EMBO reports

Dynamic subcellular compartmentalization ensures fidelity of piRNA biogenesis in silkworms

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

1st Editorial Decision 10th Aug 2020

Dear Yuki,

Thank you for the submission of your manuscript to EMBO reports. We have now received the full set of referee reports that is pasted below.

As you will see, all referees note that the study is rather descriptive and that the functional relevance of subcellular piRNA pathway component localization remains unclear. They also rate the novelty and general interest of the findings "medium" or "low" in the manuscript summary table that is directly sent to the editor. Importantly, all referees point out that the study is based on overexpression experiments, which might confound the results. Given the somewhat lukewarm interest, I think that in order to consider the manuscript for publication here, all referee concerns will need to be addressed experimentally, especially the over- and co-expression concerns. If you prefer, we can also talk about the revisions on the phone or via video chat.

In case you decide to embark on such a revision, I would like to invite you to revise your manuscript with the understanding that the referee concerns must be fully addressed and their suggestions taken on board. Please address all referee concerns in a complete point-by-point response. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of major revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions. Please contact us if a 3-months time frame is not sufficient for the revisions so that we can discuss this further.

You can either publish the study as a short report or as a full article. For short reports, the revised manuscript should not exceed 27,000 characters (including spaces but excluding materials & methods and references) and 5 main plus 5 expanded view figures. The results and discussion sections must further be combined, which will help to shorten the manuscript text by eliminating some redundancy that is inevitable when discussing the same experiments twice. For a normal article there are no length limitations, but it should have more than 5 main figures and the results and discussion sections must be separate. In both cases, the entire materials and methods should be included in the main manuscript file.

Regarding data quantification, please specify the number "n" for how many independent experiments were performed, the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values in the respective figure legends. This information must be provided in the figure legends. Please also include scale bars in all microscopy images.

IMPORTANT NOTE: we perform an initial quality control of all revised manuscripts before re-review. Your manuscript will FAIL this control and the handling will be DELAYED if the following APPLIES:
1) A data availability section providing access to data deposited in public databases is missing. If you have not deposited any data, please add a sentence to the data availability section that explains that.
2) Your manuscript contains statistics and error bars based on n=2. Please use scatter blots in these cases. No statistics should be calculated if n=2.

When submitting your revised manuscript, please carefully review the instructions that follow below. Failure to include requested items will delay the evaluation of your revision.

1) a .docx formatted version of the manuscript text (including legends for main figures, EV figures and tables). Please make sure that the changes are highlighted to be clearly visible.

2) individual production quality figure files as .eps, .tif, .jpg (one file per figure).
See https://wol-prod-cdn.literatumonline.com/pb-assets/embo-site/EMBOPress_Figure_Guidelines_061115-1561436025777.pdf for more info on how to prepare your figures.

3) We replaced Supplementary Information with Expanded View (EV) Figures and Tables that are collapsible/expandable online. A maximum of 5 EV Figures can be typeset. EV Figures should be cited as 'Figure EV1, Figure EV2" etc... in the text and their respective legends should be included in the main text after the legends of regular figures.

- For the figures that you do NOT wish to display as Expanded View figures, they should be bundled together with their legends in a single PDF file called *Appendix*, which should start with a short Table of Content including page numbers. Appendix figures should be referred to in the main text as: "Appendix Figure S1, Appendix Figure S2" etc. See detailed instructions regarding expanded view here:
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- Additional Tables/Datasets should be labeled and referred to as Table EV1, Dataset EV1, etc. Legends have to be provided in a separate tab in case of .xls files. Alternatively, the legend can be supplied as a separate text file (README) and zipped together with the Table/Dataset file.

4) a .docx formatted letter INCLUDING the reviewers' reports and your detailed point-by-point responses to their comments. As part of the EMBO Press transparent editorial process, the point-by-point response is part of the Review Process File (RPF), which will be published alongside your paper.

5) a complete author checklist, which you can download from our author guidelines <https://www.embopress.org/page/journal/14693178/authorguide>. Please insert information in the checklist that is also reflected in the manuscript. The completed author checklist will also be part of the RPF.

6) Please note that all corresponding authors are required to supply an ORCID ID for their name upon submission of a revised manuscript (<https://orcid.org/>). Please find instructions on how to link your ORCID ID to your account in our manuscript tracking system in our Author guidelines <https://www.embopress.org/page/journal/14693178/authorguide#authorshipguidelines>

7) Before submitting your revision, primary datasets produced in this study need to be deposited in an appropriate public database (see https://www.embopress.org/page/journal/14693178/authorguide#datadeposition). Please remember to provide a reviewer password if the datasets are not yet public. The accession numbers and database should be listed in a formal "Data Availability" section placed after Materials & Method (see also https://www.embopress.org/page/journal/14693178/authorguide#datadeposition). Please note that the Data Availability Section is restricted to new primary data that are part of this study. * Note - All links should resolve to a page where the data can be accessed. *

If your study has not produced novel datasets, please mention this fact in the Data Availability Section.

8) We would also encourage you to include the source data for figure panels that show essential data. Numerical data should be provided as individual .xls or .csv files (including a tab describing the data). For blots or microscopy, uncropped images should be submitted (using a zip archive if multiple images need to be supplied for one panel). Additional information on source data and instruction on how to label the files are available at <https://www.embopress.org/page/journal/14693178/authorguide#sourcedata>.

9) Our journal also encourages inclusion of *data citations in the reference list* to directly cite datasets that were re-used and obtained from public databases. Data citations in the article text are distinct from normal bibliographical citations and should directly link to the database records from which the data can be accessed. In the main text, data citations are formatted as follows: "Data ref: Smith et al, 2001" or "Data ref: NCBI Sequence Read Archive PRJNA342805, 2017". In the Reference list, data citations must be labeled with "[DATASET]". A data reference must provide the database name, accession number/identifiers and a resolvable link to the landing page from which the data can be accessed at the end of the reference. Further instructions are available at https://www.embopress.org/page/journal/14693178/authorguide#referencesformat

We would also welcome the submission of cover suggestions, or motifs to be used by our Graphics Illustrator in designing a cover.

As part of the EMBO publication's Transparent Editorial Process, EMBO reports publishes online a Review Process File (RPF) to accompany accepted manuscripts. This File will be published in conjunction with your paper and will include the referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript.

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I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

Best wishes,
Esther

Esther Schnapp, PhD
Senior Editor
EMBO reports

Referee #1:

In the manuscript entitled 'Dynamic subcellular compartmentalization ensures the fidelity of piRNA biogenesis in silkworms', Chung and colleagues perform co-localization analyses of piRNA pathway proteins in the silkworm cell line BmN4. BmN4 cells express two cytoplasmic PIWI proteins that

engage in post-transcriptional silencing of transposons and ping-pong processing of piRNAs. Ping-pong biogenesis of piRNAs requires the germline specific helicase Vasa/Ddx4 and is thought to take place in electron-dense perinuclear and inter-mitochondrial RNA-protein aggregates called nuage, a membrane-less organelle. Here, the authors show that a catalytical dead mutant of SIWI (Siwi-D670A) does not localize to nuage like the wild type protein. Siwi-D670A mis-localizes to processing bodies (P-bodies), a cytoplasmic site of protein-RNA aggregates and potential RNA degradation. Loss of coordinated target degradation and piRNA production in a catalytical dead mutant of the RNA helicase Vasa/DDx4 also resulted in accumulation of SIWI in piP-bodies. Furthermore, the piRNA pathway components BmSpnE and BmQin localized to piP-bodies already in the wild type situation. The authors move on to investigate the biophysical state of these piP-bodies and show that these are solid aggregates and not liquid phases. Next, the authors describe that inhibition of ping-pong either due to overexpression of catalytical dead SIWI or catalytical dead BmVasa result in loss of transposon-derived piRNAs and a relative increase in mRNA-derived piRNAs. Finally, the authors claim that dynamic changes in the subcellular localization of piRNA pathway components re-assign mRNAs to become piRNA precursors and thus influence the fidelity of piRNA biogenesis. While the authors present extensive subcellular localization studies, the functional relevance of the observed changes remains purely speculative. I do not recommend this manuscript for publication without major revision.

Major points:

1. Dynamic compartmentalization or static endpoint for non-functional proteins? The authors show focal enrichment of BmSpnE, BmQin and non-functional SIWI in P-bodies in BmN4 cells. Based on these observations, the authors propose a dynamic compartmentalization. As alternative explanations, the authors should consider and discuss the following possibility: All presented experiments seem to be based on transient over-expression experiments in tissue culture. The authors should test, if the endogenous proteins behave the same way. If this is impossible due to lack of antibodies and the inability to tag the endogenous gene by CRISPR-editing, the authors could titrate the amount of transfected plasmid, test the subcellular localization of the transgene at different time points after transfection and eliminate the possibility that the transfection procedure puts the cells in a stressed state. Transfection stress could induce stress granule, which have been shown to interact with P-bodies and might change the behaviour of these cytoplasmic aggregates.

2. Pi-P-bodies were defined by the presence of MAELSTROM (MAEL) in mouse. The presence of MAEL differentiates piP-bodies from pi-bodies (likely part of nuage) in mice and loss of MAEL eliminates the localization of PIWI proteins to piP-bodies. To investigate, if the herein observed cytoplasmic granules are indeed piP-bodies, the authors should test for the presence of MAEL and for changes in the piP-body localization of piRNA pathway components upon knock-down or knock-out of MAEL (whatever is experimentally possible in the BmN4 cell culture system).

3. Changes in piRNA composition? The authors propose that upon overexpression of catalytical dead SIWI (and thus inhibition of ping-pong), piRNAs that are derived from transposons are reduced while piRNAs derived from mRNAs are increased. It is not clear from the Figure legend or the methods section, how piRNA sequencing data were normalized for this analysis. This is important, because one expects a dramatic reduction of all piRNAs upon inhibition of ping-pong. The overall reduction of piRNAs is visible in small RNA seq data, when they are correctly normalized to an independent stable population of small RNAs like miRNAs. In contrast, normalization to the total data set would artificially inflate the fraction of low-abundant background fragments and is not appropriate for this analysis. Inappropriate normalization could result in the perception of an increase in mRNA-derived small RNA fragments. However, this increase would only be relative to

the total population, which might be dramatically reduced. The authors should clarify to avoid misinterpretation. Finally, the authors should immune-precipitate SIWI and BmAgo3 and analyze associated small RNAs to examine, if the observed RNA fragments are indeed bona-fide piRNAs.

