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The exoribonuclease Dis3L2 defines a novel eukaryotic RNA degradation pathway

Michal Malecki, Sandra C. Viegas, Tiago Carneiro, Pawel Golik , Clémentine Dressaire, Miguel G. Ferreira and Cecília M. Arraiano

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

Editor: Anne Nielsen

1st Editorial Decision 29 November 2012

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 referees highlight the impact and novelty of your findings, however they do also raise a number of technical concerns that will need to be addressed in full.

Given the referees' positive recommendations, we offer you the opportunity 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 or rejection of your manuscript will therefore depend on the completeness of your responses to the full satisfaction of the referees in this revised version. Please do not hesitate to contact me if you have questions related to the review process and the requests made by the referees.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Peer-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

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed.

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

REFEREE REPORTS:

Referee #1:

This manuscript reports the characterisation of a novel exoribonuclease in S. pombe, denoted Dis3L2. The authors report that Dis3L2 does not interact with the exosome complex, unlike the homologous protein Dis3. This is expected, since Dis3L2 lacks the PIN domain required for Dis3 to interact with the exosome. In contrast to Dis3, Dis3L2 is localized to the cytoplasm. Genetic analyses reveal that lack of Dis3L2 is synthetic lethal with an xrn1 null allele, and causes a synergistic slow growth phenotype with an lsm1 allele. The dis3L2 lsm1 double mutant accumulates a select group of mRNAs and the transcripts that are accumulated have oligo-U tails. Consistent with a direct role in mRNA degradation, recombinant Dis3L2 shows a preference for substrates with an oligo-U tract at the 3' end.

The experimental findings are well supported by the data and the results shown provide a coherent piece of work that delineates a novel pathway in eukaryotic mRNA degradation. As such, the work should be of significant interest to a wide readership. The authors should address the following minor points upon revision:

1. The authors should include an SDS-PAGE analysis of the purified Dis3L2 wild-type and mutant proteins in the supplemental data.
2. The authors show data that support the conclusion that dis3l2 xrn1 double mutants are nonviable, and argue that the "cytoplasmic exosome is unable to compensate for the absence of Dis3L2". If this is true, the ski2 xrn1 double mutant should be viable in S. pombe. They should show data to demonstrate that this is the case.
3. In Fig S3B the protein is incorrectly labelled Dis3-TAP; it should be Dis3-GFP.

Referee #2:

The authors have identified a third variant of the Dis3 exonuclease, Dis3L2, and characterized its function in S. pombe. They demonstrate ribonuclease activity of the enzyme and suggest that it functions in the cytoplasm, independently of the exosome. A deletion is synthetically lethal with a deletion of Xrn1. This provides strong evidence for an involvement of Dis3L2 in mRNA decay. However, increased mRNA abundance or half-life can only be detected when a deletion of Dis3L2 is combined with a deletion of Lsm1. Under these conditions, the authors observe an accumulation of 3' shortened, oligouridylated mRNAs, which are likely to be normally degraded either by Dis3L2 or by Lsm1-dependent decapping. In fact, Dis3L2 appears to prefer oligouridylated RNAs as substrates in vitro.

The data are very interesting and, overall, convincing. I have a few technical comments for the authors to consider.

Comments:
1. Nuclease assays (Fig. 1 and elsewhere): More information must be provided. Report, for every assay, either concentrations of substrate and enzyme or amounts and assay volume. Currently, amounts of enzyme and substrate are reported only for Fig. 7, and here an excess of enzyme over substrate was used in several assays. For obvious reasons, it is normal to use excess substrate over enzyme.
In Fig. 1, the substrates are not described clearly. I assume that, in the ds substrate, only one strand was labeled, and this should be stated. The use of 5' labeling is important for the interpretation of the figure; it should thus be mentioned in the main text. According to the Methods section, the double-stranded substrate contained one DNA strand. This is unusual and should be mentioned. The length of the product is the same with ss and ds substrate. Is it a mononucleotide? Do the authors propose that the enzyme displaces the complementary DNA strand? I could not find lengths and sequences of the RNA or DNA strands. A reference (Matos et al.) is given, but that paper used several different substrates.
The authors have purified their enzyme over an additional ion exchange column after the first GSH

