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Hen1 is required for oocyte development and piRNA stability in zebrafish

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1st Editorial Decision 21 May 2010

Thank you for submitting your manuscript for consideration by The EMBO Journal. It has been evaluated by three referees and I enclose their reports below. As you will see from their comments the referees provide mixed recommendations regarding publication and as a consequence further experimental analysis is required before it can be further considered for the EMBO Journal. While, overall referee #1 and #2 are quite positive, referee #3 finds that the current study does not provide sufficient new insight into the mechanism of piRNA stability, which is a point that I also agree with. S\He states a number of ways that the current study could be expanded to make it suitable for The EMBO Journal. I do not think that all these points need to be addressed but some further novel insight how piRNAs are uridylated and/or the mechanism involved in their turnover should be provided. I would urge you to identify and characterize the poly(U) polymerase as has been done in a number of recent papers in other organisms. Given the interest in the study should you be able to address these issues, we would be wiling to consider a revised manuscript.

I should remind you that it is EMBO Journal policy to allow a single round of revision only and that, therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript. When you submit a revised version to the EMBO Journal, please make sure you upload a letter of response to the referees' comments. Please note that when preparing your letter of response to the referees' comments 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 your revision.

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1 (Remarks to the Author):

In this manuscript Kamminga et al explore the role HEN1 plays in zebrafish development. The authors convincingly show that the function of this protein is critical for germline development in female fish, where it is essential for oogenesis. Similar to previous findings, the authors show that HEN is essential for accumulation of piRNA that are methylated and are thereby prevented from undergoing adenylation and uridynlation (with the "anti uridynation" activity important for piRNA stabilization).

In general, the work presented in this paper is a very interesting and the results are convincing. I would therefore support publishing this work following some corrections and addressing one more central issue that has to do with the interpretation / presentation of the role Hen1 plays in sex determination.

1. The are several places where the language / writing should be improved. e.g. (there are more in the text)- In the Abstract- "We show zebrafish hen1 is specifically"- add "that" after "show". "..while dispensable for a functional.." - add "it is" after "while". In the Introduction "vertenrates" - "vertebrates". "As the mode of piRNA biogenesis responds to the actual presence of transposon transcripts, see below, this bias towards retro-elements likely reflect the inactivity of DNA transposons in vertenrates (Feschotte & Pritham, 2007)." - put the "see below" in parenthesis.

2. "The likely zebrafish homologue of the hen1 gene had previously been identified through bioinformatics analysis as..." I would not use the word "likely" unless there are specific strong reasons (that should be presented then) to doubt that it is the homologue.

3. The quality of Figure 3 is disappointing, in particular, the Hen1:GFP upper right panel where a lot of material appears out of the granules. Demonstrating the localization of Hen1 to the granules would maybe work better by co-expressing Hen1:GFP with another protein marker for these structures that is labeled with a different fluorescent protein.

4. "As shown in Figure 3C, only the CTD of Hen1 directs GFP to nuage, indicating that this region of the protein is responsible for the observed co-localisation of Hen1:GFP with Ziwi, possibly through direct interaction between the CTD and Ziwi (see Discussion)." - the fact that the N-term did not localize the GFP to the nuage is not a proof that it does not participate in doing that in the "non-GFP fusion" case of the normal protein (could also be a result of interference by the GFP fusion). I would simply state that the results of this experiment indicate that the CTD contains protein sequences sufficient for localizing the GFP to the nuage. Alternatively, change the "indicating" into "consistent with the notion that", which is more careful.

5. Referring to Figure 4A, the authors claim that the presented gel proves complete lack of splicing. This point would be strengthened by performing the RT PCR using the same 5' primer and a nearby 3' primer from the intron. If such a product cannot be obtained, presumably through nonsense mediated RNA decay, it would provide further support for the notion that the hen1 mutation and the resulting phenotype reflect the null situation. If the non-spliced RNA is retained, a truncated protein lacking only the CTD would be produced. The intracellular localization of such a protein was not examined. It would be interesting to determine if such a protein is localized to the nuage. If it does,

considering that the mutant protein cannot carry out its function, it means that the CTD is responsible for a function beyond the localization of the enzyme.
6. The results in Figure 5B and C require more explanation including a clear definition of the biological material used.

7. For Figure 6C, include statistical analysis, since in the current presentation, it is not clear which differences are significant.