4. Integration of recent data by their own group and others. Mechanisms of piRNA biogenesis have been extensively studied over the past years. The authors should relate their current findings to existing data: (4.1.) The helicase Armitage couples piRNA amplification in nuage and piRNA production on the surface of mitochondria (Ge et el., Mol Cell 2019, PMID: 31076285). The authors should examine the subcellular location of Armi in their experimental set up and integrate their findings in the established framework. (4.2.) Phased piRNA biogenesis has been shown to occur upon induction of piRNA processing by ping-pong piRNAs (Mohn et al., Science 2015, PMID: 25977553; Han et al., Science 2015, PMID: 25977554). The authors should probe, if changes in subcellular localization of piRNA pathway components affect coupling of ping-pong to the primary piRNA processing machinery, and if changes in this coupling and phasing could explain the proposed differences in piRNA composition. (4.3.) The authors' group has recently identified consensus motifs that determine piRNA production in BmN4 cells (Izumi et al., Nature 2020, PMID: 31996847). The authors could use their existing small RNA sequencing data or additional bona-fide piRNA data (suggested in point 2) to characterize potential effects on the consensus motif. Does this consensus motif discriminate the processed mRNAs from unprocessed mRNAs?

Referee #2:

Chung et al. present studies on the subcellular localization of piRNA pathway components in the Bombyx mori system; to be precise in cultured BmN4 cells. They find evidence for an organization that has been described in mice: an interplay between the germ cell specific nuage and P-bodies that contain some piRNA factors (hence are named piP bodies). A role for Siwi slicing in separating nuage and piP bodies is described, as well as a function of RNA helicase Vasa. Finally, a function of such de-mixing in discrimination between self and non-self is proposed.

These studies are novel for the BmN4 system, but largely parallels published work on mice. Fact that BmN4 cells do show this parallel, while Drosophila does not appear to do so, is an interesting finding. In addition, the proposed role for Siwi slicing and the role of de-mixing in self-identification are intriguing. However, the presented studies are very descriptive and do not really prove these points. I would still think EMBO reports would be a proper venue to publish this, but the authors need to tune down the strength of their wording. In particular, the fact that they (by necessity) use overexpression to look at sub-cellular localization is a factor that may convolute the results, and this should be clearly acknowledged. Finally, Figure 5 needs to be improved by a different experimental design that takes out endogenous Siwi activity, in order to draw the presented conclusion.

Some specifics that would improve the manuscript:
Figure 1:
Minor: Validation of microscopy (co-localization): BmDcp2 immunoprecipitation studies followed by Western Blot on otherP body components to validate presence of BmDcp2 in P-bodies.
More serious concern: how do we know if Dcp2 overexpression does not affect its localization? Are there antibodies to known P body components that can be used to assess this?

Is the localization of Siwi to piP bodies dependent on its loading status? This can be easily tested

by generating a piRNA-loading deficient mutant.

Figure 2:
2C: It should be checked if SpnE and Qin are expressed at similar levels in both Siwi wt and D670A transfected cells.

2D: The depletion of dsBmQin or BmSpnE leads to decreased co-localization between Siwi-D670A and BmDcp2. Knockdowns should be verified on endogenous levels either by WB or immunostaining. Also, in M&M details are lacking on the dsRNA treatment. How much dsRNA was transfected, for how long and how often?

Figure 3:
3A +B: When Siwi is depleted BmQin and BmSpnE are not found in piP bodies, do they now co-localize with BmVasa (ie nuage) instead?
3C: Siwi is not found in nuage when Vasa is depleted. This can simply be due to the fact that the overexpressed Siwi does not get loaded under Vasa kd conditions (see comment on Figure 1).

Figure 4:
"We could also confirm the colocalization of BmVasa-E339Q with Siwi, BmAGO3 and BmQin in our hands)data not shown)". There is enough space to show this in EV2. Would be good to show as it would indicate that BmQin shuttles between piP bodies and nuage as well.

"We propose that the slicer activity of Siwi and the ATPase activity of BmVasa act together to keep remodeling RNP complexes...". This statement is based on the observation that with overexpression of the respective mutants bigger foci are formed. Even though the term 'proposed' is rather weak, I feel that more careful wording would be warranted.

Figure 5:
How were TEs defined. Please describe this.
More importantly, the whole set-up is in presence of wild-type endogenous Siwi. This may imply that TE transcripts are properly processed. Any additional production of piRNAs would therefore by definition need to come from endogenous (self) genes. Hence, a proper experiment to assess if self-recognition is affected would be to first deplete endogenous Siwi and to then come in with the expression of tagged versions of specific mutants. Without such more controlled experiments, the conclusion drawn from this figure ("We concluded that the subcellular compartmentalization of the silkworm piRNA pathway is critical for self-non self-discrimination during piRNA precursor acquisition") is not valid. (BTW, Figure 5G is not present).

Referee #3:

The manuscript by Chung et al. deals with the problem of Piwi-interacting (pi)iRNA biogenesis in the silkworm. Specifically, this paper focuses on the dynamics of the cytoplasmic distribution of piRNA pathway proteins and the relationship to piRNA biogenesis. Prior studies in flies and mammals have identified various forms of perinuclear "nuage" and cytoplasmic piRNA protein aggregates/condensates in germ cells. Some such aggregates contain proteins characteristic of

processing bodies (P-bodies), raising the possibility of crosstalk between piRNA and RNA regulatory pathways in germ cells. However, despite the evident alterations in piRNA protein localization patterns in piRNA deficient germ cells, the functional meaning and significance of this co-localization are not well understood. The present study uses a relative newcomer in the piRNA field, silkworm Bombyx mori, to gain insights into the relationship of various cytoplasmic aggregates and piRNA biogenesis and functions.

The study uses live-cell imagining in cultured BmN4 cells, which have a fully functional piRNA biogenesis pathway known as the ping-pong cycle. To follow protein localization, the authors overexpressed proteins of interest using the epitope-tagging strategy.
Epitope-tagged wild-type Siwi and BmAgo3 proteins, the primary effector proteins in the piRNA pathway, exhibited comparable localization patterns with BmVasa in nuage. Using this experimental setup, the authors explore the localization of piRNA and P-body proteins and the dependency of observed localization patterns on each other as determined using gene-specific knockdowns in BmN4 cells.

This study's main observations agree strongly with prior studies in several model organisms and provide further mechanistic links between the subcellular cytoplasmic localization of piRNA proteins and piRNA biogenesis. This work also suggests the critical role of proper localization of piRNA proteins for the correct targeting of transposons rather than genic mRNAs.

Overall, this is a well designed and executed study that certainly deserves being published. There are no serious experimental flaws or significant sticking points. The two minor points are as follows:

1. All observations were obtained on the background of wild-type piRNA proteins. Consequently, the presence of wild-type proteins impacts piRNA biogenesis (as is suggested by the authors based on small RNA sequencing data). It might also impede or alter the localization of ectopically expressed epitope-tagged proteins. Similarly, the presence of mutant proteins may interfere with the normal functioning of wild type proteins. Consequently, some observations might reflect actual and other artifactual outcomes of the experimental setup. Can the authors acknowledge and discuss these shortcomings when introducing their experimental system?

2. It would be beneficial if the authors stated the specific advancement in our understanding of the piRNA system that this study provides. The authors include pictures interpreting and summarizing some of their findings. Still, I wonder if the final model could depict the generalized view of cytoplasmic compartmentalization and functionality of the piRNA system across model organisms.

Referee #1:

In the manuscript entitled 'Dynamic subcellular compartmentalization ensures the fidelity of piRNA biogenesis in silkworms', Chung and colleagues perform co-localization analyses of piRNA pathway proteins in the silkworm cell line BmN4. BmN4 cells express two cytoplasmic PIWI proteins that engage in post-transcriptional silencing of transposons and ping-pong processing of piRNAs. Ping-pong biogenesis of piRNAs requires the germline specific helicase Vasa/Ddx4 and is thought to take place in electron-dense perinuclear and inter-mitochondrial RNA-protein aggregates called nuage, a membrane-less organelle. Here, the authors show that a catalytical dead mutant of SIWI (Siwi-D670A) does not localize to nuage like the wild type protein. Siwi-D670A mis-localizes to processing bodies (P-bodies), a cytoplasmic site of protein-RNA aggregates and potential RNA degradation. Loss of coordinated target degradation and piRNA production in a catalytical dead mutant of the RNA helicase Vasa/DDx4 also resulted in accumulation of SIWI in piP-bodies. Furthermore, the piRNA pathway components BmSpnE and BmQin localized to piP-bodies already in the wild type situation. The authors move on to investigate the biophysical state of these piP-bodies and show that these are solid aggregates and not liquid phases. Next, the authors describe that inhibition of ping-pong either due to overexpression of catalytical dead SIWI or catalytical dead BmVasa result in loss of transposon-derived piRNAs and a relative increase in mRNA-derived piRNAs. Finally, the authors claim that dynamic changes in the subcellular localization of piRNA pathway components re-assign mRNAs to become piRNA precursors and thus influence the fidelity of piRNA biogenesis. While the authors present extensive subcellular localization studies, the functional relevance of the observed changes remains purely speculative. I do not recommend this manuscript for publication without major revision.

We thank the Referee for the valuable comments.

Major points:

1. Dynamic compartmentalization or static endpoint for non-functional proteins? The authors show focal enrichment of BmSpnE, BmQin and non-functional SIWI in P-bodies in BmN4 cells. Based on these observations, the authors propose a dynamic compartmentalization. As alternative explanations, the authors should consider and discuss the following possibility: All presented experiments seem to be based on transient over-expression experiments in tissue culture. The authors should test, if the endogenous proteins behave the same way. If this is impossible due to lack of antibodies and the inability to tag the endogenous gene by CRISPR-editing, the authors could titrate the amount of transfected plasmid, test the subcellular localization of the transgene at different time points after transfection and eliminate the possibility that the transfection procedure puts the cells in a stressed state. Transfection stress could induce stress granule, which have been shown to interact with P-bodies and might change the behaviour of these cytoplasmic aggregates.

