THE EMBO JOURNAL

Manuscript EMBO-2011-77885

Tdrd1 acts as a molecular scaffold for Piwi proteins and piRNA targets in zebrafish

Hsin-Yi Huang, Saskia Houwing, Lucas J. T. Kaaij, Amanda Meppelink , Stefan Redl, Sharon Gauci, Harmjan Vos, Bruce W. Draper, Cecilia B. Moens, Boudewijn M. Burgering, Peter Ladurner, Jeroen Krijgsveld, Eugene Berezikov and René F. Ketting

Corresponding author: René F. Ketting, Hubrecht Institute

Review timeline:

Submission date:	13 April 2011
Editorial Decision:	10 May 2011
Revision received:	22 May 2011
Editorial Decision:	08 June 2011
Revision received:	09 June 2011
Accepted:	10 June 2011

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 10 May 2011

Thank you for submitting your manuscript for consideration by The EMBO Journal. It has been now been evaluated by three referees and I enclose their reports below. As you will see the referees find the role of Tdrd1 in piRNA biogenesis and especially the identification the TATs to be interesting, however, they do require further experimental analysis to make the study suitable for The EMBO Journal. The main issues are the clarification of the role of Tdrd1 in binding RNA, while the interaction with Ziwi/Zili and piRNA seems to be the most likely scenario, it seems prudent to demonstrate that Tdrd1 can not directly bind RNA as suggested by referee #1. Given the interest in the study, should you be able to address the concerns we would be happy to consider a revised version of the manuscript for publication in The EMBO Journal.

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 #1:

In this manuscript, Huang et al. describe zebrafish Tdrd1 for the potential function in the production of piRNA. As reported for mouse Tdrd1, zebrafish Tdrd1 also localizes to perinuclear nuage, where Piwi-family proteins involved in ping-pong cycle to generate piRNAs are found. Though the effect on germ-line development is rather mild compared to ziwi and zili mutants, tdrd1 mutant gonads exhibit clear defects in the generation of piRNAs. As reported in mice, zebrafish Tdrd1 binds to Ziwi and Zili, and is found to piRNA. Furthermore, they report a novel RNA population bound to Tdrd1 (TAT). Those are derived from the piRNA producing loci, and the authors argue that piRNAs and TATs represent guide-taget-RNA interaction.

In summary, this study provides substantial evidence to support the molecular function of zebrafish Tdrd1 in piRNA generation, confirming the previous findings of mouse Tdrd1. Though the manuscript would be a great interest of the field, it appears lacking further understanding of molecular function of Tdrd1, which has been reported in mice. The author may wish to streamline to get more focus on their new findings which could give impact to the field. There is no good link connecting domain analysis using GST-Tdrd1 and in vivo analyses. I suggest extending the domain analysis (not only tudor domains but also MYND domain) to examine if they can bind to TATs, which distinguishes this manuscript from other studies. Though Zili and Ziwi can bind to Tdrd1, why no TATs are found with Zili IP? Even with longer exposure? Their model appears predicting a ternary complex of TATs, Tdrd1 and Ziwi or Zili with piRNA in the process of piRNA production. Then Tdrd1 does not bind directly to piRNA, but it holds piRNA only via interaction with Ziwi and Zili, although the interaction is not RNA-dependent manner. The author could examine whether purified Tdrd1 can bind piRNA as well as long RNA in vitro. How about stoichiometric ratio of the Tdrd1-Piwi family protein complex, and the relationship among them with respects to binding ability to TATs? In my personal opinion, MYND domain should be examined for any function, too. Although tudor domain is reported to bind sDMA on the Piwis, there's no reason why a possibility that MYND interacts with those can be excluded. Overall, the text could be much improved. Some phrases are not clear to convey the authors' points. I hope the authors find this general comments and specific comments listed below useful to improve the manuscript.

Major comments

Pg 7, During development, Tdrd1 protein becomes detectable in the primordial germ cells (PGCs) at four days post fertilization (4dpf; Fig. 1C and S1D).
Does Tdrd1 go to nucleus? Or are they on the surface of the nuclear envelope showing perinuclear foci?

Pg 8, MYND domain should be briefly introduced, at least as a zinc finger domain. As commented above, this domain should be also examined for the interaction with Piwi-family proteins.

What about Tdrd1 localization in ziwi mutant? Reciprocally, is cytoplasmic Ziwi affected in tdrd1 mutant?

Figure 2E-F, mitochondria on EM images is not visible. Pictures with better resolution should be provided. What is the sample size in F?

