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# Molecular basis for cytoplasmic RNA surveillance by uridylation-triggered decay in Drosophila

Madalena M. Reimão-Pinto, Raphael A. Manzenreither, Thomas R. Burkard, Pawel Sledz, Martin Jinek, Karl Mechtler and Stefan L. Ameres

*Corresponding author: Stefan L. Ameres, IMBA*

**Review timeline:**

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|---|---|
| Submission date: | 20 July 2016 |
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*Editor: Anne Nielsen*

## 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 08 August 2016

Thank you for submitting your manuscript for consideration by the EMBO Journal. It has now been seen by three referees whose comments are shown below.

As you will see from the reports, all three referees express interest in the findings reported in your manuscript although they also raise a number of points relating to both textual and experimental concerns that you will have to address before they can support publication of the manuscript.

Given the referees' positive recommendations, I would like to invite you to submit a revised version of the manuscript, addressing the comments of all three reviewers. 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. Given the recent publication of competing work I would encourage you to submit this revision as soon as possible and also ask you to send me an estimate of the timeline for this.

For the revised manuscript I would particularly ask you to focus your efforts on the following points:
-> Please address/clarify the concerns from Ref #2 on potential artefacts of dmDis3l2CM over-expression
-> Please comment on the endogenous interaction of Tailor/Dis3L2 and add data, if possible.
-> Please clarify points about significance and assay quantification raised by refs #1 and #3

 

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

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REFEREE COMMENTS

Referee #1:

This manuscript from Reimão-Pinto identifies the nuclease responsible for uridylation-mediated decay in Drosophila (dmDis3L2). Consistent with work on the mammalian orthologue, elegant in vitro biochemistry reveals that this enzyme prefers uridyl-containing substrates, and this preference is reflected in the tailing of its in vivo substrates. Using high-throughput sequencing, the authors identify the substrates of this so-called TRUMP complex-in addition to previously-identified mirtrons, a variety of RNA polymerase III transcripts are targeted for decay by this pathway. Finally, the author contrast the activity of dmDis3l2 with E. coli RNase R and propose that these two enzymes function analogously in mRNA decay pathways in eukaryotes and prokaryotes, respectively. In general, this manuscript presents a convincing characterization of the TRUMP complex with thoughtful experiments. This manuscript will be of interest to the community, and I support its publication once the following issues have been addressed.

Major points:
1) In general, the authors downplay the biological and conceptual contributions of the TRAMP pathway and RNase R literature. For instance, the authors stress the idea that RNase R is a conserved 'reader' of epitranscriptomic marks; however, in the TRAMP pathway, the exosome is the associated nuclease. Similarly, there is a rich body of work underlying the role of adenylation, RNase R, etc. in prokaryotic mRNA decay that should be more properly cited in the introduction and discussion (especially of Figure 7).
2) p. 15: "uridylated pre-miRNAs usually carry short one- to three-nucleotide-long uridine tails...most certainly because longer U-tails trigger rapid decay in cells." Were longer tails observed on mirtrons in the pull-downs described in Figure 5? As it stands, this statement is unsupported and should be softened as well as moved to the discussion.
3) Figure 4: Of all the figures in the paper, this is the most unconvincing. Because the authors make no comparison to either their pure in vitro assays or their in vivo sequencing, they have not demonstrated that this lysate "quantitatively recapitulates post-transcriptional uridylation of cellular RNA." For instance, can nucleotide analogues be used to label and select these uridylated transcripts so they can be sequenced? Similarly, showing the entire gel for the ATP labeling would be a better control than showing only a portion of it.
4) Figure 5C: What is the enrichment of dmDis3l2 targets in the IP versus the input sample?

Minor points:
1) In Figure 1D/E, the protein interaction constructs are very hard to follow.
2) In Figure 3,C/D the addition of uridine residues both increases the single-strandedness and the U content for the substrates. What is the increase in decay rate for substrates with 3' (A)5, (A)10, etc.?
3) Figure 5E: this figure is hard to follow.
4) 'ecoRNase R' is not defined in the text.