We thank the Referee for the critical feedback regarding the overexpression experiments. To validate our original co-localization data obtained with the baculovirus-derived OpIE2 promoter, we performed western blotting with antibodies against endogenous proteins and compared the expression levels between the exogenously expressed proteins and their endogenous counterparts. We confirmed that all exogenous proteins were expressed at a level less than or comparable to endogenous proteins (Fig EV2A and B). To further avoid a potential artifact of overexpression, we replaced the OpIE2 promoter with the P_{TRE3G} Tet-On inducible promoter, with which the expression level can be adjusted by Doxycycline concentration. All proteins from the Tet-On constructs were expressed at a level markedly lower than their endogenous counterparts (Fig EV2A and B). Still, we observed co-localization of Siwi-D670A, BmSpnE, BmQin with piP-bodies and co-aggregation between Siwi-D670A and BmVasa-E339Q (Fig EV2C). Thus, the co-localization patterns we originally observed with OpIE2 promoter are reproducible with expression levels much lower than

endogenous proteins. These results are now included to the manuscript. Please refer to Extended View Figure 2.

Regarding the concern about transfection stress, we followed the Referee's suggestion and performed a time-course experiment with medium change at day 4 post-transfection. As shown in Figure R1 below, the localization of Tet-On-driven Siwi-D670A to piP-bodies showed the same patterns on day 4, 7 and 10 post-transfection, suggesting that the originally observed colocalization pattern is unlikely to be caused by transient transfection stress.

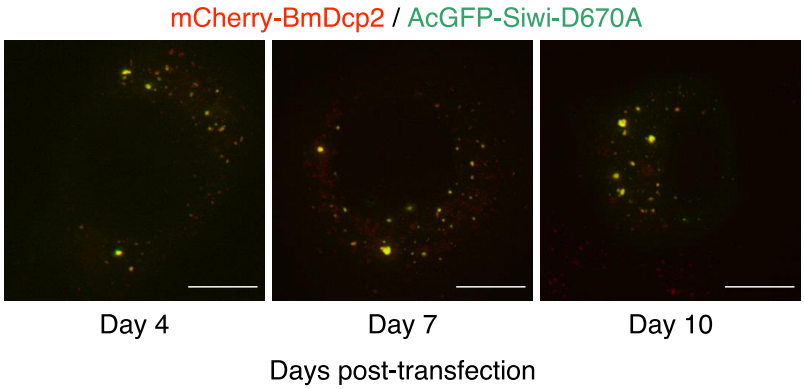

Fig R1. Time-course analysis of colocalization between BmDcp2 and Siwi-D670A slicer mutant. Expression of mCherry-BmDcp2 and AcGFP-Siwi-D670A were driven by an OpIE2 promoter and a Tet-On promoter respectively. Scale bar, 10 μm.

2. Pi-P-bodies were defined by the presence of MAELSTROM (MAEL) in mouse. The presence of MAEL differentiates piP-bodies from pi-bodies (likely part of nuage) in mice and loss of MAEL eliminates the localization of PIWI proteins to piP-bodies. To investigate, if the herein observed cytoplasmic granules are indeed piP-bodies, the authors should test for the presence of MAEL and for changes in the piP-body localization of piRNA pathway components upon knock-down or knock-out of MAEL (whatever is experimentally possible in the BmN4 cell culture system).

According to the Reviewer's constructive suggestion, we performed a comprehensive analysis on BmMael, the silkworm homolog of mouse MAEL. In our hands, endogenous BmMael (detected by a polyclonal antibody) was found not only in BmDcp2-positive piP-bodies but also more strongly in Siwi-positive nuage. This dual localization pattern suggested a marked difference between silkworm BmMael and mouse MAEL, which was shown to localize in piP-bodies but not in pi-bodies (Aravin et al, *PLoS Genet.* 2009). Nevertheless, in the presence of Siwi-D670A, BmMael strongly localized in piP-bodies together with Siwi-D670A. Moreover, knock-down of BmMael reduced the co-localization ratio between Siwi-D670A and BmDcp2, just like knock-down of BmSpnE and BmQin. Thus, BmMael may play an important role in recruiting Siwi to piP-bodies. These data are now shown in the newly added Fig EV1D, EV3C and D.

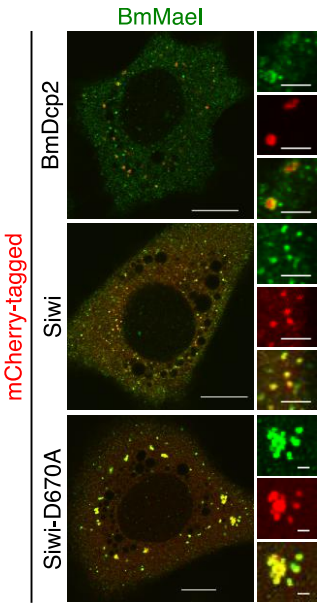

Fig EV1D. Colocalization of endogenous BmMael with BmDcp2, Siwi and Siwi-D670A.

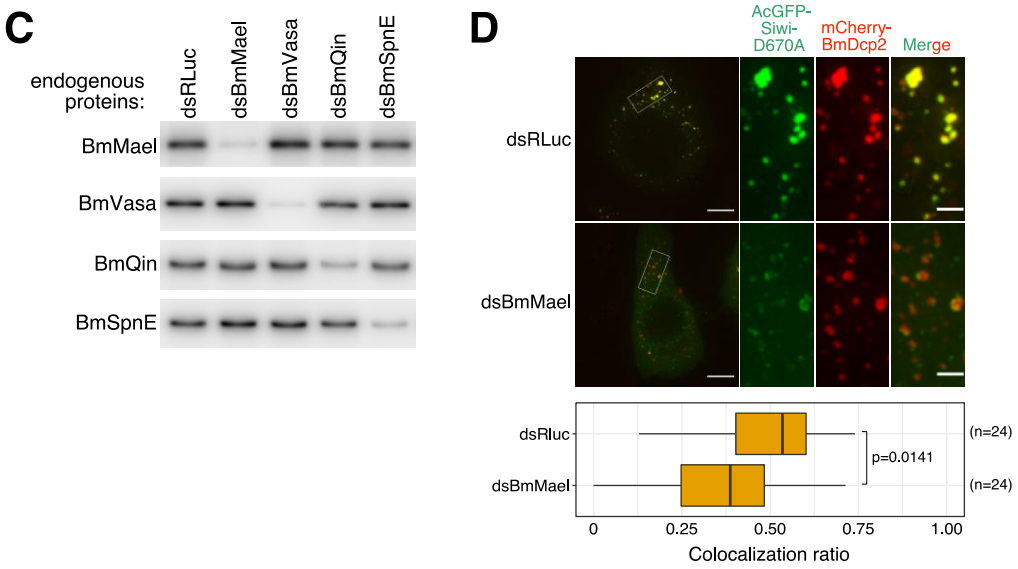

Fig EV3C. Knockdown of endogenous BmMael, BmVasa, BmQin and BmSpnE detected with western blotting.
Fig EV3D. Knockdown of endogenous BmMael impairs piP-body localization of Siwi-D670A.

3. Changes in piRNA composition? The authors propose that upon overexpression of catalytical dead SIWI (and thus inhibition of ping-pong), piRNAs that are derived from transposons are reduced while piRNAs derived from mRNAs are increased. It is not clear from the Figure legend or the methods section, how piRNA sequencing data were normalized for this analysis. This is important, because one expects a dramatic reduction of all piRNAs upon inhibition of ping-pong. The overall reduction of piRNAs is visible in small RNA seq data, when they are correctly normalized to an independent stable population of small RNAs like miRNAs. In contrast, normalization to the total data set would artificially inflate the fraction of low-abundant background fragments and is not appropriate for this analysis. Inappropriate normalization could result in the perception of an increase in mRNA-derived small RNA fragments. However, this increase would only be relative to the total

population, which might be dramatically reduced. The authors should clarify to avoid misinterpretation. Finally, the authors should immune-precipitate SIWI and BmAgo3 and analyze associated small RNAs to examine, if the observed RNA fragments are indeed bona-fide piRNAs.

We apologize for the confusion caused by our insufficient description of the normalization method. In our original manuscript, we normalized all reads to the total mapping reads on Silkbase GeneModel library (Kawamoto et al, *Insect Biochem. Mol. Biol.* 2019), which contains both transposon and non-transposon genes. To address the Reviewer's concern, we have now normalized the sequencing data using both highly abundant miRNAs and total mapping reads (as performed in Izumi et al, *Cell* 2016) and replotted all the panels for small RNA sequencing data. We did not find any major discrepancy between the two normalization methods, which is also consistent with the fact that the intensity of total piRNA bands were comparable in naive, Siwi and Siwi-D670A-expressing samples when observed on gels (Fig R2 below). Therefore, we concluded that the increase of non-TE mRNA-derived reads is not caused by an overall reduction in total piRNA production. We would like to clarify that TE-derived piRNAs are not reduced but remain the same upon Siwi-D670A expression (Fig 5B); non-TE piRNAs (which are only lowly expressed in normal cells) are specifically increased by Siwi-D670A (Fig 5B).

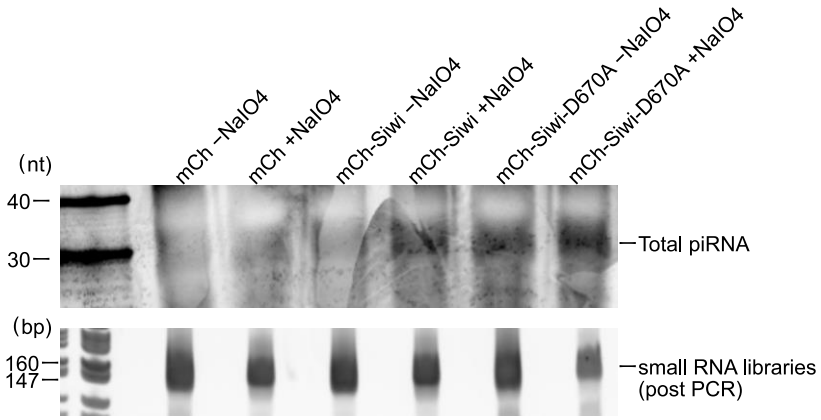

Fig R2. Gel analyses of pre-sequencing small RNA samples.