Pg 11, in Figure 3B, it might be better to compare normalized transcript levels rather than fold-change as there appears to be only a 2- to 3-fold increase in transposon levels for EnSpmN1, Ngaro1 and Polinton1, and t tests should be conducted. Why were these five transposons selected? Which ones are RNA- and DNA-elements?

Along with this line, the author stated as the extent of transcript upregulation and piRNA decrease for these elements match rather well (Fig. S5), although we cannot make general statements regarding such correlation based on five individual elements.
If the authors have mapped their piRNA sequence reads to retrotransposons and DNA transposons, they could identify specific transposons that possess the most severely affected piRNA reads, and examine the concomitant upregulation of the specific transposons.

Pg 12, Interestingly, the Tdrd1 bound piRNA length profile is intermediate between the Ziwi and Zili profiles (Fig. 5B), suggesting roughly equimolar amounts of Ziwi- and Zili-bound piRNAs in Tdrd1 IPs.
How can the authors exclude the possibility that Tdrd1 binds both to Ziwi and Zili at the same time? Do Zili and Ziwi compete the binding to Tdrd1?

Minor comments

Pg 3, an electron dense, germ cell-specific structure associated with mitochondria...
The predominant subcellular localization of the nuage in all metazoans studied so far is perinuclear, and its association with mitochondria is not well documented in some systems.

Pg. 3, Nuage is known to associate with clusters of nuclear pores (Pitt et al, 2000), suggesting that nuage has a function in the trafficking of molecules between the nucleus and the cytoplasm.
Nuage association with clusters of nuclear pores has only been reported in C. elegans so far. In my opinion, the trafficking function of the nuage cannot be generalized until this association is confirmed in more systems.

Pg. 7, Using Western blotting analysis we can detect Tdrd1 expression in testis and immature oocytes (Fig. 1A and S1A-C).
S1A-C do not show Western blotting, but only RT-PCR and immunostaining.

Fig.1C incorrectly labeled as Fig.1B in statement. Tdrd1 immunohistochemistry on tdrd1 mutant cells shows no detectable staining (Fig.1B)

Fig.1C, it will be better to use a different pseudocolour for Tdrd1 as Vasa-GFP is shown in the same figure and might be confusing at first. Label as Vas::EGFP in the images would be helpful, in the other images as well (eg FigS1).

Figure 2D, what is in green? It was not stated in the legend. Vas::EGFP? If this is not relevant, the authors can take it out.

Pg.9, First mention of C. elegans should be spelt out in full.

Pg. 11, Add 'with testis lysate' to, Co-IP experiments using zebrafish Tdrd1-specific antibodies confirmed that Tdrd1 binds, directly or indirectly,

Having schematic drawings for Fig C and D would be a great help for readers.

Figure 4D, signal for GST-tagged protein appears to be saturated (white spots in the bands). The authors can appropriately adjust them.

Pg 13, a spelling error "therefor"

Referee #2:

A diverse set of Tudor domain containing proteins play essential roles in the animal piRNA pathway. These proteins are proposed to bind to key factors of the pathway (PIWI proteins and VASA) via their Tudor domains, which recognize symmetrically methylated Arginine peptides.
In this manuscript, Ketting et al. characterize zebrafish TDRD1 genetically and

molecularly/biochemically and present the following key findings:

1. Different Tudor domains of TDRD1 appear to exhibit specificity to methylated peptides conferred by the sequence surrounding the methylated Arginine. This extends previous findings by the Siomi lab and suggests that there is a sequence code for the Tudor domain - sDMA-peptide interaction.
2. TDRD1 complexes contain both zebrafish PIWI proteins and importantly, RNA molecules that likely represent processing intermediates and/or target RNAs.

This manuscript has an overall high quality. The findings are well documented, the conclusions are adequately supported by the presented data and the results are discussed in light of the recent literature in an overall accurate manner.

Given the above mentioned major findings, I support publication of this manuscript after the following minor revisions:

1. "We show that Tdrd1 binds both RNA and protein components of the Piwi pathway and that in absence of Tdrd1 the Piwi pathway operates with a much lower efficiency."
this sounds as if TDRD1 interacts directly with RNA, a rather unlikely scenario. The authors should be more specific in terms of the envisioned model how TDRD1 complexes interact with target RNAs.

2. Some graphs in the Supplement show small RNA length and ping-pong signatures as continuous lines. These should be bar diagrams as there are no intermediate values.

3. Figure 6C: The authors state in their text that the frequent overlap of piRNAs and TATs starting from nt 23 onwards could result from PIWI-target interactions where the target has not yet been sliced. Does the distribution in 6C remain at high values after 29nt? And if not, what would that mean?