8. "The results we present here describe zebrafish Hen1 as a factor required for ovary development, thus marking it as a protein involved in zebrafish sex determination." - I would not conclude that Hen1 is involved in zebrafish sex determination without additional evidence such as differences between presumptive males and females in its expression pattern before or during sex determination. Alternatively, if enhancing Hen1 activity would bias the sex ratio to have more females, it would support a role in determining the sex. As it stands, the results suggest that Hen1 function is critical for early ovary development and when that does not progress properly, the still plastic germline forms testis and the fish develop into males. In the discussion, the authors take the finding that Hen1 have germ cells and yet develop as fertile males as support for the statement that "This again contradicts a necessity for germ cells per se for female development.". The papers the authors cite clearly show that germ cells are absolutely necessary for female development (in contrast to the sentence taken from the discussion). Those previous finding do not mean that when germ cells are present you will necessarily differentiate into a female fish. Indeed, when the still bi-potential gonad is forced to develop as a male gonad (e.g. by hormone treatment (testosterone) or due to inability to differentiate into a female germline (in Hen1 mutants)), a fertile male is invariably formed. Reading this part of the discussion I am sure the authors understand this point. I would simply put Hen1 as a protein essential/required for proper early oocyte differentiation at a stage that is still plastic as far as sex determination is concerned, rather than claiming that the protein is involved in sex determination, which given the presented data would be a misleading statement.

9. It would be interesting (in the future rather than as part of revising this work) to determine if Hen1 mutant germ cells transplanted into wild-type hosts can develop only into testis (i.e. how autonomous Hen1 is in this context) the and if so whether such "forced" testis dictate male gonad and somatic development of the host.

10. In the discussion -"We found that the C-terminal region of Hen1, the CTD, is responsible for this localisation." -The CTD was found to be sufficient (not responsible) for this localization

11. The paper would benefit from an additional figure or panels with a schematic presentation of the role Hen1 plays in the piRNA pathway in zebrafish and the consequences of lacking Hen1 function on piRNAs and germ-cell development and sex differentiation.

Referee #2 (Remarks to the Author):

The authors of this manuscript describe the function of the Hen1 methyltransferase in zebrafish. They identify Hen1 as the enzyme that modifies piRNAs and they elucidate its function in germ cell development and sex determination in zebrafish. They show that Hen1 localizes to the nuage and this important finding solidifies the model that shows that piRNA biogenesis occurs in the cytoplasm and in association with nuage.

Furthermore, by deep sequencing of piRNAs from hen1 mutants they demonstrate that Hen1 is required for protection of the 3'-ends of piRNAs from uridilation. By a clever analysis of their sequencing data they show that hen1 protects piRNAs that actively target transposons. Overall the data are of high quality. There are many novel findings about the role of Hen1 described in this paper that are of interest to a broad audience.

Referee #3 (Remarks to the Author):

This manuscript continues the Ketting lab's analysis of piRNA biogenesis in zebrafish germ cells. piRNA production is Dicer-independent. Two models for piRNA biogenesis have been proposed: a Slicer-dependent "ping-pong" cycle and a ping-pong-independent "primary processing" pathway. Previously the authors showed that the ping-pong cycle of piRNA production is conserved in zebrafish. Extending their previous analysis of piRNAs, the authors investigate requirements of zebrafish HEN1, an RNA methytransferase, for piRNA production in germ cells. Hen1 in animals has been demonstrated to mediate 2'-O-methylation at the 3' end of piRNAs. The authors show that piRNA synthesis per se is not affected by loss of Hen1. However, loss of Hen1 results in marked reduction of piRNA levels. In addition, piRNAs in Hen1 mutants tend to become shorter and modified at their 3' end with A or U-tails.

This manuscript is technically quite good. However, with a few relatively small exceptions, this paper mostly repeats data already published by the authors' and other groups including Zamore, Mourelatos and Siomi labs though, with the use of next-generation sequencing technology, piRNA sequences in Hen1 mutants are more finely defined here.

Experiments demonstrating, for example, how piRNAs in Hen-1 mutants are uridylated at their 3' ends and how piRNAs with poly (U) tails are destabilized would only be suitable for EMBO J if they brought us closer to understanding molecular basis for the recognition of piRNAs by Hen1 and degradation of piRNAs by a poly (U) dependent mechanism. Unfortunately, this manuscript leaves all the big questions about the mechanism of action of Hen1 in piRNA recognition and degradation unanswered. How might Hen1 be recruited onto piRNAs to modify their 3' ends? How is poly(U) added to their 3' ends? How and where in the cell do polyuridylated piRNAs get degraded?