To examine whether the observed non-TE-derived small RNA reads are *bona fide* piRNA or not, we performed small RNA sequencing by introducing an extra step: treating gel-excised small RNAs with sodium periodate ($NaIO_4$). This step prevents 3′ adaptor ligation of any small RNA fragments without 3′ modification (including miRNAs) but genuine piRNAs, which are 2′-*O*-methylated. We were able to reproduce the global up-regulation of non-TE gene-derived piRNAs in Siwi-D670A overexpressed cells (now shown in Fig EV5A and B). The up-regulated non-TE piRNAs were not affected by $NaIO_4$ treatment, suggesting that these piRNAs were faithfully 2′-*O*-methylated at their 3′ ends (Fig EV5C). Please note that small RNAs were normalized to total mapped reads in Fig EV5, because $NaIO_4$-mediated oxidation prohibits the use of miRNAs as normalization factors (Fig EV5D).

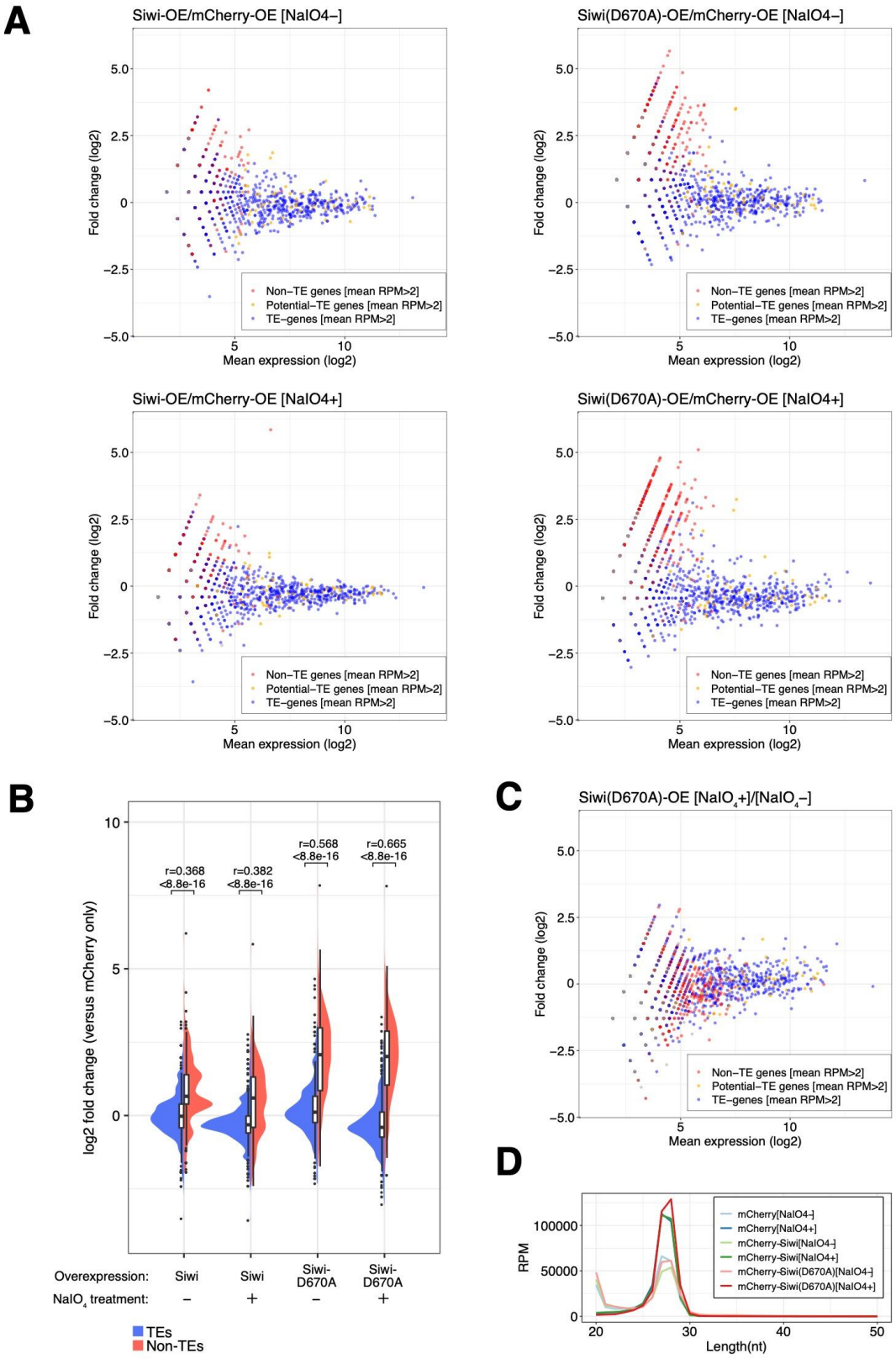

Fig EV5. Up-regulated non-TE piRNAs in Siwi-D670A expressed cells are 2′-*O*-methylated. (A–C) $NaIO_4$ oxidation did not affect the upregulation of Siwi-D670A-OE induced non-TE piRNAs. (D) $NaIO_4$ oxidation depleted non-methylated siRNA, causing reduction in 20 nt small RNA peaks and relative increase in 28 nt piRNA peaks.

4. Integration of recent data by their own group and others. Mechanisms of piRNA biogenesis have been extensively studied over the past years. The authors should relate their current findings to existing data: (4.1.) The helicase Armitage couples piRNA amplification in nuage and piRNA production on the surface of mitochondria (Ge et el., Mol Cell 2019, PMID: 31076285). The authors should examine the subcellular location of Armi in their experimental set up and integrate their findings in the established framework.

We thank the Reviewer for the fruitful suggestion. We have now probed the colocalization with Siwi-D670A and Armi, using the stable GFP-Armi cell-line established in our previous paper (Izumi et al, *Nature* 2020). We found that GFP-BmArmi partially, if not completely, co-localizes with both Siwi and Siwi-D670A granules (newly added Fig EV1E). Armitage is known to shuttle between nuage and mitochondria (Ge et al, *Mol. Cell* 2019; Ishizu et al, *Cell Rep.* 2019), but not in P-bodies at least in the steady state. It is therefore intriguing that BmArmi was found associated also with Siwi-D670A trapped in piP-bodies. This observation is in line with the idea that BmArmi not only shuttles between nuage and mitochondria but also transiently enters piP-bodies for piRNA biogenesis in silkworm cells. We have started a thorough search on the identification of other piP-body components, and we hope to pursue this direction in the future.

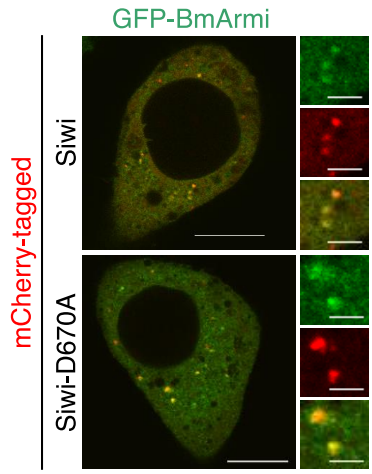

Fig EV1E. Colocalization of GFP-BmArmi, Siwi and Siwi-D670A.

(4.2.) Phased piRNA biogenesis has been shown to occur upon induction of piRNA processing by ping-pong piRNAs (Mohn et al., Science 2015, PMID: 25977553; Han et al., Science 2015, PMID: 25977554). The authors should probe, if changes in subcellular localization of piRNA pathway components affect coupling of ping-pong to the primary piRNA processing machinery, and if changes in this coupling and phasing could explain the proposed differences in piRNA composition.

Silkworms lack a homolog of *Drosophila* Piwi, the phased piRNA-dedicated PIWI protein that doesn't have the ping-pong partner; in silkworms, Siwi can participate in both the phased piRNA biogenesis and the ping-pong amplification together with its partner BmAgo3. Accordingly, production of phased piRNAs can not only spread the piRNA-generating region but also initiate new ping-pong cycles. Indeed, unlike in *Drosophila*, almost all phased piRNAs have complementary ping-pong pairs in BmN4 cells (Izumi et al, *Nature* 2020) as well as in silkworm ovaries (our unpublished data). In other words, once the piRNA-generating regions have been established (on existing TEs) in silkworms, phased piRNA production does not tightly couple with the ping-pong cycle any more, making these two pathways somewhat redundant. This loose system should allow flexible and robust piRNA

biogenesis, which is presumably beneficial for silkworms to combat transposons by using only two PIWI proteins (Izumi et al, *Nature* 2020).

In agreement with such robustness of TE-derived piRNA biogenesis that has been established in silkworms, we observed no apparent changes in the levels of phased TE piRNAs (those starting downstream of putative Zuc-cleavage sites, defined in Izumi et al, *Nature* 2020) by Siwi-D670A expression (Fig. R3). This is expected because Siwi-D670A does not affect the expression of TE piRNAs in general (Fig. 5B), but reiterates the fact that Siwi-D670A causes specific up-regulation of non-TE piRNAs. As discussed in 4.3 below, non-TE piRNAs are likely generated independently of well-defined phased/ping-pong piRNA biogenesis mechanisms. Such promiscuous production of non-TE piRNAs is usually suppressed in normal cells, but is aberrantly activated when proper subcellular compartmentalization is disrupted by Siwi-D670A expression. We have clarified these points in the revised manuscript.