column, and they also show that a point mutation abolishes the activity. This is very good evidence that the nuclease activity they see is in fact due to Dis3L2 rather than a contaminating enzyme. Nevertheless, I could cite cases where even this good evidence has been misleading. It would be nice if the authors provided additional evidence by demonstrating that the peak of nuclease activity in the ion exchange column corresponds to the protein peak.
The authors conclude from Fig. 1B that Dis3L2 is an exonuclease (p. 6). While this is obviously a very reasonable assumption, the assay only demonstrates nuclease activity but not exonuclease activity: No intermediates of degradation can be seen, and the authors have not attempted to inhibit the enzyme by modifying the RNA ends. (The effects of 3'-terminal U's reported later in the paper provide some, although not conclusive, direct support for 3' exo activity.)

2. Fig. 3: I agree with the authors' conclusions. However, the contrast of Dcp2-RFP in the glucose panel is quite poor. Even on the screen, the 'docking' of Dis3L2 structures to P bodies was hard to see for many pairs, it was worse after printing. I am aware that P bodies are not so easily visible under some conditions. Still, an enhanced contrast would make it easier for the reader. It would also be helpful if, in 3B, cell boundaries were visible as in A and C.

3. Fig. 4A: Again, the conclusions are probably ok. Nevertheless, the authors should provide a marker for the nucleus (DAPI staining) and, more importantly, for the nucleoli in order to substantiate their claim that Rrp43 is enriched in nucleoli (p. 8).

4: Lack of exosome association of Dis3L2: It is not very informative to document poor solubility of Dis3L2 (supplement); I suggest to delete these data.
As the recombinant enzyme is apparently well behaved, insolubility of TAP-tagged Dis3L2 is a bit surprising. The authors might try to integrate a small epitope tag into the genome and assay for exosome association either by co-IP or by sedimentation through a glycerol gradient. As it stands now, the conclusions that Dis3L2 is not associated with the exosome is based on localization data and the negative results of Fig. 4B, which is ok but not entirely conclusive.

5. It is my impression that Suppl. Fig. S4 duplicates data in Fig. 5B. Delete?

6. Fig. 6: State whether the RNAs examined by Northern blot correspond to the red crosses in 6A.
The use of phenanthroline for blocking transcription is problematic: To my knowledge, the compound works by chelating Zn2+, and it is hard to believe that this should not have multiple effects on the cells. Can any of the other methods used in other cells (tet-regulated promoter, carbon source-regulated promoter, ts mutation in pol II) be used in S. pombe?
The evidence for a stability effect on adh1 and pgk1 seems convincing, but I am not so sure for nmt1: First, the two lines seem to run quite parallel; if they did not originate from the same point, the slopes would be identical. In other words, the apparent stability difference rests on a single time point (0 min). Second, everything else being equal, a change in the decay rate should have a proportional effect on the steady-state level. While this is in fact visible for the first two RNAs, I do not see an increase in the steady-state level of nmt1 in the double mutant.
I also noticed (p. 19) that cells were resuspended at a fairly high density for the decay experiments. I see no reason why this was done, and I would be concerned that this affects cell behavior during the decay experiment.
The authors conclude (p. 12) that the data of Fig. 6C indicate a direct involvement of Dis3L2 in mRNA decay. While the interpretation is perfectly reasonable, the experiment actually does not prove that the effect is direct.

7. Fig. 7: These data are quite striking, and the interpretation is perfectly adequate.
It is surprising that the enzyme activity appears to stop after a few minutes. The authors suggest (p. 14) that the enzyme 'can go through on reaction cycle and then its activity is inhibited'. I suggest one little control experiment, which is to preincubate the enzyme under reaction conditions in the absence of substrate; maybe it is simply inactivated quite fast.

