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An in vivo RNAi assay identifies major requirements for primary piRNA biogenesis in Drosophila

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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. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 04 August 2010

Thank you for submitting your manuscript for consideration by The EMBO Journal. Your manuscript has been reviewed by two referees and I enclose their comments below. I am pleased to inform you that both referees recommend publication once the minor issues below are addressed in text.

When you send us your revision, please include a cover letter with an itemised list of all changes made, or your rebuttal, in response to comments from review.
When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process initiative, please visit our website: http://www.nature.com/emboj/about/process.html

Thank you for the opportunity to consider your work for publication. I look forward to reading the revised manuscript.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1 (Remarks to the Author):

Manuscript Number: EMBOJ-2010-75460

"An in vivo RNAi assay identifies major genetic and cellular requirements for somatic piRNAs biogenesis in Drosophila"
Daniel Olivieri, Martina M. Sykora, Ravi Sachidanandam, Karl Mechtler, and Julius Brennecke
Advertising to the contrary, we know almost nothing about how piRNAs are made and loaded into functional complexes in either the soma or the germ line. This manuscript reports both a significant advance in our understanding of the somatic piRNA pathway in flies and important new tools for studying the pathway. The work is appropriate for publication in EMBO Journal and will be of considerable interest to those who study RNA silencing, germ-line development, and transposon control. Just a few revisions (and no new experiments) are required to ready it for publication. I strongly recommend publication of a revised version that addresses the minor flaws detailed below.
(1) Abstract: "...is understood at the genetic, cellular and molecular level" implies that to be understood,. all three levels are required. Perhaps the authors mean or rather than and?
(2) Page 3: What is the evidence that "mutated and immobile transposon copies...are a considerable genetic burden"? In an organism where DNA synthesis rates no longer limit generation time, is this still true? Also, I don't understand what the authors mean by "several dozen different types of transposable elements." The Drosophila melanogaster genome harbors more than 200 distinct transposon families, but no more than four types (DNA, LTR-retrotransposons, non-LTR retrotransposons, and perhaps viral retrotransposons) that I can think of.
(3) Page 4: miss: lack is more idiomatic (remains uncorrected from a previous version)
(4) Page 5: flies lacking ovaries are not carcasses (i.e., one can genetically manipulate flies to lack ovaries and still get viable flies, as Table S1 demonstrates); carcasses are the fly after the ovary is manually removed.
(5) Page 6: "loss of...and vasa does not"; I think the authors mean or rather than and, which would imply combining five mutations simultaneously.
(6) Page 9 and page 12: Rather than interact biochemically, the authors might consider writing interact physically.
(7) Page 10: Figure 7B does not contain the sequencing data; it is in Fig. 5B.
(8) Page 11 and legend to Figure S4: lose (i.e., was lost) not loose (i.e., needs tightening; this remains uncorrected from a previous version).
(9) A description of the method of primer validation and the method of analysis or simply a reference is needed (e.g., Livak et al., 2001). How was the error of the control (actin or rp49) propagated through the error of the experimental values?
(10) Figure 7C is missing x- and y-axes. Were only uniquely mapping piRNAs used to generate this figure?
(11) Supplemental figures: does Mio mean million (M is more standard)? In Figure S2: HeT-A, not Het-A; in Figure S8: HeT-A, not Het-A or HetA
(12) Table S2 should report (1) the total number of reads in addition to the total number of reads analyzed; and (2) the statistics should be provided for species, not just reads.

Referee #2 (Remarks to the Author):

Olivieri et al present a manuscript in which three players are positioned in the somatic piRNA pathway in Drosophila. The proteins concerned are Armi, Zuc and Yb, and a clear picture is presented of how these proteins interact with Piwi. A number of novelties is presented:
First, Yb is presented as a factor required for nuclear Piwi localization. The same is true in zuc mutants, and interestingly, Armi and Piwi start to accumulate in the Yb bodies in zuc mutant cells, suggesting zuc is required for Piwi transit through the Yb body into the nucleus. Finally, Armi is required to get Piwi into the Yb body. As a small bonus, the authors present evidence for tissue specific isoforms of Armi. However, the functional relevance of the two forms remain unclear. The same scenario is proposed to hold true for the germ cells, only there likely another Yb paralogue plays a role, although that is not experimentally addressed. In fact, the systems appear to not be quite the same if zuc loss-of-function phenotypes are considered: in the follicle cells Piwi accumulates into Yb bodies, which are clearly distinct granules. In the germ cells however loss of zuc results in clouds of Piwi and Armi, not granules. This might indicate that the potential germ cell alternatives for Yb behave quite distinct from Yb iteslf (also see my point 1 below).