Referee #2:

A variety of cytoplasmic eukaryotic RNAs are tailed by 3' uridylation by terminal U-transferase (TUT) enzymes. 3' oligouridylated RNAs are preferentially degraded by the processive 3'-5' exonuclease DIS3L2, and these aspects of RNA metabolism are known to be conserved across distantly related eukaryotic phyla. Earlier work from these authors demonstrated that, in Drosophila, a uridylation-mediated decay pathway involving the TUT Tailor is important in limiting the accumulation of intron-derived hairpin RNAs, which might otherwise enter the microRNA pathway. Other studies have shown that pre-let-7 microRNAs are substrates of the TUT-DIS3L2 RNA turnover pathway, as well as showing that a wide variety of mRNAs are TUT substrates.

 

Here, the authors describe in detail several further aspects of uridylation-mediated RNA decay in Drosophila. Surprisingly, they show that the cytoplasmic Tailor is stably associated via a coiled-coil interaction with the Drosophila DIS3L2 homolog dmDis3l2. In line with its role in degradation, the heterodimer is somewhat topically referred to as the TRUMP (terminal RNA uridylation-mediated processing) complex. No such stable interaction has been described between a TUT and DIS3L2 in any other system; consistent with this point, a comparatively poorly conserved N-terminal domain of unknown function in Tailor is shown to be essential for formation of the coiled-coil. An elegant and thorough in vitro approach is used to characterize dmDis3l2 substrate specificity: a 3' U is found to be strongly preferred, with additional 3' U residues increasing degradation rate, in line with recent biochemical and structural studies of DIS3L2 reported elsewhere. The TRUMP complex is shown to promote degradation of structured RNA substrates in a manner analogous to that previously described for the nuclear TRAMP complex, which instead uses oligoadenylation to generate preferred substrates for the nuclear exosome. As in other systems, TUT-DIS3L2 (TRUMP complex-mediated) RNA decay is shown here to be a negative regulator of miRNA biogenesis. An affinity capture approach using catalytically inactive dmDis3l2 is further used to identify candidate TRUMP complex substrates in vivo; these are predominantly highly abundant, structured non-coding RNAs, particularly Pol III transcripts, bearing 3' U-tails. The manuscript concludes with a comparative in vitro analysis of dmDis3l2 and its bacterial relative RNAseR, which will be of particular value to those readers interested in the evolution of this family of nucleases, though it stands a little apart from the main body of the study.
Together, these data provide further insight into TUT-DIS3L2-mediated RNA decay generally, as well as highlighting the novel stable interaction of Tailor and dmDis3l2 in particular. The data are of a very high quality, and the clear, concise manuscript will in my view be suitable for publication if the authors are able to address the following related points:

1. The catalytically inactive dmDis3l2CM is clearly very highly over-expressed relative to endogenous dmDis3l2 (Figure 4D). Is there a concern that dmDis3l2CM expressed at this level may simply act as an affinity-purification 'sponge' for all RNAs bearing 3'oligo-U stretches, regardless of whether or not they are authentic dmDis3l2 substrates?

2. Does expression of dmDis3l2CM induce a cell cycle phenotype analogous to that previously described as resulting from DIS3L2 knock-down in human cells (Astuti et al., 2012)? If so, does this raise concerns about the physiological state of the cells in which the candidate RNA substrates were identified (Figure 5)?

Referee #3:

This study uses a set of pull down assays, in vitro decay and a high throughput analysis of 3' variable RNA substrates to identify the TUTase Tailor as a binding partner of the Drosophila Dis3l2 homolog (which the authors aptly named the 'TRUMP' complex, a particularly timely name given the current US presidential race). They also determine that terminally uridylated RNAs - in particular Pol III products are preferred substrates. Similar studies using E. coli RNAse R indicate that the related prokaryotic exonuclease is a similar 'reader' of terminal extensions on RNAs targeted for degradation (in this case adenine extensions).
The data are overall robust and in general provide strong support for the conclusions that are drawn. My only major concern is that the study does not break a large amount of new mechanistic ground, as previous work by several other labs clearly demonstrated a preference for terminally uridylated RNA substrates by Dis3l2 in other species and recent work by Labno et al demonstrates the activity of human Dis3l2 on Pol III substrates. However to my knowledge this is the first broad-based characterization of Dis3l2 function in Drosophila.

Other Points

1. Fig. 1: The Tailor/Dis3L2 complex is characterized using FLAG-tagged exogenous proteins. Can the complex be confirmed/validated using available antibodies and endogenous proteins? In addition, what percentage of each protein is part of the complex (i.e. can the proteins also act independently)?