Minor comments:
Abstract: 'Deletion of dis3l2+ is synthetically lethal with xrn1+.' This should be either '...with a deletion of xrn1+' or '....with xrn1 '.
Discussion, p. 15: '...the plant Dis3L2 homologue SOV was found to suppress phenotypes of decapping mutants....' What does that mean? Does overexpression of SOV suppress the phenotypes?

p. 17, first line: 'extent' (not 'extend'); 'Dis3L2-dependent degradation' would be more precise than 'Dis3L2 degradation'.

Referee #3:

This is a very interesting manuscript that effectively implicates Dis3L2 as a 3'-5' exonuclease that targets mRNAs in S. pombe, may function in concert with Xrn1 to mediate overall cytoplasmic mRNA decay, and is stimulated by terminal uridylation. The data in general support the conclusions that are drawn and the study should have high impact to a broad audience. The study represents an exciting new twist on our appreciation of mechanisms of cytoplasmic mRNA decay. I only have a few minor comments to polish the study:

1. Throughout the manuscript, the writing/grammar could be sharpened for clarity.
2. The use of the term 'prove' should be avoided since the experiments shown largely indicate or suggest rather than provide 100% evidence for the conclusion drawn.
3. Pg. 8, lines 234,237 - I believe that the authors wish to refer to panel 3C, not 3B
4. Fig. 1B: What is the sequence of the RNA substrate employed in these experiments? Given the sequence influences shown later in the study, showing this could have relevance.

1st Revision 14 February 2013

Answers to the reviewers' comments

Referee #1

This manuscript reports the characterisation of a novel exoribonuclease in S. pombe, denoted Dis3L2. The authors report that Dis3L2 does not interact with the exosome complex, unlike the homologous protein Dis3. This is expected, since Dis3L2 lacks the PIN domain required for Dis3 to interact with the exosome. In contrast to Dis3, Dis3L2 is localized to the cytoplasm. Genetic analyses reveal that lack of Dis3L2 is synthetic lethal with an xrn1Δ null allele, and causes a synergistic slow growth phenotype with an lsm1Δ allele. The dis3L2Δ lsm1Δ double mutant accumulates a select group of mRNAs and the transcripts that are accumulated have oligo-U tails. Consistent with a direct role in mRNA degradation, recombinant Dis3L2 shows a preference for substrates with an oligo-U tract at the 3' end.

The experimental findings are well supported by the data and the results shown provide a coherent piece of work that delineates a novel pathway in eukaryotic mRNA degradation. As such, the work should be of significant interest to a wide readership. The authors should address the following minor points upon revision:

1. The authors should include an SDS-PAGE analysis of the purified Dis3L2 wild-type and mutant proteins in the supplemental data.

The SDS-PAGE analysis of the purified proteins together with the UV profiles from two purification steps are now included in the Supplementary Figure 2.

2. The authors show data that support the conclusion that dis3l2Δ xrn1Δ double mutants are nonviable, and argue that the "cytoplasmic exosome is unable to compensate for the absence of Dis3L2". If this is true, the ski2Δ xrn1Δ double mutant should be viable in S. pombe. They should show data to demonstrate that this is the case.

We thank the referee for this comment. To clarify this, we crossed *xrn1*Δ and *ski2*Δ strains. Similarly to the situation in *S. cerevisiae*, deletion of both of these genes is lethal. We found it interesting since these results suggest that the functions of Dis3L2 and exosome in 3'-5' mRNA degradation at least in some respects do not overlap. In the absence of XRN1 both of them have to be present for viability. The new cross results are now shown in Supplementary Figure 6. We have included this new information in the text. The respective sentences were changed as follows:

"Lethality of double mutant (*xrn1*Δ*dis3l2*Δ) suggests that cytoplasmic exosome cannot compensate for Dis3L2 function. Similarly, in the *xrn1*Δ strain Dis3L2 cannot fully compensate for loss of cytoplasmic exosome function since *xrn1*Δ*ski2*Δ mutant is inviable (Supplementary Figure S6)."