The manuscript is well presented, and clearly presents significant results that are of interest for the EMBO J readership. I only have two items that should be addressed by the authors:

1) The authors present data on zuc loss-of-function material that differs from data published previously by Brennecke (Malone et al. 2009). The explanation of genotyping difficulties needs some further explanation for non-Drosohila geneticists. Genotyping issues would have resulted in heterogeneous results, not in an absence of a phenotype. Related to this: how does nuage behave in the zuc mutants? In the Malone et al paper it is described that Piwi still localizes to nuage, but with these new insights: is nuage present in zuc mutants (using for example a vasa or tudor staining)? This is relevant in relation to the perinuclear Piwi clouds observed in zuc mutant germ cells. Is this disintegrating nuage? As zuc may well play a role in the ping-pong cycle start-up, nuage may be severely affected. As in general, the precise appearance of nuage is quite sensitive to how one performs the experiment, it would be good to also check a background in which it is believed there is no nuage anymore (aub for example). This analysis would strengthen the comparison the authors try to make between the somatic and germ cell piRNA pathways in Drosophila.

2) The authors describe a partially RNA dependent interaction between Piwi and Armi. As an argument that the Rnase treatment worked well they say that no piRNA are observed in those samples. However, RNaseA is extremely stable and can remain a problem also after phenol extraction etc. Thus, the lack of piRNAs does not mean the RNase treatment went to completion during the IP. Actually, it doesn't mean a whole lot if no proper controls are taken along, such as synthetic RNA that is added to the samples after RNA isolation from the IP. I do not suggest the authors should do such controls, but I suggest this argument be removed from the text.

1st Revision - authors' response — 09 August 2010

Response to Reviewers.

We thank both reviewers for their very helpful comments. We incorporated all their comments in the revised version, which clearly improved the manuscript.

Reviewer 1:
(1) Abstract: "...is understood at the genetic, cellular and molecular level" implies that to be understood,. all three levels are required. Perhaps the authors mean or rather than and?
This has been changed accordingly.

(2) Page 3: What is the evidence that "mutated and immobile transposon copies...are a considerable genetic burden"? In an organism where DNA synthesis rates no longer limit generation time, is this still true? Also, I don't understand what the authors mean by "several dozen different types of transposable elements." The Drosophila melanogaster genome harbors more than 200 distinct transposon families, but no more than four types (DNA, LTR-retrotransposons, non-LTR retrotransposons, and perhaps viral retrotransposons) that I can think of.
We agree that there is no evidence that the transposon load in animals constitute a genetic burden and therefore deleted this part. As for the transposon types, we changed this into "over a hundred different transposon families". The catalog at BDGP for natural transposons contains after removal of elements from other Drosophilid species a bit more than 100 elements.

(3) Page 4: miss: lack is more idiomatic (remains uncorrected from a previous version)
Changed accordingly.

(4) Page 5: flies lacking ovaries are not carcasses (i.e., one can genetically manipulate flies to lack ovaries and still get viable flies, as Table S1 demonstrates); carcasses are the fly after the ovary is manually removed.
Changed accordingly.

(5) Page 6: "loss of...and vasa does not"; I think the authors mean or rather than and, which would imply combining five mutations simultaneously.
Changed accordingly.

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(6) Page 9 and page 12: Rather than interact biochemically, the authors might consider writing interact physically.
Changed accordingly.

(7) Page 10: Figure 7B does not contain the sequencing data; it is in Fig. 5B.
Not changed as the data is shown in Figure 7B.

(8) Page 11 and legend to Figure S4: lose (i.e., was lost) not loose (i.e., needs tightening; this remains uncorrected from a previous version).
Changed accordingly.