 

2. Fig. 4E: The results section regarding these data states that uridylation signals were 'significantly' elevated across the time course. These data are qualitatively presented - how was significance determined?
3. Minor Points:
a. Pg. 6, line 5 from the top: UTR1 should be URT1; truncataed = truncated
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d. Pg. 16, line 7: dmDis3l2 in (is the 'in' a misprint?)
e. Pg. 20, middle of the page: .... to this were tRNA - should read either was tRNA or were tRNAs
f. Pg. 25: associated = associated

1st Revision - authors' response 25 August 2016

Point-by-point response to reviewers related to the manuscript
"Molecular basis for cytoplasmic RNA surveillance by uridylation-triggered decay in *Drosophila*"
Reimão-Pinto *et al.*

Referee #1:

*This manuscript from Reimão-Pinto identifies the nuclease responsible for uridylation-mediated decay in Drosophila (dmDis3L2). Consistent with work on the mammalian orthologue, elegant in vitro biochemistry reveals that this enzyme prefers uridyl-containing substrates, and this preference is reflected in the tailing of its in vivo substrates. Using high-throughput sequencing, the authors identify the substrates of this so-called TRUMP complex-in addition to previously-identified mirtrons, a variety of RNA polymerase III transcripts are targeted for decay by this pathway. Finally, the author contrast the activity of dmDis3l2 with E. coli RNase R and propose that these two enzymes function analogously in mRNA decay pathways in eukaryotes and prokaryotes, respectively. In general, this manuscript presents a convincing characterization of the TRUMP complex with thoughtful experiments. This manuscript will be of interest to the community, and I support its publication once the following issues have been addressed.*

We thank the reviewer for insightful comments and the positive evaluation of our manuscript.

*Major points:*

1) *In general, the authors downplay the biological and conceptual contributions of the TRAMP pathway and RNase R literature. For instance, the authors stress the idea that RNase R is a conserved 'reader' of epitranscriptomic marks; however, in the TRAMP pathway, the exosome is the associated nuclease. Similarly, there is a rich body of work underlying the role of adenylation, RNase R, etc. in prokaryotic mRNA decay that should be more properly cited in the introduction and discussion (especially of Figure 7).*

It was far from our intention to downplay any contribution to the admittedly very dense literature on exonucleolytic degradation by the TRAMP complex in the nucleus of eukaryotes and RNaseR in bacteria. We have now expanded the citations to what we believe involves the key relevant studies in the discussion.

2) *p. 15: "uridylated pre-miRNAs usually carry short one- to three-nucleotide-long uridine tails...most certainly because longer U-tails trigger rapid decay in cells." Were longer tails observed on mirtrons in the pull-downs described in Figure 5? As it stands, this statement is unsupported and should be softened as well as moved to the discussion.*

While we believe that this hypothesis is valid, the low recovery of pre-miRNAs compared to other TRUMP-complex substrate in the dmDis3l2$^{CM}$-IP do not provide sufficient experimental evidence to validate this hypothesis. Hence, we have removed this speculation from the manuscript.

3) *Figure 4: Of all the figures in the paper, this is the most unconvincing. Because the authors*

*make no comparison to either their pure in vitro assays or their in vivo sequencing, they have not demonstrated that this lysate "quantitatively recapitulates post-transcriptional uridylation of cellular RNA." For instance, can nucleotide analogues be used to label and select these uridylated transcripts so they can be sequenced? Similarly, showing the entire gel for the ATP labeling would be a better control than showing only a portion of it.*

The main purpose of the experiments described in Figure 4 was to introduce a quantitative in vitro assay that enabled the evaluation of Tailor's contribution to the post-transcriptional uridylation of cellular RNA and test the proposed functional interaction between Tailor and dmDis3l2 in an in vitro assay that mimics the more complex intracellular situation. While it is true that the assay does not permit any comment on the identity of uridylated endogenous RNA, it enabled us to quantitatively visualize the post-transcriptional modification of cellular RNA in whole lysate and to dissect a genetic interaction of Tailor and Dis3l2 under experimental conditions that we believe to more faithfully recapitulate the cellular environment. While we understand the concerns of the Referee, we believe that this assay provides a quantitative experimental framework to studying post-transcriptional uridylation and may represent a relevant experimental entry-point also for other model organisms. In principle we also agree that the employment of nucleotide analogues may represent an interesting experimental extension of this assay. However, instead of identifying TRUMP complex substrates, this assay – if experimentally feasible – would rather uncover Tailor-substrates but not necessarily TRUMP-targets. Notably, we show in the revised version of the manuscript that Tailor exists both in a dmDis3l2-bound and –unbound state, implying that Tailor-directed uridylation may trigger possible functional consequences other than degradation (see also discussion of the revised manuscript). We consider the identification of Tailor substrates to be beyond the scope of this study. Instead, we believe that the RIP-seq approach using a catalytic-inactive version of dmDis3l2 represents a more pertinent experimental avenue to identify TRUMP-complex substrates. Finally, as requested we now show the entire gel for the ATP labeling (originally placed in the respective EV figure) in the main Figure 4.