3. In Fig S3B the protein is incorrectly labelled Dis3-TAP; it should be Dis3-GFP.
This Figure was removed from the final manuscript version according to suggestion of referee 2.

Referee # 2

The authors have identified a third variant of the Dis3 exonuclease, Dis3L2, and characterized its function in S. pombe. They demonstrate ribonuclease activity of the enzyme and suggest that it functions in the cytoplasm, independently of the exosome. A deletion is synthetically lethal with a deletion of Xrn1. This provides strong evidence for an involvement of Dis3L2 in mRNA decay. However, increased mRNA abundance or half-life can only be detected when a deletion of Dis3L2 is combined with a deletion of Lsm1. Under these conditions, the authors observe an accumulation of 3' shortened, oligouridylated mRNAs, which are likely to be normally degraded either by Dis3L2 or by Lsm1-dependent decapping. In fact, Dis3L2 appears to prefer oligouridylated RNAs as substrates in vitro.

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Comments:
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In this new version of the manuscript we have included information about the amount of both the enzyme and substrate in each experiment. In general, we have applied the same amount of enzyme in each assay either with around twice less or a 4 time excess of substrate concentration. Along the manuscript we used the word "around" when referring to the enzyme concentration - due to the high degree of enzyme proteolysis we could only roughly estimate enzyme concentration based on the protein concentration in the sample. The standard reaction volume was 40 µl and for the time course reactions samples of 6 or 8 µl were collected at the indicated times. This information was now added to the Materials and Methods section.

In Fig. 1, the substrates are not described clearly. I assume that, in the ds substrate, only one strand was labelled, and this should be stated. The use of 5' labelling is important for the interpretation of the figure; it should thus be mentioned in the main text. According to the Methods section, the double-stranded substrate contained one DNA strand. This is unusual and should be mentioned. The length of the product is the same with ss and ds substrate. Is it a mononucleotide? Do the authors propose that the enzyme displaces the complementary DNA strand? I could not find lengths and sequences of the RNA or DNA strands. A reference (Matos et al.) is given, but that paper used several different substrates.

We appreciate the referee's comment. Indeed we agree that Figure 1 needed additional description. We now describe the position of the labelled nucleotide in the Figure legend, in the picture and in the respective Results section. We performed an additional experiment showing the length of the end product resultant from the substrate's degradation by Dis3L2 enzyme (Figure 1B). The sequences of all the oligonucleotides used in this study were included in Supplementary Table 2. We also mention now the use of a DNA oligonucleotide in the main text and in the Figure 1 legend. We additionally provide the sequences of the substrates used in the Figure 1 legend.
In the case of the double stranded substrate we propose that Dis3L2 displaces the DNA strand while it is degrading the RNA strand. The end product observed is the product of degradation of the 5'-end labelled RNA strand. The ability to displace the annealed strand (DNA or RNA) was shown for some proteins of RNase II family and, recently, a mechanism for this displacement was proposed [Ref.1].

1. Lee et al "Elastic coupling between RNA degradation and unwinding by an exoribonuclease"2012. *Science* Jun 29;336(6089):1726-9.

The authors have purified their enzyme over an additional ion exchange column after the first GSH column, and they also show that a point mutation abolishes the activity. This is very good evidence that the nuclease activity they see is in fact due to Dis3L2 rather than a contaminating enzyme. Nevertheless, I could cite cases where even this good evidence has been misleading. It would be nice if the authors provided additional evidence by demonstrating that the peak of nuclease activity in the ion exchange column corresponds to the protein peak.
The authors conclude from Fig. 1B that Dis3L2 is an exonuclease (p. 6). While this is obviously a very reasonable assumption, the assay only demonstrates nuclease activity but not exonuclease activity: No intermediates of degradation can be seen, and the authors have not attempted to inhibit the enzyme by modifying the RNA ends. (The effects of 3'-terminal U's reported later in the paper provide some, although not conclusive, direct support for 3' exo activity.)