Several typographical errors:
1. 4 lines from the top in page 4: vertenrates -> vertebrates
2. 8 lines from the top of the 2nd paragraph in page 17: (Ibrahim et al) -> (Ibrahim et al. 2010)
3. References: in both Katoh T. et al. and Ohara T et al, the senior author's name (Suzuki T) is missing.

Additional correspondence (from author) 25 May 2010

Again, thanks for the fast review process. I could not have hoped for things to move faster. As briefly mentioned last week, I would contact you to discuss potential experiments we can do to address the reviewers. I thought I would put it into writing so you can look at it when it suits you best. However, I would be happy to discuss this further by phone. Please let me know what you would prefer.

Reviewer 1 raises a number of relatively minor issues regarding Hen1 localization. This we will address these with Hen1 fragments tagged with GFP at the N-terminus, instead of the currently used C-terminus, to diminish the chances that the GFP tag has a strong impact on the observed subcellular localizations. The rest we can address textually.

As you mention in your letter, reviewer 3 has the most substantial comments when it comes to suitability for The EMBO J. This reviewer suggests that the novelty of the current manuscript may not be sufficient and describes our sequencing analysis as refinement of already published data. While we clearly build on published work, I would first like to stress the novelties of the current manuscript and then continue to discuss what we could do to further extend our work.

First, the papers referred to by the reviewer describe piRNA destabilization in hen1 mutants, but do not experimentally link this to 3'end modification by terminal transferase activities. We describe both adenylation and uridylation as previously unknown processes acting on piRNAs in the germ cells of animals. Furthermore, we link uridylation, and not adenylation, with the observed piRNA destabilization. These findings may have been expected, based, for example, on our own work on C. elegans siRNAs, but this has never been experimentally shown. Whenever people describe uridylation and piRNAs they would have to cite this manuscript. In addition, we place Hen1 at the nuage, targeted there through the C-terminal tail of Hen1, again a previously unknown property of

this poorly conserved Hen1 domain. These are the reasons why we felt, and still feel EMBO J is suitable for this manuscript. However, to further boost novelty, reviewer three suggests that we should provide more insight into how Hen1 is recruited to piRNAs, how uridylation takes place and/or how piRNA destabilization works. These are certainly relevant questions and actually we partially already meet those in the current manuscript. Below I will explain that and describe possibilities how we can push novelty further along the suggested lines.

Regarding the first point: we describe Hen1 is localized in sub-cellular compartments where piRNA processing is believed to take place (also mentioned by reviewer 2). This refines the co-IP experiments between Piwi and Hen1 published before, by placing this interaction in nuage, mediated by C-terminal Hen1 sequences.

With regard to how piRNAs are uridylated you suggest in the decision letter to identify the transferase enzyme. However, this is no easy thing, and will not be possible within the allotted (and desirable) timeframe. If one wants to do this conclusively, this would require identification and breeding of mutants in all terminal transferase candidates, based on published work (for example zebrafish mutants of homologues to the C. elegans cde-1 enzyme), and hope you hit the right gene. Apart from the fact that we would have to be lucky to find all mutant alleles in our current zebrafish mutant library, this would take up to 9 months to complete if everything works in our favor.
In fact, we have already screened for alleles of one terminal transferase enzyme but unfortunately were not able to find a mutant allele in our library. The fast alternative, morpholino oligos, are not possible since we have to target germ cells in the adult, or at its earliest at 3 weeks of development, long past the active period of morpholino oligos (5 days).
What we can do is RNA expression analysis of potential candidates and hope one/some display(s) a striking gonad specific expression pattern (like cde-1 in C. elegans). Recombinant expression of the relevant catalytic domain(s) may then show nucleotide preference of the enzyme(s). Protein localization studies, which we may attempt by injecting GFP fusion constructs into primordial germ cells, may actually show the likely place of piRNA uridylation. All this would also remain correlative, but would pinpoint future genes of interest with regard to piRNA uridylation and adenylation.

Regarding the last point raised by the reviewer (piRNA breakdown) we can further dissect our piRNA sequencing data to see whether we can see evidence for 3'-5' or 5'-3' directional degradation. For example, a 3'-5' degradation signature would imply the cytoplasmic exosome in piRNA degradation. However, biochemical verification that indeed the exosome targets the uridylated piRNAs in vivo will be impossible to obtain.