4) *Figure 5C: What is the enrichment of dmDis3l2 targets in the IP versus the input sample?*

While we agree that enrichment-over-input-analyses could in principle be a meaningful addition to the analysis of RIPseq data, our analysis indicates that the vast majority of 3´ ends recovered by dmDis3l2$^{CM}$-IP do not overlap with abundantly detected 3´ ends of equivalent RNA species in the input (i.e. total RNA) sample (e.g. exemplified in Fig. 6A, B and EV6H, K, L and M). This indicates that the direct comparison between species would be inappropriate if grouped merely according to mapping category. The problem of determining enrichment over input is further illustrated by the fact that apparently underrepresented RNA species recovered in dmDis3l2CM-IP libraries clearly represent bona-fide TRUMP-complex substrate. For example, while tRNAs only contribute 1.6 % of dmDis3l2-bound RNA, they are far more abundantly detected in input libraries (11%). Nevertheless, a set of validation experiments clearly confirm tRNAs (together with other unprocessed RNA polymerase III transcripts) as TRUMP complex substrates. Hence, we believe that simple IP over input enrichment analyses would actually be misleading. Given this background, we chose to evaluate dmDis3l2-bound RNA species according to their post-transcriptional modification status (Figure 5D), an experimental strategy that finds support in the fact that dmDis3l2 apparently operates exclusively in association with the terminal uridylyltransferase Tailor (see Figures 4A and EV6B). In fact, further analysis of tail nucleotide identities of modified RNA species revealed a clear enrichment in uridine, reminiscent of the products of Tailor-activity (Figure 5D). This analysis revealed that the majority of RNA species bound by dmDis3l2 exhibit more frequent 3´ terminal uridine additions compared to similar mapping categories in total RNA and therefore provide in our view a more appropriate evaluation compared to simple enrichment over input analyses.

*Minor points:*

1) *In Figure 1D/E, the protein interaction constructs are very hard to follow.*

While we are open to suggestions that would facilitate the comprehension of the protein interaction studies shown in Figure 1, we believe that the current version conveys all necessary information in a compact format.

2) *In Figure 3,C/D the addition of uridine residues both increases the single-strandedness and the U content for the substrates. What is the increase in decay rate for substrates with 3'*

 

*(A)5, (A)10, etc.?*

To test if the addition of a single-stranded extension irrespective of its nucleotide composition is sufficient for the initiation of structured RNA degradation by dmDis3l2, we compared decay rates of pre-miR-1003 variants containing an $(A)_{10}$ or a $(U)_{10}$ extensions (Figure EV3). While deca-adenylation enhanced the degradation of pre-miR-1003 merely by 4.8-fold, a uridine-tail of similar length was 8.3-times more efficient in triggering dmDis3l2-directed decay, resulting in a 40-fold increase in decay rates compared to untailed pre-miR-1003 (Figure EV3). We concluded that primary sequence composition of Tailor-directed tailing is the major criterion for the efficient degradation of structured RNA by dmDis3l2.

*3) Figure 5E: this figure is hard to follow.*

While we are certainly open to suggestions that would facilitate the comprehension of Figure 5E, we believe that the current version conveys all necessary information in a compact format.

*4) 'ecoRNase R' is not defined in the text.*

We now introduce ecoRNase R in the context "To this end, we incubated recombinant *E. coli* RNase R (ecoRNase R) with 5′ radiolabeled RNA substrate…" in the first paragraph of the last results section.