We have now included the chromatography profiles from GST and ion exchange column purifications in Supplementary Figure 2. The protein was eluted from an ion exchange column as a single peak and the nuclease activity corresponded to the peak.
We are confident that the observed activity is due to Dis3L2. First, because single amino acid substitution of the conserved residue completely blocks the activity; second because the activity itself is characteristic of the RNaseII family of proteins. To support this, we have included additional *in vitro* assays in this corrected version of the manuscript. (a) The assays in Figure 1B and in Supplementary Figure 3 suggest that substrate degradation by Dis3L2 is exonucleolytic and 3-5' directed since the final reaction products are 5'-end labelled oligonucleotide and mononucleotides. (b) Figure1B shows that there are no degradation by-products longer than the end product even if the reaction is performed with an excess of the substrate. That again strongly suggests that activity is exonucleolytic. For comparison, we included a picture of substrate degradation by an endonuclease. In this case, even in the presence of an excess of enzyme we can see the characteristic enrichment of different size degradation by-products in point "0". (c) The different migration of the end products of the reactions performed by Dis3L2 and RNase I suggest a hydrolytic mode of degradation characteristic of the RNaseII family. Hydrolytic enzymes leave an OH group at the 3'-end of their degradation product while many endonucleases, including RNase I, leave a 2'-3' cyclic phosphate. Short oligonucleotides and mononucleotides with a cyclic phosphate have a stronger charge and migrate faster in the gel.

2. Fig. 3: I agree with the authors' conclusions. However, the contrast of Dcp2-RFP in the glucose panel is quite poor. Even on the screen, the 'docking' of Dis3L2 structures to P bodies was hard to see for many pairs, it was worse after printing. I am aware that P bodies are not so easily visible under some conditions. Still, an enhanced contrast would make it easier for the reader. It would also be helpful if, in 3B, cell boundaries were visible as in A and C.

We agree with the referee. Actually due to the journal policy of image format our images lost much of its quality. In this newer version we have converted the pictures to CMYK colour code taking care not to have significant quality loss. Most characteristic docked signals are now marked with a circle in Figure 3, which we hope will help. We also include two additional microscopy pictures collected from independent experiments (Supplementary Figure 5B) to give more strength to our data set. These new pictures have panels with visible cell boundaries.

3. Fig. 4A: Again, the conclusions are probably ok. Nevertheless, the authors should provide a marker for the nucleus (DAPI staining) and, more importantly, for the nucleoli in order to substantiate their claim that Rrp43 is enriched in nucleoli (p. 8).

We have included a new supplementary figure (Supplementary Figure 5A) showing the Hoechst nucleus staining in the cells expressing Dis3L2-GFP. As for the comment regarding the nucleoli, since it was not our goal in this work to investigate the localization of Rrp43 inside the nucleus, we decided to rephrased the sentence as: "Similar to *S. cerevisiae*, the exosome complex is localized mainly in the nucleus".

4: Lack of exosome association of Dis3L2: It is not very informative to document poor solubility of Dis3L2 (supplement); I suggest to delete these data.
As the recombinant enzyme is apparently well behaved, insolubility of TAP-tagged Dis3L2 is a bit surprising. The authors might try to integrate a small epitope tag into the genome and assay for exosome association either by co-IP or by sedimentation through a glycerol gradient. As it stands now, the conclusions that Dis3L2 is not associated with the exosome is based on localization data and the negative results of Fig. 4B, which is ok but not entirely conclusive.

We have removed the Figure as advised by the referee (previous Supplementary Figure 3). In this case we believe that insolubility is not due to the tag attached to the protein but to Dis3L2 localisation in cytoplasmic aggregates that can be pulled down during centrifugation because of its size like it was described by others (Ref.2). We mention purification difficulties in the manuscript since it justifies our choice of using exosome pull-downs for examination of exosome-Dis3L2 interaction.
As stated in the previous version text we faced similar difficulties when trying to purify a GFP fused protein, we also attempted to make a pull-down from a yeast strain with TAP tagged exosome and Dis3L2 fused with HA tag. These experiments were not included in the manuscript since we could not detect the HA fusion in the soluble fraction which we believe is again due to the poor solubility of the protein.