I hope I have made clear how we can extend our work to meet the raised concerns. I'd appreciate it if you could let me know whether you think the suggested additions are likely to make our manuscript acceptable for The EMBO J.

Additional correspondence (from editor) 28 May 2010

Sorry it has taken so long to get back to you, but it has been a horrifically busy week, I am happy to have time to sit down and carefully read through your email and give it some thought. I appreciate that identifying the transferase enzyme may be difficult and that morpholinos are not amenable to germ cells and I agree that the approach that you suggest based on expression, localisation and some biochemical activity of candidate transferases would be sufficient. In addition some further insight into the degradation patterns would be acceptable. So I agree that the additions that you suggest would address the concerns of referee #3. If you would like to discuss this further by phone I am in the office Mon before leaving for CSHL for a week.

Additional correspondence (from author) 29 May 2010

Thanks for your mail. We'll do these expts.

1st Revision - authors' response 26 August 2010

Rebuttal to reviewers' comments on manuscript EMBOJ-2010-74688.

First we wish to thank all three reviewers for their thoughtful comments on our work. We have revised our manuscript using the comments of reviewers 1 and 3 as a guideline and below we have dealt with the individual comments individually.

Reviewer 1

1) Adjusted textually.

2) We prefer to maintain the term 'likely homologue' until proven it functions as such.

3) Figures 3A and B have been updated with new experiments, now with GFP fused to the N-terminus of Hen1 and Hen1 fragments. This results in better expression, and thus clearer pictures. Co-localisation with a known nuage component, as the reviewer proposed, is shown in Figure 3B.

4) Adjusted textually.

5) We now include in Figure 4A RT-PCR analysis of the 5' region of the transcript showing this part of the *hen1* mRNA is still detectable, showing it is not subject to nonsense-mediated decay. The protein resulting from such an mRNA would be very similar to the N-terminal fragment tested in Figure 3C, which does not localize to nuage.

6) Adjusted textually.

7) Statistical analysis is now included.

8) The text dealing with the issue of sex determination has been adapted to take out the misunderstanding pointed out by the reviewer.

9) We agree and the germ cell transplantation technique is currently being applied to a number of mutants in our laboratory.

10) Adjusted textually.

11) We have considered this but we could not find a clear way to illustrate the quite subtle phenotypic effects triggered by loss of Hen1, and we thus refrain from capturing our results in a simplified model.

Reviewer 3

This reviewer has questions about the novelty or impact of this work in relation to previously published data. First, I wish to note that the papers referred to by the reviewer describe piRNA destabilisation in *hen1* mutants, but do not experimentally link this to 3'end modification by terminal transferase activities. We describe both adenylation and uridylation as previously unknown processes acting on piRNAs in the germ cells of animals. Furthermore, we link uridylation, and not adenylation, with the observed piRNA destabilisation. These findings may have been expected, based, for example, on our own work on *C. elegans* siRNAs, but adenylation and uridylation of RNAi-related small RNAs is still a very poorly covered topic. We for the first time place these processes firmly in the piRNA pathway in a vertebrate.

Furthermore, we answer, at least to some extent, the three questions posed by the reviewer that would in his/her eyes make the work suitable for The EMBO Journal:

1) How might Hen1 be recruited onto piRNAs to modify their 3' ends?

We place Hen1 at nuage, making that a likely site for 3' end methylation. In addition, newly added data in Figure 3A indicate that in fact a C-terminally truncated Hen1 protein may be produced by the *hen1(sa0026)* allele. As we show that Hen1 lacking the CTD does not localise to nuage and *hen1(sa0026)* does not support piRNA methylation, this suggests that nuage localisation is in fact required for its function.

2) How is poly(U) added to their 3' ends?

We now include expression analysis of potential candidates involved in piRNA uridylation and show that three good candidates involved in adenylation/uridylation (TUTase2, 4 and 7) are indeed expressed in germ cells (TableS4, Figure S6 and Figure S7). Showing which of these enzymes is indeed involved requires mutational analysis, and the extent of the work and analysis required would make it a completely independent story by itself.

3) How and where in the cell do piRNAs get degraded?

We now include bio-informatic analysis strongly suggesting that degradation of unmethylated piRNAs occurs in a 3'-5' direction (Figure 6B). This suggests that the cytoplasmic exosome is involved. Detailed testing of this idea would require experiments currently not feasible in any vertebrate system, partly due to the fact that *in vitro* cell culture systems faithfully executing the Piwi-pathway are not available.

Typographical errors have been corrected.