Referee #2:

*A variety of cytoplasmic eukaryotic RNAs are tailed by 3′ uridylation by terminal U-transferase (TUT) enzymes. 3′ oligouridylated RNAs are preferentially degraded by the processive 3′-5′ exonuclease DIS3L2, and these aspects of RNA metabolism are known to be conserved across distantly related eukaryotic phyla. Earlier work from these authors demonstrated that, in Drosophila, a uridylation-mediated decay pathway involving the TUT Tailor is important in limiting the accumulation of intron-derived hairpin RNAs, which might otherwise enter the microRNA pathway. Other studies have shown that pre-let-7 microRNAs are substrates of the TUT-DIS3L2 RNA turnover pathway, as well as showing that a wide variety of mRNAs are TUT substrates.*
*Here, the authors describe in detail several further aspects of uridylation-mediated RNA decay in Drosophila. Surprisingly, they show that the cytoplasmic Tailor is stably associated via a coiled-coil interaction with the Drosophila DIS3L2 homolog dmDis3l2. In line with its role in degradation, the heterodimer is somewhat topically referred to as the TRUMP (terminal RNA uridylation-mediated processing) complex. No such stable interaction has been described between a TUT and DIS3L2 in any other system; consistent with this point, a comparatively poorly conserved N-terminal domain of unknown function in Tailor is shown to be essential for formation of the coiled-coil. An elegant and thorough in vitro approach is used to characterize dmDis3l2 substrate specificity: a 3′ U is found to be strongly preferred, with additional 3′ U residues increasing degradation rate, in line with recent biochemical and structural studies of DIS3L2 reported elsewhere. The TRUMP complex is shown to promote degradation of structured RNA substrates in a manner analogous to that previously described for the nuclear TRAMP complex, which instead uses oligoadenylation to generate preferred substrates for the nuclear exosome. As in other systems, TUT-DIS3L2 (TRUMP complex-mediated) RNA decay is shown here to be a negative regulator of miRNA biogenesis. An affinity capture approach using catalytically inactive dmDis3l2 is further used to identify candidate TRUMP complex substrates in vivo; these are predominantly highly abundant, structured non-coding RNAs, particularly Pol III transcripts, bearing 3′ U-tails. The manuscript concludes with a comparative in vitro analysis of dmDis3l2 and its bacterial relative RNAseR, which will be of particular value to those readers interested in the evolution of this family of nucleases, though it stands a little apart from the main body of the study.*
*Together, these data provide further insight into TUT-DIS3L2-mediated RNA decay generally, as well as highlighting the novel stable interaction of Tailor and dmDis3l2 in particular. The data are of a very high quality, and the clear, concise manuscript will in my view be suitable for publication if the authors are able to address the following related points:*

We thank the reviewer for insightful comments and the positive evaluation of our manuscript.

1. *The catalytically inactive dmDis3l2CM is clearly very highly over-expressed relative to endogenous dmDis3l2 (Figure 4D). Is there a concern that dmDis3l2CM expressed at this level may simply act as an affinity-purification 'sponge' for all RNAs bearing 3′ oligo-U stretches, regardless of whether or not they are authentic dmDis3l2 substrates?*

We had similar concerns and therefore highlighted already in the initial version of the manuscript that RNA-immunoprecipitation of dmDis3l2$^{CM}$ followed by thigh-throughput sequencing merely provides "the basis for a systematic annotation of transcripts subjected to post-transcriptional uridylation and decay via the TRUMP complex in flies." In fact, in Figure 6 and EV6 we provide a step-by-step instruction for the experimental validation of TRUMP-complex substrates: Employing loss-of-function alleles of *dmdis3l2* in S2 cells and in vivo in flies, combined with Northern hybridization experiments we validated the function of the TRUMP-complex in the degradation of unprocessed RNA polymerase III substrates and revealed the accumulation of 3′ end processing-deficient transcripts, such as 3′ trailer-containing tRNA. We furthermore employed in vitro reconstitution experiments to show the rapid degradation of unprocessed RNA polymerase III transcripts. We believe that the extensive molecular validation of putative substrates provided strong evidence for a function of the TRUMP complex in the degradation of unprocessed RNA polymerase III transcripts, and serves as a guideline for the future experimental evaluation of TRUMP complex substrates.