2. Teixeira at al. “Processing bodies require RNA for assembly and contain nontranslating mRNAs” 2005 RNA Apr;11(4):371-82

5. It is my impression that Suppl. Fig. S4 duplicates data in Fig. 5B. Delete?
We thank the referee for this comment. We have now removed this figure from the manuscript.

6. Fig. 6: State whether the RNAs examined by Northern blot correspond to the red crosses in 6A.

No, they do not. Microarray data concerns to differences between the wild-type and a single Dis3L2 deletion and they prove that the single Dis3L2 deletion does not have much impact on mRNA metabolism. The Northern blot analysis of the differences in mRNA levels between *ΔLsm1* and *ΔLsm1ΔDis3L3* were performed before we had access to the microarray results. As far as we have checked, there is no correlation between mRNAs up regulated in single Dis3L2 deletion and in *ΔLsm1ΔDis3L2* deletion.

The use of phenanthroline for blocking transcription is problematic: To my knowledge, the compound works by chelating Zn2+, and it is hard to believe that this should not have multiple effects on the cells. Can any of the other methods used in other cells (tet-regulated promoter, carbon source-regulated promoter, ts mutation in pol II) be used in S. pombe?

We are aware of the drawbacks of phenanthroline usage, but we aimed to stop transcription globally to have the opportunity to check any yeast transcript that we will choose. We believe that investigating turnover of transcripts under their natural level is important and overexpressing of transcripts from a plasmid or by using inserted promoters can also be considered as a disadvantage. Even considering phenanthroline side effects we are comparing degradation rate between the strains under exactly the same conditions in several repetitions and the only variable should be the lack of Dis3L2.
Even though, not to base our conclusions only on phenanthroline transcription blockage results, we have included an experiment regarding *nmt1* mRNA degradation rate. Blocking transcription of *nmt1* message by addition of thiamine is natural phenomenon giving great opportunity for half live measurements without perturbing the overall metabolism. The results support the ones obtained from experiments with phenanthroline.

The evidence for a stability effect on adh1 and pgk1 seems convincing, but I am not so sure for nmt1: First, the two lines seem to run quite parallel; if they did not originate from the same point, the slopes would be identical. In other words, the apparent stability difference rests on a single time point (0 min). Second, everything else being equal, a change in the decay rate should have a proportional effect on the steady-state level. While this is in fact visible for the first two RNAs, I do not see an increase in the steady-state level of nmt1 in the double mutant.

Although lines seem to run paralel, based on the data we can see an increase on stability difference between points 40 min (12% difference) and 80 min (17% difference). Later on, this difference decreases due to the slowing down of the degradation rate in *ΔLsm1* strain. We hypothesize that this is due to a lower accessibility of some fraction of the messenger that is most resistant to degradation. A similar slowing down is also observed in the two other cases (adh1 and pgk1) in the later time points . At the same time the half-life measurements show that the stability of *nmt1* is significantly different between the two strains. (*ΔLsm1*- 65,1±12,9 min. *ΔLsm1ΔDis3L2*- 101,03±3,7)
The graph presented shows the average data from three independent measurements, together with standard deviations. We decided to represent our data in graph where we joined obtained points. Although it is not standard way of presenting degradation data it shows dynamics of degradation.

As for the second concern, we did not noticed statistically significant difference in steady state level of nmt1 transcript between *ΔLsm1* and *ΔLsm1ΔDis3L2* strains, even if we report a difference in the messenger half-life. There are several possible explanantions for this situation with a possible difference in transcription efficiency being the most obvious. Still we believe this does not invalidate the conclusions that we present.

...also noticed (p. 19) that cells were resuspended at a fairly high density for the decay experiments. I see no reason why this was done, and I would be concerned that this affects cell behaviour during the decay experiment.

We used advices derived from the literature in transcription block experiments [Ref. 3]. Sample concentration in half-life comparison experiments is commonly used in yeast, reducing the volume helps in a faster time points collection.

