was found plus/minus Flag-Siwi expression by the Fisher's exact test ($P < 0.05$).*"

Accepted **8th Aug 2020**

Thank you again for submitting the final revised version of your manuscript and sending the source data. I am pleased to inform you that we have now accepted it for publication in The EMBO Journal.

EMBO PRESS

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: The EMBO Journal
Manuscript Number: EMBOJ-2020-105130

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 iustified
- ➔ 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 $\chi 2$ 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.	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.	No.
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.	No.
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. Shapiro-Wilk test to check nomal distribution.
Is there an estimate of variation within each group of data?	Yes.

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Is the variance similar between the groups that are being statistically compared?	Yes.

C- Reagents

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).	Anti-Siwi, Ago3 and Vret antibodies were produced from immunized mouse, respectively. Anti-myc (catalog number: 9E10), Anti-DDDDK-tag (Anti-Flag antibody) (catalog number: FLA-1) and anti-Tubulin antibodies (catalog number: E7) were purchased from DSHB, MLB and DSHB, respectively. Y12 antibody was gifted from Dr. Gideon Dreyfuss.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	BmN4 cells were gifted from National Institute of Agrobiological Sciences (NIAS). BmN4 cells were not tested for mycoplasma contamination.

* 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.	NA
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
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15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
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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	RNA-Seq data: Gene Expression Omnibus GSE150444.
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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

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