(9) A description of the method of primer validation and the method of analysis or simply a reference is needed (e.g., Livak et al., 2001). How was the error of the control (actin or rp49) propagated through the error of the experimental values?
Thanks for pointing out this very valuable paper. We re-calculated all values based on that paper and cited it in the materials and method section.

(10) Figure 7C is missing x- and y-axes. Were only uniquely mapping piRNAs used to generate this figure?
Yes, Genome-unique mappers were used and we indicated this now in text and Figure legend; axes were added;

(11) Supplemental figures: does Mio mean million (M is more standard)? In Figure S2: HeT-A, not Het-A; in Figure S8: HeT-A, not Het-A or HetA
Changed accordingly.

(12) Table S2 should report (1) the total number of reads in addition to the total number of reads analyzed; and (2) the statistics should be provided for species, not just reads.
Changed accordingly.

From an earlier review round at a different journal, it was pointed out that our initial statement that Yb localization is less dependent on Armi than vice versa was not strongly supported by our data as we analyzed this in cells where we only depleted Armi by RNAi. We therefore analyzed Yb-localization in full armi mutants (armi[1] / armi[1]). This indeed showed that Yb accumulation in cytoplasmic foci strongly depends on Armi. This section was therefore changed accordingly in the revised manuscript and a new supplementary Figure (S5) was added.

Reviewer 2:
1) The authors present data on zuc loss-of-function material that differs from data published previously by Brennecke (Malone et al. 2009). The explanation of genotyping difficulties needs some further explanation for non-Drosophila geneticists. Genotyping issues would have resulted in heterogeneous results, not in an absence of a phenotype.
We were also puzzled by this obvious discrepancy to the Malone paper. Upon knowing the problem, we realized how difficult it is to genotype homozygous mutants with absolute certainty. We now give a more detailed explanation of this in the revised manuscript. We do agree that such a mixture (while explaining the molecular results in Malone et al. 2009) should have been obvious in the IF-stainings, but we do not have a better explanation.

Related to this: how does nuage behave in the zuc mutants? In the Malone et al paper it is described that Piwi still localizes to nuage, but with these new insights: is nuage present in zuc mutants (using for example a vasa or tudor staining)? This is relevant in relation to the perinuclear Piwi clouds observed in zuc mutant germ cells. Is this disintegrating nuage? As zuc may well play a role in the ping-pong cycle start-up, nuage may be severely affected. As in general, the precise appearance of nuage is quite sensitive to how one performs the experiment, it would be good to also check a background in which it is believed there is no nuage anymore (aub for example). This analysis would strengthen the comparison the authors try to make between the somatic and germ cell piRNA pathways in Drosophila.
We agree that this is an interesting issue. Our main point is that both, in germline and in somatic cells, loss of Zuc leads to a considerable co-localization of Armi and Piwi. In the soma, this is most apparent in the Yb-bodies. In the germline, the strongest signal arises from clouds around the

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nucleus, which we believe are distinct from nuage (see below). Even in wildtype germline cells, Armitage is not uniformly distributed in the cytoplasm and similar clouds are present (see also Cook et al. 2004). We therefore do not think that these clouds are caused by the loss of Zucchini per se. Indeed, nuage formation does not seem to be strongly perturbed in zuc mutants, as both, Aubergine and AGO3 still show the characteristic ring-like localization around the nucleus. This is now added as Supplementary Figure S10. We note, however, that AGO3 also strongly accumulates in cytoplasmic foci under these conditions, a pattern that is often seen in piRNA pathway mutants (e.g. spn-E or krimp mutants). Altogether, the new data indicates that the clouds stained positively for Piwi and Armi in zuc mutants are not disintegrating nuage.

2) The authors describe a partially RNA dependent interaction between Piwi and Armi. As an argument that the Rnase treatment worked well they say that no piRNA are observed in those samples. However, RNaseA is extremely stable and can remain a problem also after phenol extraction etc. Thus, the lack of piRNAs does not mean the RNase treatment went to completion during the IP. Actually, it doesn't mean a whole lot if no proper controls are taken along, such as synthetic RNA that is added to the samples after RNA isolation from the IP. I do not suggest the authors should do such controls, but I suggest this argument be removed from the text.
We agree that this is a difficult argument to make and therefore deleted it in the revised text.

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