4. The title is rather strong in suggesting that TDRD1 interacts directly with RNAs. Would it not be better to stress the scaffolding function of TDRD1?

5. The authors state in their manuscript that the libraries from RNA obtained from TDRD1 immunoprecipitates have not been size selected. How can the authors in this case state that the discrete bands observed in the gel are TATs and not degradation fragmen's of abundant cellular RNAs? For example, the library composition shows a substantial amount of ribosomal RNA. It would be rather important to clarify this issue as currently, the authors discuss a model in which some unknown activity trims TATs to a rather specific size.

Referee #3:

Small RNA-mediated transposon silencing is critical for germ cell lineages in worms, insects and chordates. Piwi proteins, Piwi-interacting RNAs (piRNAs) and a cadre of accessory proteins (many of which contain tudor-domains) comprise a conserved piRNA pathway. The study by Huang et al. identifies zebrafish tudor-domain containing Tdrd1 as an important component of transposon silencing in this organism and provides new insights by nicely revealing subcellular localization of nuage structures and identifying specific roles of individual tudor domains for interaction with Zili. Of particular significance, however, is the identification of Tdrd1-associated transcripts (TATs) that hint at possible upstream events in the process of piRNA biogenesis. Overall, this work expands our understanding of the mechanism of piRNA biogenesis and transposon silencing, and could be accepted for publication without further revisions following minor proofreading of the supplemental material text.

1st Revision - authors' response 22 May 2011

First, we would like to thank the reviewers for their thoughtful comments on our work. They have cearly helped to improve our manuscript.

Reviewer 1

General comments

We would like to stress that while important work on Tdrd1 in mice has indeed been pubished, none of those studies provided evidence for a scaffolding function like we propose, nor did they report the potential piRNA precursors, as we do. We therefore disagree with the suggestion that we just confirm previous findings of mouse Tdrd1. We do, of course, confirm some of it to show the relevance of our model in this field.

One of the novel aspects is our demonstration of differential affinity for the different tudor domains of Tdrd1 for Zili and different methylated arginine sites in Zili for Tdrd1. These two approaches complement each other and we think they fit well in the paper.

The reason we (and others) have not see TATs in direct Piwi IPs may be due to the fact that small RNA libraries are usually size selected. One of our Tdrd1 IP libraries was not. Whether TATs would be visible in straight Piwi IPs is an open question. They could very well be. We describe them in association with Tdrd1 and we show that they are good candidates for piRNA biogenesis intermediates. This makes them very interesting and this is why we thing they should be published as such. Biochemical data claiming that TAT-like molecules can indeed be processed to mature piRNAs is circulating and we do not want to prolong publication of our findings.

We do not believe that Tdrd1 binds TATs directly. This is now explicitly state in the discussion, and reflected in a model. However, to experimentally prove that Tdrd1 does NOT bind RNA would be very difficult. First, an experiment showing that *in vitro* made Tdrd1 does not significantly bind RNA may be cocnsidered of little value for the *in vivo* situation. Second, none of the individual domains of Tdrd1 likely bind RNA.

Stoichiometric analysis of Tdrd1 complex components is most surely interesting, but using our IPs from whole tissues and analyzing protein contents with western blot this will not be possible. The cell-based systems now available to study the Piwi pathway may enable experiments addressing such issues.

In our opinion, detailed analysis of the MYND domain deserves much more attention than we can give in the context of this manuscript. We consider that outside the scope of this work.

We have made adjustments throughout the text to improve clarity.

Specific comments

-Tdrd1 is predominantly cytoplasmic. We describe this inthe text and add a novel FigS2 to illustrate this better. We cannot exclude nuclear localization of a fraction of the Tdrd1 pool.

-We now introduce the MYND domain in the introduction.

-Unfortunately, the very strong maternal load of Ziwi (i.e. present before Tdrd1 is expressed and lasting until two weeks of development) prevents meaningful localizaiton experiments in *ziwi* mutants. Unfortunately, analysis of *tdrd1* mutant animals is currently not possible due to problems

with our animals. Despite recurrent trials, the available *tdrd1* mutant family does not provide offspring from incrosses for unknown reasons. We are raising a new family but it will take at least 10-12 weeks before these will be fertile. While potentially interesting to look at we do not think this experiment is important enough to hold back publication this work for this long. Especially given the circulating work mentioned above on the *in vitro* processing of TAT-like molecules into mature piRNAs.

-We include an additional supplemental figure (current Fig. S4) to show mitochondria. The numbers of oocytes analyzed are now indicated in the legend.