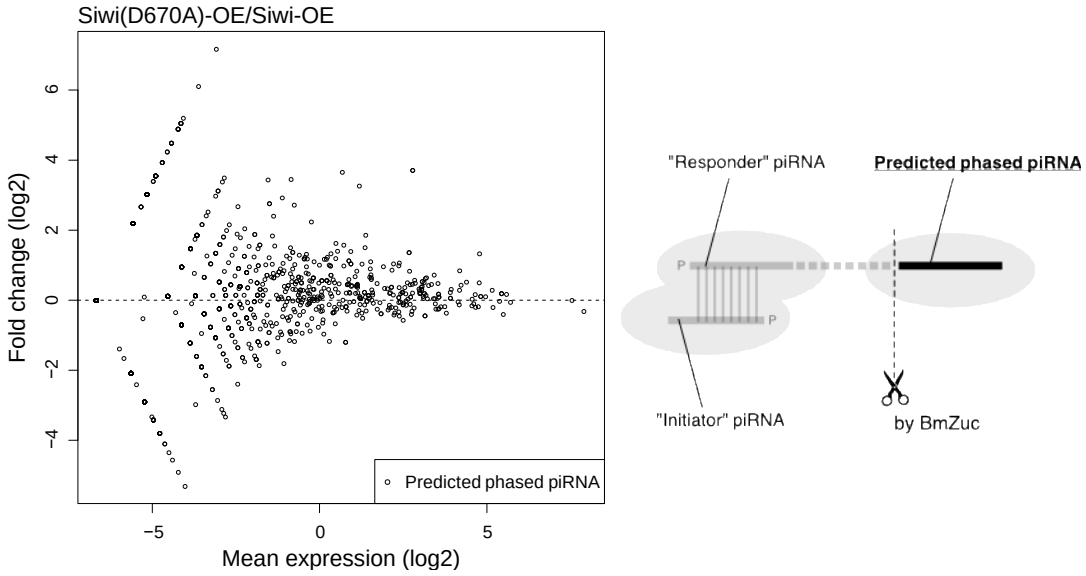

Fig R3. Differential expression analysis of phased piRNAs immediately downstream of the 3′ ends of predicted pre-piRNAs (“Responder”) with Zuc-cleavage sites in BmN4 cells (Siwi-D670A-OE versus Siwi-OE).

(4.3.) The authors' group has recently identified consensus motifs that determine piRNA production in BmN4 cells (Izumi et al., Nature 2020, PMID: 31996847). The authors could use their existing small RNA sequencing data or additional bona-fide piRNA data (suggested in point 2) to characterize potential effects on the consensus motif. Does this consensus motif discriminate the processed mRNAs from unprocessed mRNAs?

Based on the Referee’s constructive suggestion, we probed for the BmZuc consensus motif in non-TE mRNAs. To do this, we calculated the similarity scores with the previously defined BmZuc consensus motif (Izumi et al, *Nature* 2020) and counted the numbers of high score sites (top 1%) on each mRNA. Fig. R4A shows that there is no apparent correlation between the Zuc motif counts and piRNA production from non-TE mRNAs. Instead, the increase of non-TE piRNAs is global and all non-TE mRNAs seem to be subjected to piRNA production. Indeed, we confirmed that highly expressed mRNAs (top 25% read counts in wildtype BmN4 total RNA RNA-seqs) account for most of the detected non-TE piRNAs (Fig. R4B). Although we admit that these analyses are preliminary (definition of BmZuc score may not have a sufficient detection power; the sequencing depth may not be sufficient to quantitatively detect non-TE piRNAs that are only lowly expressed in general), but they do suggest that

non-TE piRNA production is correlated simply with the transcript level and therefore there is no discrimination among non-TE mRNAs for piRNA processing. In other words, unlike TE piRNAs, non-TE piRNAs are likely generated independently of well-defined phased/ping-pong piRNA biogenesis mechanisms. This is also evident from the fact that, while TE-piRNAs are mapped to both strands, non-TE piRNAs are exclusively mapped to the sense strand without ping-pong partners (Figs. 5D and EV4D). We envision that promiscuous production of non-TE piRNAs is usually suppressed in normal cells but is aberrantly activated when proper subcellular compartmentalization is disrupted by Siwi-D670A expression. We have clarified these points in the revised manuscript.

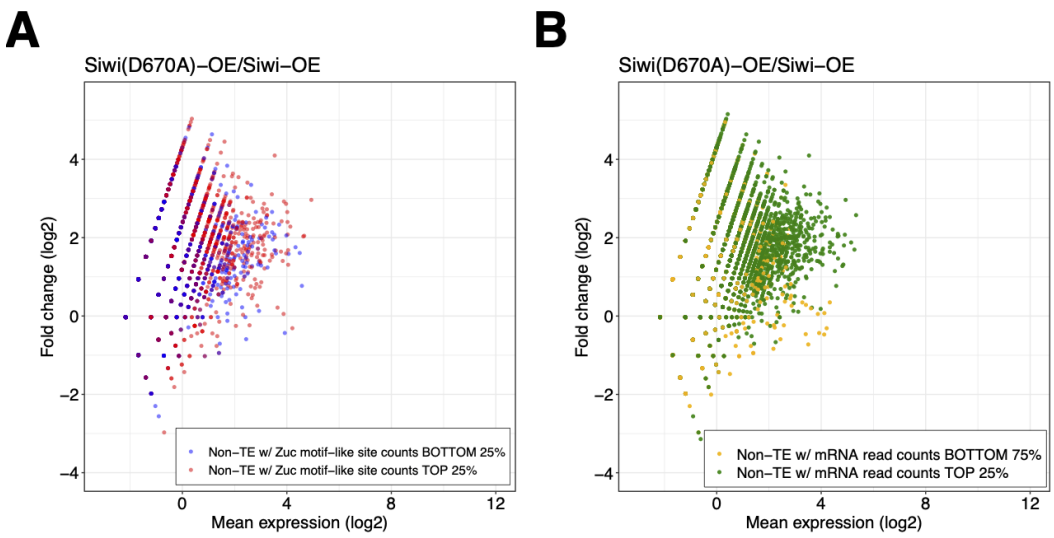

Fig R4. (A) Presence or absence of Zucchini-recognition motif (Zuc motif) does not affect the increase of non-TE piRNA expression in Siwi-D670A-OE cells. (B) All non-TE mRNAs accompany increased non-TE piRNAs, where most highly expressed mRNAs (green) generated the majority of the detected non-TE piRNAs in Siwi-D670A-OE cells. Each dot represents the piRNAs mapped on to a predicted gene.

Referee #2:

Chung et al. present studies on the subcellular localization of piRNA pathway components in the Bombyx mori system; to be precise in cultured BmN4 cells. They find evidence for an organization that has been described in mice: an interplay between the germ cell specific nuage and P-bodies that contain some piRNA factors (hence are named piP bodies). A role for Siwi slicing in separating nuage and piP bodies is described, as well as a function of RNA helicase Vasa. Finally, a function of such de-mixing in discrimination between self and non-self is proposed.

These studies are novel for the BmN4 system, but largely parallels published work on mice. Fact that BmN4 cells do show this parallel, while Drosophila does not appear to do so, is an interesting finding. In addition, the proposed role for Siwi slicing and the role of de-mixing in self-identification are intriguing. However, the presented studies are very descriptive and do not really prove these points. I would still think EMBO reports would be a proper venue to publish this, but the authors need to tune down the strength of their wording. In particular, the fact that they (by necessity) use overexpression to look at sub-cellular localization is a factor that may convolute the results, and this should be clearly acknowledged. Finally, Figure 5 needs to be improved by a different experimental design that takes out endogenous Siwi activity, in order to draw the presented conclusion.

We share the Referee's concern about overexpression. To validate our original co-localization data obtained with the baculovirus-derived OpIE2 promoter, we performed western blotting with antibodies against endogenous proteins and compared the expression levels between the exogenously expressed proteins and their endogenous counterparts. We confirmed that all exogenous proteins were expressed at a level less than or comparable to endogenous proteins (Fig EV2A and B). To further avoid a potential artifact of overexpression, we replaced the OpIE2 promoter with the P_{TRE3G} Tet-On inducible promoter, with which the expression level can be adjusted by Doxycycline concentration. All proteins from the Tet-On constructs were expressed at a level markedly lower than their endogenous counterparts (Fig EV2A and B). Still, we observed co-localization of Siwi-D670A, BmSpnE, BmQin with piP-bodies and co-aggregation between Siwi-D670A and BmVasa-E339Q (Fig EV2C). Thus, the co-localization patterns we originally observed with OpIE2 promoter are reproducible with expression levels much lower than endogenous proteins. These results are now included to the manuscript. Please refer to Extended View Figure 2.

Some specifics that would improve the manuscript:
Figure 1:
Minor: Validation of microscopy (co-localization): BmDcp2 immunoprecipitation studies followed by Western Blot on otherP body components to validate presence of BmDcp2 in P-bodies. More serious concern: how do we know if Dcp2 overexpression does not affect its localization? Are there antibodies to known P body components that can be used to assess this?

We thank the Referee for raising this fundamental point. We have now confirmed that BmDcp2 was co-immunoprecipitated with BmMe31B (homolog of DDX6, the core P-body factor) (Fig R5A below). Moreover, the Tet-On version of BmDcp2, which has a 16-fold reduced expression level compared to the previous OpIE2 promoter version (Fig R5B for reviewers), showed consistent colocalization with BmMe31B (Fig R5C). Thus, we believe that BmDcp2 can be used as a marker for P-bodies in BmN4 cells.

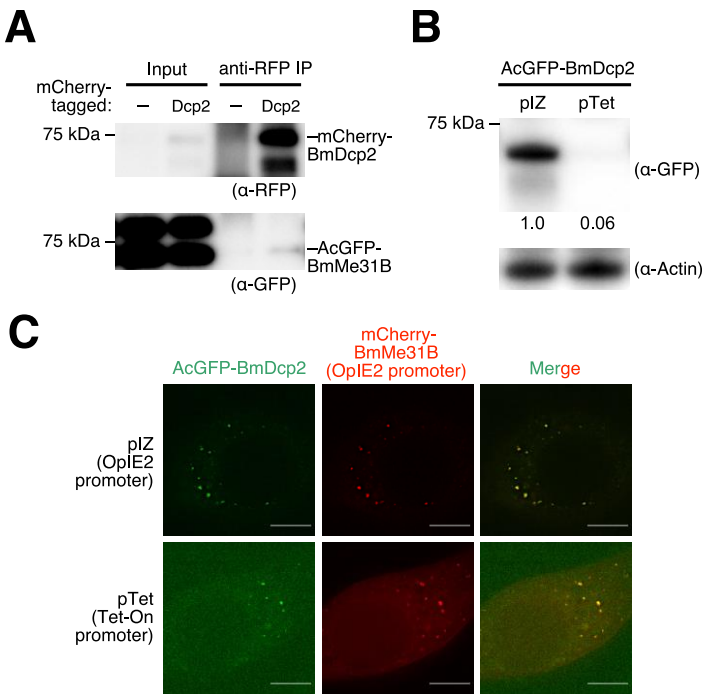

Fig R5. Validation of BmDcp2 as a P-body marker in BmN4 cells. (A) Co-immunoprecipitation of BmMe31B and BmDcp2. (B) pTet construct reduces BmDcp2 expression level. Relative amounts are indicated below the α-GFP gel image. (C)

Colocalization between BmDcp2 and BmMe31B in BmN4 cells with pIZ and pTet constructs. Exposure of the GFP panels in the pTet set is enhanced for 5-folds. Scale bar, 10 μm.