2. *Does expression of dmDis3l2CM induce a cell cycle phenotype analogous to that previously described as resulting from DIS3L2 knock-down in human cells (Astuti et al., 2012)? If so, does this raise concerns about the physiological state of the cells in which the candidate RNA substrates were identified (Figure 5)?*

We determined viability of cells upon overexpression of dmDis3l2$^{CM}$ and depletion of dmDis3l2 and did not detect any defect in cell viability compared to wild-type cells (in both cases viability was >95%). Furthermore, we would like to stress that the independent evaluations of putative dmDis3l2-substrates as described in Figure 6 and EV6 have been performed in cultured cells and in vivo in flies under experimental conditions that are distinct from the ones used to identify TRUMP-complex substrates (i.e. depletion of dmDis3l2, as well as editing of the endogenous *dmdis3l2* locus in vivo, not resulting in strong overexpression). We have now also performed a proliferation assay in cultured S2 cells that are depleted of dmDsi3l2 by genome editing, and in contrast to the observations by Astuti *et al.* 2012, we did not observe any major difference in cell growth. Notably, this observation is in agreement with similar studies in *S. pombe* published by Malecki *et al.*, 2013, where no effect on cell growth in the absence of Dis3l2 was observed. If required, we would be happy to add these data to the manuscript.

Referee #3:

*This study uses a set of pull down assays, in vitro decay and a high throughput analysis of 3' variable RNA substrates to identify the TUTase Tailor as a binding partner of the Drosophila Dis3l2 homolog (which the authors aptly named the 'TRUMP' complex, a particularly timely name given the current US presidential race). They also determine that terminally uridylated RNAs - in particular Pol III products are preferred substrates. Similar studies using E. coli RNAse R indicate that the related prokaryotic exonuclease is a similar 'reader' of terminal extensions on RNAs targeted for degradation (in this case adenine extensions).*
*The data are overall robust and in general provide strong support for the conclusions that are drawn. My only major concern is that the study does not break a large amount of new mechanistic ground, as previous work by several other labs clearly demonstrated a preference for terminally uridylated RNA substrates by Dis3l2 in other species and recent work by Labno et al demonstrates the activity of human Dis3l2 on Pol III substrates. However to my knowledge this is the first broad-based characterization of Dis3l2 function in Drosophila.*

We thank the reviewer for insightful comments and for acknowledging the high quality of our work.

Other Points

1. *Fig. 1: The Tailor/Dis3L2 complex is characterized using FLAG-tagged exogenous*

 

*proteins. Can the complex be confirmed/validated using available antibodies and endogenous proteins? In addition, what percentage of each protein is part of the complex (i.e. can the proteins also act independently)?*

We now added additional data confirming the complex formation at the level of endogenous protein (Figure EV1C). We immunoprecipitated endogenous dmDis3l2 from wild-type *Drosophila* S2 cell lysate using a monoclonal antibody generated against an N-terminal unstructured fragment of dmDis3l2 (amino acids 97 to 259), followed by Western blot analysis of Tailor and dmDis3l2 (Figure EV1C). This data confirms the interaction of endogenous dmDis3l2 and Tailor. Because immunodepletion of dmDis3l2 did not result in complete recovery of Tailor, we concluded that Tailor may exist both in a dmDis3l2-bound and -unbound state. Because of the semi-quantitative nature of Western blot analysis, we refrain from making any statement on what percentage of Tailor exists in complex with dmDis3l2. Furthermore, based on genetic evidence, which revealed that depletion of Tailor resulted in co-depletion of dmDis3l2, while depletion of dmDis3l2 does not affect Tailor protein levels (note, that we now additionally provide experimental evidence for this observation in vivo in flies, using ovary lysate; see Figure EV6B), we concluded that dmDis3l2 but not Tailor protein stability is dependent on TRUMP-complex formation. Hence, we propose that dmDis3l2 exists solely in a Tailor-bound form, while Tailor may exist also in a dmDis3l2-unbound form. This finding raises the possibility that Tailor-directed uridylation may have other functions than triggering dmDis3l2-directed RNA decay, a possibility that we now mention in the discussion of the revised manuscript.