3. Passos and Parker “Analysis of cytoplasmic mRNA decay in Saccharomyces cerevisiae” 2008 *Methods Enzymol* 448:409-27

The authors conclude (p. 12) that the data of Fig. 6C indicate a direct involvement of Dis3L2 in mRNA decay. While the interpretation is perfectly reasonable, the experiment actually does not prove that the effect is direct.

We thank referee for this comment and we agree that this phrase was too strong. It was now rephrased for: “These results suggest an involvement of Dis3L2 in the degradation of the transcripts analysed.”.

7. Fig. 7: These data are quite striking, and the interpretation is perfectly adequate.
It is surprising that the enzyme activity appears to stop after a few minutes. The authors suggest (p. 14) that the enzyme 'can go through on reaction cycle and then its activity is inhibited'. I suggest one little control experiment, which is to preincubate the enzyme under reaction conditions in the absence of substrate; maybe it is simply inactivated quite fast.

We would like to especially thank for this comment. The experiment proposed was performed and indeed it appeared that under these reaction conditions the enzyme is fastly inactivated . This information was included into the main text as
“We noticed that efficiency of the reaction slowed down with time which was due to inactivation of the enzyme ”
and the results were included in the Supplementary materials as Supplementary Figure 10A.

Minor comments:
Abstract: 'Deletion of dis3l2+ is synthetically lethal with xrn1+.' This should be either '...with a deletion of xrn1+' or '....with xrn1Δ'.
The sentence has been changed.

Discussion, p. 15: '...the plant Dis3L2 homologue SOV was found to suppress phenotypes of decapping mutants....' What does that mean? Does overexpression of SOV suppress the phenotypes?
We used the term suppression since the name of Dis3L2 homologue (SOV) derives from Suppressor of Varicose (VCS). But indeed we think we could make it more understandable, so it was rephrased as follows:

"…mutation of SOV, the *A. thaliana* Dis3L2 homologue, gives strong phenotypes with defects on the decapping complex scaffold protein VCS".

p. 17, first line: 'extent' (not 'extend'); 'Dis3L2-dependent degradation' would be more precise than 'Dis3L2 degradation'.
Both sentences have been changed.

Referee # 3

This is a very interesting manuscript that effectively implicates Dis3L2 as a 3'-5' exonuclease that targets mRNAs in S. pombe, may function in concert with Xrn1 to mediate overall cytoplasmic mRNA decay, and is stimulated by terminal uridylation. The data in general support the conclusions that are drawn and the study should have high impact to a broad audience. The study represents an exciting new twist on our appreciation of mechanisms of cytoplasmic mRNA decay. I only have a few minor comments to polish the study:

1. Throughout the manuscript, the writing/grammar could be sharpened for clarity.
This new version of the manuscript was revised in terms of writing/grammar by Professor Sarah Newbury.

2. The use of the term 'prove' should be avoided since the experiments shown largely indicate or suggest rather than provide 100% evidence for the conclusion drawn.
The referee is right. The term "prove" was avoided in the corrected version.

3. Pg. 8, lines 234,237 - I believe that the authors wish to refer to panel 3C, not 3B.
We acknowledge the remark. It was a mistake and we have corrected it.

4. Fig. 1B: What is the sequence of the RNA substrate employed in these experiments? Given the sequence influences shown later in the study, showing this could have relevance.
Sequences of all oligonucleotides used in this work are included in Supplementary Table 2.

Accepted 26 February 2013

We have heard back from two of the original referees, whose comments are included below, and given their positive recommendations I am pleased to inform you that your manuscript has now been accepted for publication in the EMBO Journal pending a few editorial additions as indicated below.

REFEREE REPORTS:

Referee 2:

This is a revised manuscript. The first version was already quite good. While I am not 100 % happy with every detail, I do think that the authors have given satisfying responses to my suggestions. The paper should now be published.

Referee 3:

The authors have done an effective job in addressing the points raised in the previous critiques. The manuscript is improved, the conclusions well supported, and I believe that the study should have substantial impact in the field.