Is the localization of Siwi to piP bodies dependent on its loading status? This can be easily tested by generating a piRNA-loading deficient mutant.

According to the Reviewer's fruitful suggestion, we have generated a piRNA-loading deficient mutant by mutating a previously validated residue (Tyr607) at the 5′ recognition pocket (Kawaoka et al, *Mol. Cell* 2011). In the newly added Fig EV1C, we confirmed that both Siwi-Y607E mutant (deficient in piRNA-loading) and Siwi-Y607E-D670A double mutant (deficient in both piRNA loading and slicer) were found largely dispersed in the cytoplasm and do not colocalize with piP-body marker BmDcp2. This suggested that the localization of Siwi-D670A to piP-bodies requires the piRNA-loaded state.

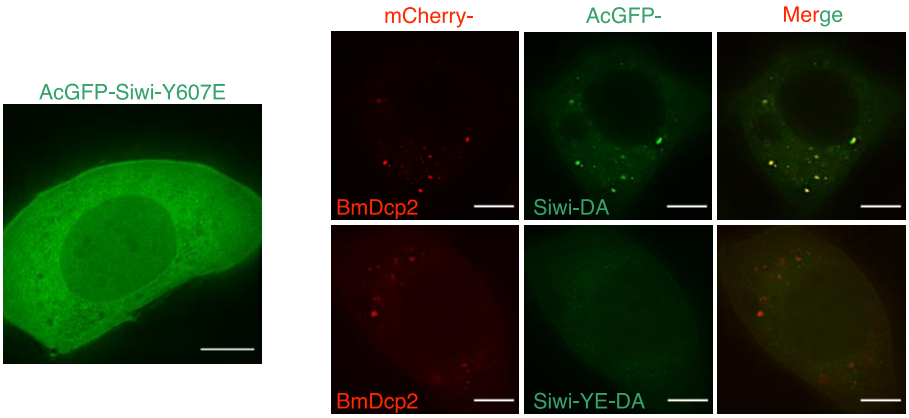

Fig EV1C. 5′ recognition pocket mutation compromises piP-body localization of Siwi-D670A.

Figure 2:
2C: It should be checked if SpnE and Qin are expressed at similar levels in both Siwi wt and D670A transfected cells.

We confirmed by western blotting that BmSpnE and BmQin are expressed at similar levels in both Siwi and Siwi-D670A transfected cells. Figure EV3A (below) is now added to the manuscript.

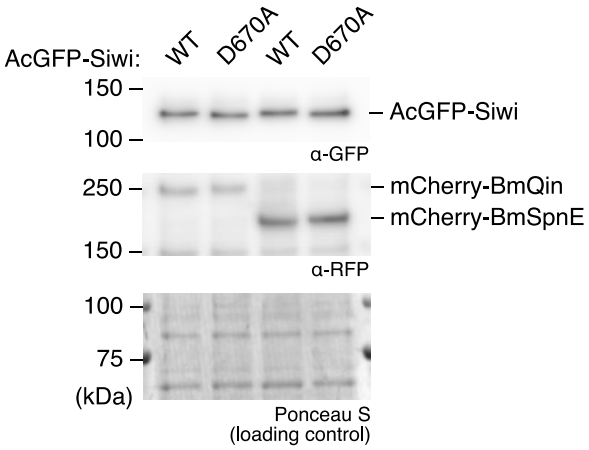

Fig EV3A. Western blotting of AcGFP-Siwi, wildtype (WT) or D670A (DA), and the co-expressed mCherry-BmQin or mCherry-BmSpnE.

2D: The depletion of dsBmQin or BmSpnE leads to decreased co-localization between Siwi-

D670A and BmDcp2. Knockdowns should be verified on endogenous levels either by WB or immunostaining. Also, in M&M details are lacking on the dsRNA treatment. How much dsRNA was transfected, for how long and how often?

We have now confirmed depletion of the endogenous proteins when treated with dsBmQin and dsBmSpnE (newly included Fig EV3C). We apologize for missing the details of dsRNA treatment, which is now added in the method section.

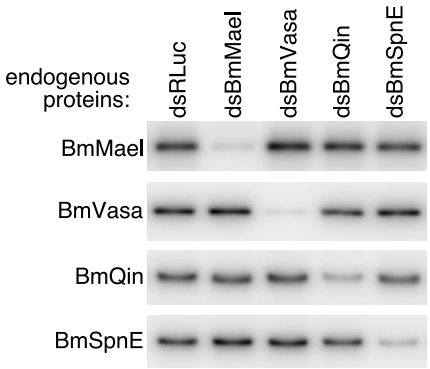

Fig EV3C. Knockdown of endogenous BmQin and BmSpnE confirmed by western blotting.

Figure 3:
3A+B: When Siwi is depleted BmQin and BmSpnE are not found in piP bodies, do they now co-localize with BmVasa (ie nuage) instead?

Yes, when Siwi is depleted, BmQin and BmSpnE are co-localized with BmVasa, as originally shown in Fig 3C.

3C: Siwi is not found in nuage when Vasa is depleted. This can simply be due to the fact that the overexpressed Siwi does not get loaded under Vasa kd conditions (see comment on Figure 1).

We believe that the Referee is referring to Fig 3E. Unlike the co-localization between Siwi and Dcp-2 in the Vasa-depleted condition, unloaded Siwi (Siwi-Y607E-D670A double mutant) was largely dispersed in the cytoplasm (Fig EV1C). Thus, the piRNA loading state of Siwi does not explain the observation in Fig. 3E.

Figure 4:
"We could also confirm the colocalization of BmVasa-E339Q with Siwi, BmAGO3 and BmQin in our hands (data not shown)". There is enough space to show this in EV2. Would be good to show as it would indicate that BmQin shuttles between piP bodies and nuage as well.

We now show the data of BmVasa-E339Q colocalizing with BmQin and BmAgo3 in the newly added Fig EV3E.

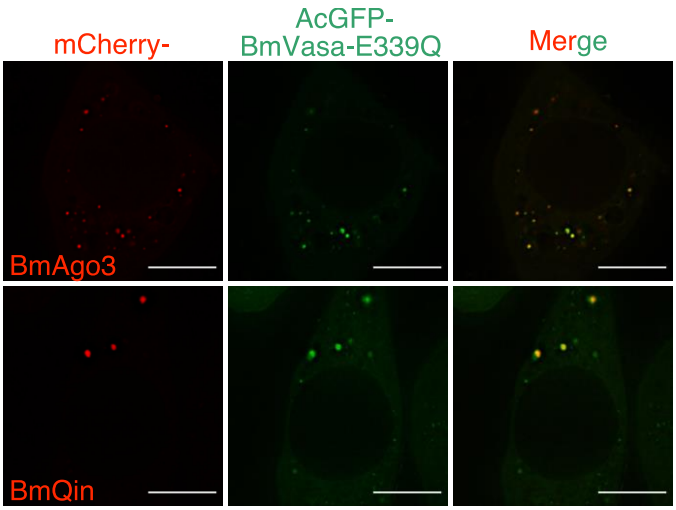

Fig EV3E. Colocalization of BmAgo3, BmQin in BmVasa-E339Q aggregates.

"We propose that the slicer activity of Siwi and the ATPase activity of BmVasa act together to keep remodeling RNP complexes...". This statement is based on the observation that with overexpression of the respective mutants bigger foci are formed. Even though the term 'proposed' is rather weak, I feel that more careful wording would be warranted.

We agree with the Referee and have toned down the statement as (“slicer activity of Siwi and the ATPase activity of BmVasa are likely to have non-redundant roles in remodeling RNP complexes”).

Figure 5:
How were TEs defined. Please describe this.

We apologize for any confusion caused by insufficient explanation. We performed tblastx between existing silkworm transposon libraries (a total of 1,811 transposons defined by Osanai-Futahashi et al, *Insect Biochem. Mol. Biol.* 2008) and the GeneModel library (a total of 16,880 predicted genes including putative transposons; modeled by Kawamoto et al, *Insect Biochem. Mol. Biol.* 2019). In the latter library, 4,136 genes which had an e-value smaller than the threshold 1e-10, were defined as transposable element (TE) genes. 811 TE genes with at least 2 piRNA reads per gene on average were selected for downstream analyses. We have now added these details in the method section.

More importantly, the whole set-up is in presence of wild-type endogenous Siwi. This may imply that TE transcripts are properly processed. Any additional production of piRNAs would therefore by definition need to come from endogenous (self) genes. Hence, a proper experiment to assess if self-recognition is affected would be to first deplete endogenous Siwi and to then come in with the expression of tagged versions of specific mutants. Without such more controlled experiments, the conclusion drawn from this figure ("We concluded that the subcellular compartmentalization of the silkworm piRNA pathway is critical for self-non self-discrimination during piRNA precursor acquisition") is not valid.

We fully agree with the Referee that depletion of endogenous Siwi and complementation with tagged mutants could yield more conclusive results. However, our preliminary results suggest that, unfortunately, Siwi knockout is lethal in BmN4 cells, and we believe that our current experimental scheme is the best we can do at this point. We have weakened our statement in the revised manuscript as following: “We concluded that the presence of Siwi-D670A or BmVasa-E339Q in BmN4 cells impairs self-nonself discrimination during piRNA

precursor acquisition, most likely by causing abnormal RNP aggregation and disrupting the subcellular compartmentalization of silkworm piRNA pathway."

(BTW, Figure 5G is not present).

We apologize for the mistake and have amended the manuscript.

Referee #3:

The manuscript by Chung et al. deals with the problem of Piwi-interacting (pi)iRNA biogenesis in the silkworm. Specifically, this paper focuses on the dynamics of the cytoplasmic distribution of piRNA pathway proteins and the relationship to piRNA biogenesis. Prior studies in flies and mammals have identified various forms of perinuclear "nuage" and cytoplasmic piRNA protein aggregates/condensates in germ cells. Some such aggregates contain proteins characteristic of processing bodies (P-bodies), raising the possibility of crosstalk between piRNA and RNA regulatory pathways in germ cells. However, despite the evident alterations in piRNA protein localization patterns in piRNA deficient germ cells, the functional meaning and significance of this co-localization are not well understood. The present study uses a relative newcomer in the piRNA field, silkworm Bombyx mori, to gain insights into the relationship of various cytoplasmic aggregates and piRNA biogenesis and functions.

