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A systematic analysis of Drosophila TUDOR domain-containing proteins identifies Vreteno and the Tdrd12 family as essential primary piRNA pathway factors

Dominik Handler, Daniel Olivieri, Maria Novatchkova, Franz Sebastian Gruber, Katharina Meixner, Karl Mechtler, Alexander Stark, Ravi Sachidanandam and Julius Brennecke

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Transaction Report:

(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 13 July 2011

Thank you for submitting your manuscript for consideration by the EMBO Journal. It has now been seen by two referees whose comments are enclosed. As you will see, the referees express interest in your study and are broadly in favour of publication, pending satisfactory minor revision. Given the referees' positive recommendations, I would like to invite you to submit a revised version of the manuscript. I should add that it is EMBO Journal policy to allow only a single round of revision, and acceptance of your manuscript will therefore depend on the completeness of your responses in this revised version.

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, 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 REPORTS

Referee #1:

The manuscript by Handler et al describes their comprehensive analysis of all Tudor-domain containing proteins in Drosophila. This study provides a clear classification of all the tudor domains based on available structural data and will serve as a key reference for fly tudor proteins and their mouse orthologues. They use their previously described soma-specific RNAi knock-down strategy and two new germline-specific knock-down approaches to individually deplete all the identified fly tudor proteins in the two compartments.
A novel Tudor protein named Avocado was further characterized in detail and shown to participate both in the somatic and germline piRNA pathway. Depletion of Avocado by RNAi activates both germline and soma-specific transposons. A similar observation is noted in a strong loss of function allele of avocado, which the authors generated. Deep sequencing analysis of small RNAs from avocado null mutants shows loss of reads from both soma and germline specific clusters, confirming its role in both piRNA pathways. Using a combination of genetic, cell biology and biochemical evidence they show that in the ovarian somatic cells, Avocado is in a complex with another tudor protein Yb and a putative RNA helicase called Armitage. While in the germline cells, this complex is slightly different with Yb being replaced by two new tudor proteins (jackfruit-1 and 2). The importance and redundance of the two jackfruit proteins is demonstrated by strong activation of transposons only in response to a double knock-down strategy. In sum, this study adds new components to the piRNA pathway and provides evidence for a biochemical platform built on the back of tudor proteins for proper functioning of piRNA pathway in fly ovaries.

Minor comments
1. Intro: Liu et al is repeated twice (4th line, page 2).
2. In the first para of Results, rearrange Fig S1-3 to fit with the flow. As of now Fig. S3 is described first. Also later in page 3.
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5. In Fig. 1A, add a new column to indicate the tudors implicated in the piRNA pathway. Label CG4771 as Avocado.
6. Generation of avocado allele. Fig. 1B should be Fig. 3B, Fig. 2A here should be 3B. The genomic rescue construct panel is not cited in the text. Check panel labels in Fig. 3.
7. I would say that the experiment in Fig. 7E does not show that Armi and Avocado interact with each other. One can say that they are together in a complex, which is the important point. Also the title of the section states "physically" interacts, which can be modified to indicate their presence in a complex.
8. The quantitative mass spec analysis of GFP-Avocado complex can be shown in the supplementary data.

Referee #2:

Comments on Handler et al. A systematic analysis of Tudor domain containing proteins identifies avocado and the Tdrd12 orthologs jackfruit-1 and jackfruit-2 as essential factors for primary piRNA biogenesis

The piRNA pathway has been implicated in various processes important in germline and embryonic development. Though many the tudor domain proteins have been reported to exert essential roles in piRNA pathway, their molecular roles in piRNA are not completely understood. In this study, the authors attempt to screen Drosophila tudor domain proteins

by tissue-specific knockdown approach. Among those exhibiting defects in piRNA pathway, they proceed on characterization of avocado, CG4771, which shows sever defects both in somatic and germline piRNA pathways. They show that avocado is required for primary processing of piRNAs both in the germline and somatic cells of the Drosophila ovaries. Furthermore, they also did some analysis to suggest that Jackfruit-1 and Jackfruit-2 function as the germline counterpart of the somatic Yb body. The manuscript provides comprehensive work focused on Drosophila tudor domain proteins and the subject will be interesting to readers of the EMBO Journal. Especially, detailed analysis of piRNAs and the comparison with armitage and zucchini mutants shed light on molecular mechanism of primary piRNA production of germline piRNAs, which still remains elusive and is of great interest of the field. I have some comments below, which the authors may wish to consider to improve this excellent work prior to publication.