2. *Fig. 4E: The results section regarding these data states that uridylation signals were 'significantly' elevated across the time course. These data are qualitatively presented - how was significance determined?*

Statistical analyses in Figure 4D and H are based on Student's t-test, determining the significance of observed differences in uridylation signal in lysate of genetically manipulated *Drosophila* S2 cells when compared to lysate of wild-type cells at the individual timepoints of the assay. This analysis revealed a statistically significant elevation of uridylation signal upon overexpression of wild-type Tailor (Tailor $^{OE}$, $p<0.001$, Figure 4D), and significantly reduced uridylation signal upon depletion of Tailor by CRISPR/Cas9 genome editing (*tailor*$^{ko}$, $p<0.001$, Figure 4D). Furthermore, depletion of dmDis3l2 by CRISPR/Cas9 genome editing (*dmdis3l2*$^{ko}$), or expression of a catalytic mutant version of dmDis3l2 (dmDis3l2$^{CM\ OE}$) prompted significantly elevated uridylation signal across the whole timecourse ($p<0.05$, Figure 4H).

*Minor Points:*
*a. Pg. 6, line 5 from the top: UTR1 should be URT1; truncataed = truncated*
*b. Pg. 8, line 4: promts = prompts*
*c. Pg. 10: aminoacids is two words*
*d. Pg. 16, line 7: dmDis3l2 in (is the 'in' a misprint?)*
*e. Pg. 20, middle of the page: .... to this were tRNA - should read either was tRNA or were tRNAs*
*f. Pg. 25: associated = associated*

We corrected all minor points in the revised version of the manuscript.

2nd Editorial Decision 07 September 2016

Thank you for submitting a revised version of your manuscript. It has now been seen by one of the original referees whose comments are shown below.

As you will see the referee finds that all main criticisms have been sufficiently addressed and recommend the manuscript for publication. However, before we can go on to officially accept the manuscript there are a few editorial issues concerning text and figures that I need you to address in a final revision:

-> Please incorporate the two minor remaining points raised by the referee

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REFEREE COMMENTS

Referee #2:

The authors have provided robust responses to both points raised in my review of the earlier version of their manuscript. Specifically, they clarify:

(1) the point that co-purification with the highly overexpressed, catalytically inactive Dis3L2 was only the first step in substrate identification, and that other, independent measures were then used to gauge the validity of selected candidate RNAs. I think there is some residual ambiguity in the phrase "the basis for a systematic annotation", which might better be re-worded as "the starting point for a systematic annotation", but this is clearly a very minor point.

(2) the lack of any cell cycle phenotype on loss of Dis3L2 function in this system. Given the prior description of such a phenotype in human cells, there may be sufficient interest among those working in the field to warrant inclusion of the data referred to in the authors' response as a further supplementary figure, but I don't have a strong view on this.

2nd Revision - authors' response 10 September 2016

I am enclosing the final revised version of our manuscript, “Molecular basis for cytoplasmic RNA surveillance by uridylation-triggered decay in Drosophila,” by Madalena M. Reimão-Pinto, Raphael A. Manzenreither, Thomas R. Burkard, Pawel Sledz, Martin Jinek, Karl Mechtler and Stefan L. Ameres, for consideration for publication as an article in *EMBO Journal*.

We have implemented the final minor request of the reviewer. Please find below a detailed overview of the changes.

(1) In reference to the minor reviewer requests, we have now changed the wording in the manuscript from "the basis for a systematic annotation" to "the starting point for a systematic annotation", to highlight the exploratory nature of our approach to identify TRUMP-complex targets. As to the second point, we believe that the mere inclusion of growth rate analysis insufficiently addresses the question if depletion of dmDis3l2 has an effect on cell cycle progression, hence not enabling us to draw any significant conclusion. Since we believe that a thorough analysis of this question should not be confined by a superficial analysis of growth rates, we refrained from including it in the manuscript. But we also believe that a thorough analysis would clearly exceed the current focus of the manuscript, which is in agreement with the reviewer’s assessment.

 

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| | |
|---|---|
| Corresponding Author Name: | Stefan L. Ameres, PhD |
| Journal Submitted to: | EMBO J |
| Manuscript Number: | EMBOJ-2016-95164 |

**Reporting Checklist For Life Sciences Articles (Rev. July 2015)**

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

## A- Figures

### 1. Data

**The data shown in figures should satisfy the following conditions:**

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

### 2. Captions

**Each figure caption should contain the following information, for each panel where they are relevant:**

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

**Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.**

**In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the information can be located. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable).**