The study uses live-cell imagining in cultured BmN4 cells, which have a fully functional piRNA biogenesis pathway known as the ping-pong cycle. To follow protein localization, the authors overexpressed proteins of interest using the epitope-tagging strategy. Epitope-tagged wild-type Siwi and BmAgo3 proteins, the primary effector proteins in the piRNA pathway, exhibited comparable localization patterns with BmVasa in nuage. Using this experimental setup, the authors explore the localization of piRNA and P-body proteins and the dependency of observed localization patterns on each other as determined using gene-specific knockdowns in BmN4 cells.

This study's main observations agree strongly with prior studies in several model organisms and provide further mechanistic links between the subcellular cytoplasmic localization of piRNA proteins and piRNA biogenesis. This work also suggests the critical role of proper localization of piRNA proteins for the correct targeting of transposons rather than genic mRNAs.

Overall, this is a well designed and executed study that certainly deserves being published. There are no serious experimental flaws or significant sticking points. The two minor points are as follows:

1. All observations were obtained on the background of wild-type piRNA proteins. Consequently, the presence of wild-type proteins impacts piRNA biogenesis (as is suggested by the authors based on small RNA sequencing data). It might also impede or alter the localization of ectopically expressed epitope-tagged proteins. Similarly, the presence of mutant proteins may interfere with the normal functioning of wild type proteins. Consequently, some observations might reflect actual and other artifactual outcomes of the experimental setup. Can the authors acknowledge and discuss these shortcomings when introducing their experimental system?

We thank the Referee for his/her kind comments and constructive suggestions. To validate our original co-localization data obtained with the baculovirus-derived OpIE2 promoter, we performed western blotting with antibodies against endogenous proteins and compared the expression levels between the exogenously expressed proteins and their endogenous

counterparts. We confirmed that all exogenous proteins were expressed at a level less than or comparable to endogenous proteins (Fig EV2A and B). To further avoid a potential artifact of overexpression, we replaced the OpIE2 promoter with the P_{TRE3G} Tet-On inducible promoter, with which the expression level can be adjusted by Doxycycline concentration. All proteins from the Tet-On constructs were expressed at a level markedly lower than their endogenous counterparts (Fig EV2A and B). Still, we observed co-localization of Siwi-D670A, BmSpnE, BmQin with piP-bodies and co-aggregation between Siwi-D670A and BmVasa-E339Q (Fig EV2C). Thus, the co-localization patterns we originally observed with OpIE2 promoter are reproducible with expression levels much lower than endogenous proteins. These results are now included to the manuscript. Please refer to Extended View Figure 2.

We fully agree with the Referee that depletion of endogenous wild-type proteins and complementation by mutant proteins could yield more conclusive results. However, our preliminary results suggest that, unfortunately, Siwi knockout is lethal in BmN4 cells, and we believe that our current experimental scheme is the best we can do at this point. We have now properly acknowledged the background presence of endogenous, wild-type counterparts in our current experimental system and also weakened our statements in the revised manuscript (e.g., from “subcellular compartmentalization of the silkworm piRNA pathway is critical for self-non self-discrimination” into “impairs self-nonself discrimination during piRNA precursor acquisition, most likely by causing abnormal RNP aggregation and disrupting the subcellular compartmentalization”).

2. It would be beneficial if the authors stated the specific advancement in our understanding of the piRNA system that this study provides. The authors include pictures interpreting and summarizing some of their findings. Still, I wonder if the final model could depict the generalized view of cytoplasmic compartmentalization and functionality of the piRNA system across model organisms.

We thank the Referee for the helpful suggestion. We constructed a new schematic diagram for the generalized view of cytoplasmic compartmentalization in BmN4 (Fig 6).

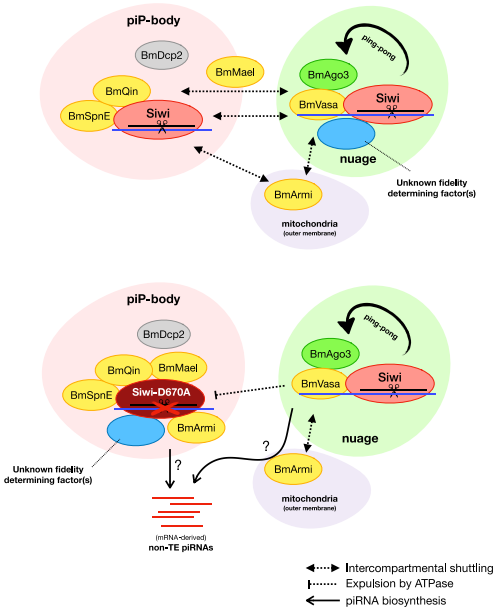

Fig 6. Proposed model for nuage/piP-body partitioning in BmN4 cells

1st Revision - Editorial Decision 25th Feb 2021

Dear Yuki,

Thank you for the submission of your revised manuscript. We have now received the enclosed reports from the referees that were asked to assess it. Referee 2 still has a minor suggestion that I would like you to incorporate before we can proceed with the official acceptance of your manuscript.

A few other editorial changes will also be required:

- Please upload all main and all EV figures as individual files and add the EV figure legends to the main manuscript file after the main figure legends. The legends need to be removed from the figure files.

- Fig EV2B+C callouts are missing and Fig EV5A-C callouts are missing. Please add.

- Please add the URLs and accession IDs to the Data Availability Section.

I attach to this email a related ms file with comments by our data editors. Please address all comments in the final manuscript.

EMBO press papers are accompanied online by A) a short (1-2 sentences) summary of the findings and their significance, B) 2-3 bullet points highlighting key results and C) a synopsis image that is exactly 550 pixels wide and 200-600 pixels high (the height is variable). You can either show a model or key data in the synopsis image. Please note that text needs to be readable at the final size. Please send us this information along with the revised manuscript.

I look forward to seeing a final version of your manuscript as soon as possible and to seeing this nice paper published!

Best wishes,
Esther

Esther Schnapp, PhD
Senior Editor
EMBO reports

Referee #1:

The authors have addressed all my concerns and the model (Fig. 6) helps to understand the descriptive nature of this study. The presented data add valuable information to the field of piRNA biology. I recommend publication of the manuscript in the revised form.

Referee #2:

Having read the replies of the authors to the issues raised, and seen the revised manuscript, I support publication of the work in EMBO Reports. It represents a valuable extension of our knowledge of the subcellular organization of the piRNA pathway, even if based on expression of transfected constructs. The experiments are controlled as well as they can be, and the conclusions are justified.

I would just ask for a small paragraph in the discussion that would mark the limitations of the study, as it remains built on transfections and overexpression. While the authors show experiments that suggest expression levels are moderate, and comparable to endogenous proteins, these are somewhat difficult to interpret. In our hands, titration of plasmid concentrations may affect the number of cells expressing the construct rather than the expression level of the construct with each cell. Using a bulk read-out such as a Western blot, this effect cannot be scored, leaving the possibility that within the imaged cells (that by definition do express the constructs) the expression levels of the transfected constructs are significantly higher than that of endogenous proteins. In addition, the suggested paragraph outlining such potential complications of the study, the authors could also consider to:
-provide information on the frequencies of transfected cells in their experiments
-provide information of the variation of expression levels in the cells based on fluorescence levels in individual cells.
Information on these two variable may enable further strengthening of the authors claim that expression of the constructs is comparable to endogenous levels.

Referee #1:

The authors have addressed all my concerns and the model (Fig. 6) helps to understand the descriptive nature of this study. The presented data add valuable information to the field of piRNA biology. I recommend publication of the manuscript in the revised form.

We thank the Referee for his/her kind comments on our revised manuscript.

Referee #2:

Having read the replies of the authors to the issues raised, and seen the revised manuscript, I support publication of the work in EMBO Reports. It represents a valuable extension of our knowledge of the subcellular organization of the piRNA pathway, even if based on expression of transfected constructs. The experiments are controlled as well as they can be, and the conclusions are justified.

We thank the Referee for his/her positive comments on our revised manuscript.

I would just ask for a small paragraph in the discussion that would mark the limitations of the study, as it remains built on transfections and overexpression. While the authors show experiments that suggest expression levels are moderate, and comparable to endogenous proteins, these are somewhat difficult to interpret. In our hands, titration of plasmid concentrations may affect the number of cells expressing the construct rather than the expression level of the construct with each cell. Using a bulk read-out such as a Western blot, this effect cannot be scored, leaving the possibility that within the imaged cells (that by definition do express the constructs) the expression levels of the transfected constructs are significantly higher than that of endogenous proteins. In addition, the suggested paragraph outlining such potential complications of the study, the authors could also consider to:
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-provide information of the variation of expression levels in the cells based on fluorescence levels in individual cells.
Information on these two variable may enable further strengthening of the authors claim that expression of the constructs is comparable to endogenous levels.

We thank the Referee for raising this critical issue. We agree that even if the protein bands appear comparable on Western blots, the number of the expressed proteins could still exceed their endogenous counterparts at a single-cell level. According to the Referee's constructive suggestion, we quantified the transfection efficiency using flow-cytometry and found that pIZ-Siwi constructs were transfected to cells at a frequency of ~8–10% (Figure R6A).

For pTet-Siwi constructs, we observed >30-folds drop of fluorescence intensity (Figure R6A) at 100 ng/mL Doxycycline. The actual particles that were gated into the GFP-positive gate (GFPpos) were found to be ~0.8–2.5%, but this percentage must be largely underestimated due to the near-background fluorescence intensity. Of note, we transfected the plasmid without any titration (i.e., at the same concentration as the pIZ constructs) and thus in theory the transfection efficiency should be comparable to that of the pIZ constructs.