-A t-test (two-tailed) has been performed on the qPCR data. All changes observed are significant ($p<0.05$). We have included these p-values in the figure. We also indicated DNA and RNA transposons in the legend.

-Relating the drop in piRNA level and upregulation of transcript level will require analysis of many transposons. We have chosen these five based on reliable qPCR results (it is not trivial to design good qPCR primers for repetitive elements). In fact, I-1 is one of the elements with the strongest drop in piRNAs and also shows the strongest reponse. We are of the opinion that our data indicate that loss of Tdrd1 mildly affects transposon transcript levels and that is what we want to bring across. Detailed analysis of transposon transcript levels genome wide will be a significant task indeed; a project on itself as not only simply piRNA and transcript levels should be studied but also transcript stability and the uniqueness of the observed transcripts and piRNAs (since we are dealing with repeats). This clearly goes beyond our current scope.

-We cannot exclude that Tdrd1 binds Ziwi and Zili simultaneously, nor can we show that Tdrd1 does so. Assessing this, for example through measuring competition between Ziwi and Zili for binding to Tdrd1, will require *in vitro* studies, something that has not yet been developed for the Piwi pathway.

Minor comments:

-We have adjusted our description of nuage to include perinuclear localization, and we have softened our wording regarding clustering and transport. We also included a reference to new work that strengthens the nuage-nuclear pore interaction observation in *C. elegans* (Updike et al, 2011).

-The text referring to FigS1A-C has been adjusted.

-Reference to Fig 1C has been corrected.

-We have added the vasa:EGFP labels to the panels with GFP (which is in grey-tones). This should provide more clarity.

-First mention of *C. elegans* is now in full.

-We adjusted the phrase concerning coIPs on page 11.

-We assume the reviewer refers to panels C and D of Fig 4. We have included schematics to clarify the experiments.

-Indeed some of the GST signals are saturated, but this only makes the point stronger that these domains interact less well with Zili than domain 4, whose signal is not saturated. We do not aim to quantify these data, as this is very difficult, if not impossible on western blots.

-Spelling error was corrected.

Reviewer 2

1. We have adjusted the wording to remove the suggestion that Tdrd1 would bind TATs directly. Indeed, indirect binding is much more likely, and this we have now specifically added to the discussion. We also added a small model figure (Fig 6F) for further clarification.

2. The graphs in question have been changed into bar diagrams.

3. By definition, the overlap between a piRNA and a TAT cannot be longer than the shortest partner (the piRNA). In this analysis we cut off piRNAs at 29 nucleotides to reduce the risk that we are matching two TAT molecules.

4. Title has been changed acording to the suggestion.

5. This is a very important point. We have fully sequenced a set of clones derived from the Tdrd1 library. This clearly revealed that indeed, as this reviewer predicted, the discrete bands high in the gel are rRNA fragments. In fact, the TAT-like sequences display a broad size distribution. Hence, we no longer adhere to the idea that TATs have discrete sizes, but rather we think that TATs are very heterogeneous in length. We have added this data to our manuscript (Figure 6 and table S4) and have adjusted the text accordingly.

Reviewer 3

Typos and labeling errors in the supplemental material have been corrected.

2nd Editorial Decision 08 June 2011

Your revised manuscript has been seen once more by referee #1 who is satisfied with your responses and recommends publication pending some minor changes with the figures. The manuscript will be accepted for publication once this issue is addressed.

Thank you for the opportunity to consider your work for publication.

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1:

In my opinion, the authors provided additional data addressing my concerns and moderately satisfactory arguments for the experiments which are currently not feasible. However, I do disagree with the authors' claim for RNA binding capability of TDRD1. They can not exclude any possibility that TDRD1 does bind to RNA in vitro without any experimental support. Furthermore, I still do feel the necessity of Piwi protein localization in tdrd1 mutant. Nevertheless, this manuscript provides novel clues and findings which would be of interest and important contribution in the field, and therefore is worthy for publication. I have a minor comment to help readers.

The authors say (response Pg. 3): We assume the reviewer refers to panels C and D of Fig 4. We have included schematics to clarify the experiments.

I meant a schematic representation of Tdrd1 protein showing where are the mutated arginine residues (in Fig 4C) and the tudor domains used for IP (in Fig 4D). The authors can delete the drawing in the revised manuscript but instead add a schematic of Tdrd1 protein.

2nd Revision - authors' response 09 June 2011

Reply to comments on EMBOJ-2011-77885R

We have adapted Figures 4 C and D to include graphics of Zili and Tdrd1 with the relevant domains and residues. The legend has been adapted accordingly. In addition, we corrected a minor mistake in the Figure: R168 was changed in R163 in Figure 4C.