## B- Statistics and general methods

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? | n/a |
| 1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used. | n/a |
| 2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established? | n/a |
| 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. | n/a |
| For animal studies, include a statement about randomization even if no randomization was used. | n/a |
| 4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe. | n/a |
| 4.b. For animal studies, include a statement about blinding even if no blinding was done | n/a |
| 5. For every figure, are statistical tests justified as appropriate? | Statistical tests were conducted as described in the main text and figure legends. Specifically this concerns figure 2G and L, Figure 3B, Figure 4D and H, Figure 7A, and C, Figure EV1J, Figure EV3C, Figure EV4G, and Figure EV6G. Number of independent experiments underlying the respective test are indicated in the figure legends. |
| Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. | Statistical tests were conducted as described in the main text and figure legends. Specifically this concerns figure 2G and L, Figure 3B, Figure 4D and H, Figure 7A, and C, Figure EV1J, Figure EV3C, Figure EV4G, and Figure EV6G. Number of independent experiments underlying the respective test are indicated in the figure legends. |
| Is there an estimate of variation within each group of data? | Statistical tests were conducted as described in the main text and figure legends. Specifically this concerns figure 2G and L, Figure 3B, Figure 4D and H, Figure 7A, and C, Figure EV1J, Figure EV3C, Figure EV4G, and Figure EV6G. Number of independent experiments underlying the respective test are indicated in the figure legends. |
| Is the variance similar between the groups that are being statistically compared? | yes in the appropriate tests |

## USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repo

http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title

http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tun

http://datadryad.org

http://figshare.com

http://www.ncbi.nlm.nih.gov/gap

http://www.ebi.ac.uk/ega

http://biomodels.net/

http://biomodels.net/miriam/
http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

## C- Reagents

| | |
|---|---|
| 6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right). | Antibody generated in this study was tested for specificity in Western blot and IF using a knockout cell line and flies generated also in this study. |
| 7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination. | Drosophila S2 cell line used in this study was derived from the Zamore Lab (University of Massachusetts Medical School, Worcester, USA). Mycoplasm tests are regularly performed (once a month), and were reported as negative throughout the course of the studies underlying this manuscript. |

* for all hyperlinks, please see the table at the top right of the document

## D- Animal Models

| | |
|---|---|
| 8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals. | n/a |
| 9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments. | n/a |
| 10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance. | n/a |

## E- Human Subjects

| | |
|---|---|
| 11. Identify the committee(s) approving the study protocol. | n/a |
| 12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report. | n/a |
| 13. For publication of patient photos, include a statement confirming that consent to publish was obtained. | n/a |
| 14. Report any restrictions on the availability (and/or on the use) of human data or samples. | n/a |
| 15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable. | n/a |
| 16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list. | n/a |
| 17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines. | n/a |

## F- Data Accessibility

| | |
|---|---|
| 18. Provide accession codes for deposited data. See author guidelines, under 'Data Deposition'.<br><br>Data deposition in a public repository is mandatory for:<br>a. Protein, DNA and RNA sequences<br>b. Macromolecular structures<br>c. Crystallographic data for small molecules<br>d. Functional genomics data<br>e. Proteomics and molecular interactions | The accession number for high-throughput sequencing datasets in this manuscript is GEO: GSE84466; For anonymous, read-only access to the data before publication please distribute the following link, if requested: http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=gfijkyeitjqxhyn&acc=GSE84466<br>Source data for protein interaction studies by Mass Spec are provided in Expanded Data information as Table S2. |
| 19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right). | The accession number for high-throughput sequencing datasets in this manuscript is GEO: GSE84466; For anonymous, read-only access to the data before publication please distribute the following link, if requested: http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=gfijkyeitjqxhyn&acc=GSE84466<br>Source data for protein interaction studies by Mass Spec are provided in Expanded Data information as Table S2. |
| 20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right). | n/a |
| 21. As far as possible, primary and referenced data should be formally cited in a Data Availability section. Please state whether you have included this section.<br><br>Examples:<br>**Primary Data**<br>Wetmore KM, Deutschbauer AM, Price MN, Arkin AP (2012). Comparison of gene expression and mutant fitness in Shewanella oneidensis MR-1. Gene Expression Omnibus GSE39462<br>**Referenced Data**<br>Huang J, Brown AF, Lei M (2012). Crystal structure of the TRBD domain of TERT and the CR4/5 of TR. Protein Data Bank 4O26<br>AP-MS analysis of human histone deacetylase interactions in CEM-T cells (2013). PRIDE PXD000208 | n/a |
| 22. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information. | n/a |

## G- Dual use research of concern

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| 23. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could. | n/a |