Major comments:
1. For the RNAi and insertion mutant experiments, it is important to show the efficiency of knockdown and discuss the possibility of potential off-target effects. For example, in Figure 3C, Figure 7D and Figure 8D, the extent of the RNA or protein present in the respective RNAi or mutant experiments could be shown in the Supplementary Data.
2. More information of the avo excision mutant, delta1, should be described in the results or material and methods. Where is the deletion located? Northern blotting, RT-PCR and/or western blotting should be done to examine the presence of Avo mRNA/protein in the mutant.
3. The rescue construct that the authors used include nearby genes like CG6985 and HP1c. Can author discuss whether inclusion of such genes can affect the rescue, or why not try the rescue experiment using only the avo transgene.
4. Is avo required for transposon silencing, piRNA production in the males? They can use Stellate and su(ste) piRNA as readouts.
5. The authors suggest that Jackfruit-1 and Jackfruit-2 function redundantly. As both have different effects on the localization/expression of Piwi in the germline cells, and both actually localize differently, it is difficult to imagine that they are redundant. Can one functionally replace the other? Furthermore, no piRNA analysis is done on those RNAi lines, how confident are the authors that they function in piRNA biogenesis?
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2. At the end of the introduction, the authors mentioned about Krimper but no corresponding reference is cited.
3. Grammatical errors can be found in entire manuscript, which need to be amended.

1st Revision - Authors' Response 01 August 2011

Response to Referee Comments:

We thank both referees for their helpful comments, which we addressed in the revised version (see point by point response below). We made several changes to the text and figures to improve the accessibility of the data and included the requested experiments (see below).

We changed the naming of the identified genes (CG4771, CG11133 and CG31755). CG4771 has been named '*vreteno'* in FlyBase in the meantime by the Lehmann group. We adopted this name and in light of this change, we also changed the naming of CG11133 and CG31755 to 'Brother of Yb' and 'Sister of Yb' to indicate their relationship to Yb.

List of significant changes to the previous submission:

1. We added additional experiments to clarify the specificity of the *CG4771[Δ1]* allele (Figure 3).
2. We removed the alignment of the CG4771 MYND domain due to space constraints, as this is not relevant for this study.
3. We added in Figure 8 data on GFP-Yb localization to illustrate expression and sub-cellular localization of all three fly Tdrd12 proteins. We further clarified the requirements of the Tdrd12 proteins for the somatic piRNA pathway.

Point by point response:

Referee #1:

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counterpart.

Corrected; more detail on the orthology assignment is given in the new text

4. Fig. 1 and Fig. S1. Please check CG17454/15930/15042 in Fig. 1 and the same in Fig. S1. Is there a mix-up?

Thanks for spotting this; corrected

5. In Fig. 1A, add a new column to indicate the tudors implicated in the piRNA pathway. Label CG4771 as Avocado.

This has been added; to avoid confusion at this point, we named CG4771 only 'vreteno' after we introduced the name in Fig 3.

6. Generation of avocado allele. Fig. 1B should be Fig. 3B, Fig. 2A here should be 3B. The genomic rescue construct panel is not cited in the text. Check panel labels in Fig. 3.

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We understand the point raised by the reviewer. However, with "physical interaction" we do not imply that two proteins interact directly with each other. We considered changing this to the suggested 'complex'. However, as multiple independent Vreteno complexes might exist in the cell with different composition, we prefer to stay with the 'physical interaction' phrase, which does not imply direct interactions.

8. The quantitative mass spec analysis of GFP-Avocado complex can be shown in the supplementary data.

Added as Table S3.

Referee #2:

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Major comments:

1. For the RNAi and insertion mutant experiments, it is important to show the efficiency of knockdown and discuss the possibility of potential off-target effects. For example, in Figure 3C, Figure 7D and Figure 8D, the extent of the RNA or protein present in the respective RNAi or mutant experiments could be shown in the Supplementary Data.

We fully agree and added several data points to indicate the knockdown efficiencies. For the systematic mini-screen presented in Figure 2, we tried to minimize off-target effects and non-functional hairpins by testing several different knockdown systems (soma versus germline and VDRC versus shRNAs).

For the more detailed analyses of CG4771, CG31755 and CG11133, we provide antibody stainings were possible (CG4771) and added QPCR measurements (CG31755 and CG11133).

2. More information of the avo excision mutant, delta1, should be described in the results or material and methods. Where is the deletion located? Northern blotting, RT-PCR and/or western blotting should be done to examine the presence of Avo mRNA/protein in the mutant.