Theoretical expression level of Siwi under the IE2 promoter (pIZ constructs) is calculated as follow:

$$\frac{GFP\ tagged\ protein_{band\ intensity}}{Endogenous\ protein_{band\ intensity}} \times \frac{1}{Transfection\ frequency} = pIZ\ expression\ level\ (folds\ of\ endogenous\ level)$$

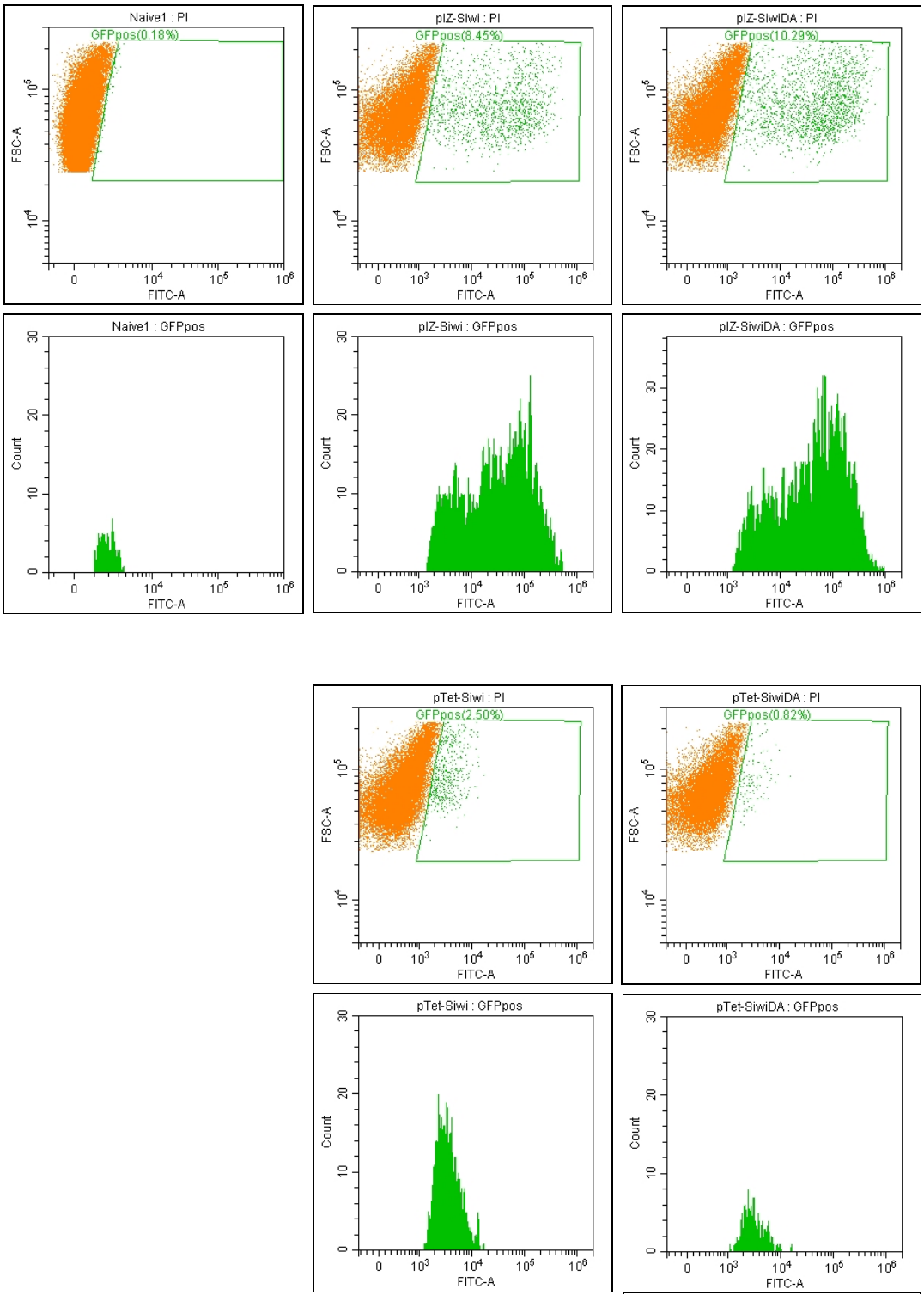
Or:

$$0.209 \times \frac{1}{0.0845} = 2.473\ folds\ of\ endogenous\ level$$

For Tet-On promoter, if we assume that the fluorescence intensity was dropped for 30 folds when compared to pIZ constructs, pTet-Siwi is estimated to be expressed at a level of 0.0824 folds relative to its endogenous counterpart.

As the gating strategy could largely affect the resultant percentage due to mixture of non-cell particles, we also attached the microscopic images captured with automatic paneling using a 20x lens for your reference (Figure R6B). Microscopic images of pTet-transfected cells captured with a 20x lens were not sensitive enough to distinguish GFP positive cells from the image background (Figure R6B), but these also demonstrated the strong reduction of the expression level. We also note that, in the colocalization experiments, more than one plasmid was transfected to the cells with the same total DNA amount, meaning that the actual expression level in most of our experiment sets should be even lower. We have now added Fig EV2C as well as a paragraph in the result section to discuss the limitation and controls of these expression systems.

A


Naive1 : PI
GFPpos(0.18%)
pIZ-Siwi : PI
GFPpos(8.45%)
pIZ-SiwiDA : PI
GFPpos(10.29%)
Naive1 : GFPpos
pIZ-Siwi : GFPpos
pIZ-SiwiDA : GFPpos
pTet-Siwi : PI
GFPpos(2.50%)
pTet-SiwiDA : PI
GFPpos(0.82%)
pTet-Siwi : GFPpos
pTet-SiwiDA : GFPpos
FSC-A
FITC-A
Count

B

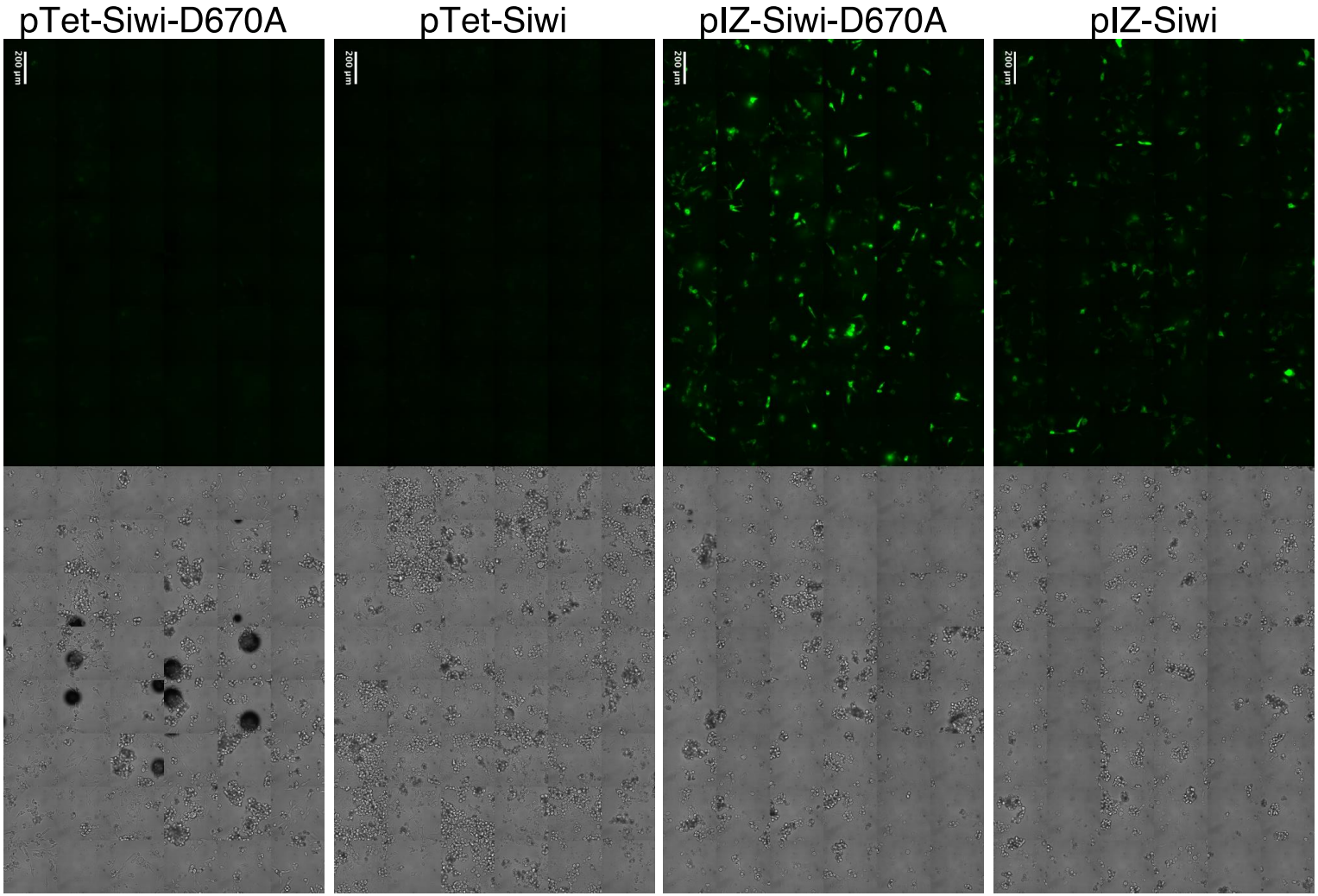


Fig R6. (A) Flow cytometry analysis of naive, pIZ- and pTet-constructs transfected BmN4 cells. FSC-A: forward scatter area. FITC-A: fluorescence intensity of GFP positive cells. Orange dots are cell-like particles with gates that removed cell debris and dead cells with propidium Iodide staining. Green dots are GFP positive cell-like particles. (B) Series of microscopic images (48 images per set) acquired with auto-paneling. Left: GFP channel. Right: Bright-field.

2nd Revision - Editorial Decision 12th Apr 2021

Prof. Yukihide Tomari
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- ➔ a statement of how many times the experiment shown was independently replicated in the laboratory.
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  - are there adjustments for multiple comparisons?
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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?	Maximum numbers of biological entities (i.e. individual silkworm BmN4 cells) were chosen to ensure adequate power to detect the statistical difference, where the numbers were limited by the equipment booking schedules and the number of parallel assays. At least 6 independent cells were observed and analyzed per experiment to ensure data accuracy.
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?	Live BmN4 cultures infected by unknown bacteria or microorganisms due to technical mistakes were excluded from the analysis.
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
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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 provided by T. Kusakabe from Kyushu University. This cell line is not authenticated and not tested for mycoplasma contamination.

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