As indicated in the materials and methods part, the deletion is internal and deletes only P-element sequences including the entire white gene. No flanking genomic regions on either side are affected. We suggest, that a weak internal promoter located downstream of the mini-white gene of the P-element is responsible for the low levels of CG4771 product in the P-insertion as has been shown for other P-insertions by Lafave & Sekelsky (2011). This is deleted in the CG4771[Δ1] allele, probably explaining the stronger phenotype.

We added a QPCR of CG4771 transcript levels in ovaries (Fig. 3) and testes (Fig. S4) showing that CG4771 RNA levels are reduced more than 20fold in the Δ1 mutant. We also added an immuno-fluorescence staining of CG4771 protein in Δ1 mutant ovaries (Fig. 3) showing barely detectable CG4771 protein levels.

3. The rescue construct that the authors used include nearby genes like CG6985 and HP1c. Can author discuss whether inclusion of such genes can affect the rescue, or why not try the rescue experiment using only the avo transgene.

We fully agree with this concern. We therefore measured by QPCR the transcript levels of the two flanking genes (which are included in the rescue construct) in ovaries of the *CG4771[Δ1]* allele in comparison to wildtype ovaries and to *CG4771[Δ1]* ovaries expressing the rescue transgene (Fig. 3). This indicated that the *CG4771[Δ1]* allele specifically affects only the *CG4771* locus.

4. Is avo required for transposon silencing, piRNA production in the males? They can use Stellate and su(ste) piRNA as readouts.

As primary piRNA biogenesis in males is only poorly understood (see for example Nagao et al; RNA, 2010), we believe that a clarification of this requires a much more in depth analysis. Nevertheless, we measured *copia* and *Stellate* transcript levels in *CG4771[Δ1]* mutants and show in Fig. S4, that *CG4771* is required for *Stellate* and *copia* silencing in males.

5. The authors suggest that Jackfruit-1 and Jackfruit-2 function redundantly. As both have different effects on the localization/expression of Piwi in the germline cells, and both actually localize differently, it is difficult to imagine that they are redundant. Can one functionally replace the other? Furthermore, no piRNA analysis is done on those RNAi lines, how confident are the authors that they function in piRNA biogenesis?

We carefully repeated the knockdown experiments for CG11133 and CG31755. This indicated that knockdowns of both genes individually result in defective Piwi localization in only 3-5% of egg chambers. This is now stated in the text. Therefore knockdown of both proteins results in similar defects in terms of Piwi localization. We agree that the different

protein localization of CG11133 and CG31755 is intriguing and argues against a strict redundancy. We therefore re-phrased the text. Nevertheless, genetically the two proteins are clearly acting redundantly in terms of Piwi localization and therefore presumably primary piRNA biogenesis. Due to the lack of genetic alleles for CG31755 and CG11133, we decided against piRNA sequencing. We agree that we cannot formally conclude that CG31755 and CG11133 are required for primary piRNA biogenesis and rephrased this to 'essential for the primary piRNA pathway'. We base this on the observation that the CG11133/CG31755 double knockdown impairs Piwi levels and localization to an extent indistinguishable to the one observed in *armi, zuc* or *CG4771* mutants.

6. The authors mentioned that Avo interacts with Jackfruit-1 and Jackfruit-2, but no data is shown. Can the authors show the data? Co-localization of Avo with Jackfruit-1 and Jackfruit-2 should be also addressed.

We added the requested data as Table S3. Due to the superiority of the Armitage antibody in comparison to the Vreteno antibody, we performed the requested co-localization experiments with Armitage, which based on Fig. 3 co-localizes with Vreteno in soma and germline.

Minor comments:

1. Recently, a number of reports had shown that piRNA pathway plays other roles in maternal mRNA degradation, chromosome condensation and segregation and canalization. To achieve balance of references, the authors can consider including those in the introduction to emphasize the importance of the piRNA pathway.

We cite 63 references in the manuscript, all of which we feel are relevant to the topic of the paper. We therefore prefer to not add the suggested additional references as these are not related to the content of the study. We hope that Reviewer 2 understands the space limitations.

2. At the end of the introduction, the authors mentioned about Krimper but no corresponding reference is cited.

Reference has been added

3. Grammatical errors can be found in entire manuscript, which need to be amended.

We hope that the revised text has addressed all of the instances that led to this comment.

2nd Editorial Decision 02 August 2011

Thank you for submitting your revised manuscript. I have read through the reports and your responses and find that you have satisfactorily addressed all the concerns raised.

I am happy to accept the manuscript for publication in The EMBO Journal. Please send us the conflict of interest and author contribution statements as soon as possible(we need this to transfer the manuscript to the publishers). You will receive the official acceptance letter by the end of tomorrow.

Yours sincerely,

Editor
The EMBO Journal

