THE EMBO JOURNAL

Manuscript EMBO-2012-83756

Exonuclease hDIS3L2 specifies an exosome-independent 3'-5' degradation pathway of human cytoplasmic mRNA

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Review timeline:

Submission date:	01 November 2012
Editorial Decision:	29 November 2012
Revision received:	11 April 2013
Editorial Decision:	19 April 2013
Revision received:	15 May 2013
Accepted:	15 May 2013

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.)

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 novelty and potential broad impact of your findings; however they also raise a number of concerns that will need to be addressed in a revised version of the manuscript.

We generally find the requests made by the referees reasonable and important, but I would like to particularly emphasize the need for you to provide mRNA decay rates following depletion of Dis3L2 as requested by all three referees. In addition, while referee #2 suggests that figure 6 be removed from the manuscript, we will not object to you leaving it in to illustrate the presumed position of Dis3L2 in cytoplasmic mRNA decay.

Given the referees' positive recommendations, we offer you the opportunity 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 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:

In addition to the exosome-associated proteins DIS3 and DIS3L, the human genome encodes a third related protein, DIS3L2. A recent report by Astuti et al. demonstrated that DIS3L2, a gene mutated in patients suffering from Perlman syndrome and associated with susceptibility to Wilms tumour disease, is a cytoplasmic 3' ribonuclease that is required for the normal expression of mitotic control proteins. The DIS3L2 protein lacks an N-terminal PIN domain and therefore is predicted not to be associated with the exosome complex. Some of the findings of this manuscript, such as the protein localization and enzyme characterisation, confirm and extend these earlier results, albeit at more detail. The more important findings, and the focus of this manuscript, link DIS3L2 more directly to cytoplasmic mRNA degradation. Specifically, DIS3L2 is shown to be associated with XRN1 in pull-down experiments and to be found in polysome fractions upon centrifugation analyses. Depletion of DIS3L2 causes an increase in P body number, which correlates with an increase in the pool of nontranslating transcripts. DIS3L2 depletion also causes an increase in the relative expression levels of specific mRNA transcripts, particularly in those encoding cell cycle factors and DNA packaging. Depletion of XRN1 or DIS3L1 reveal partial overlapping sets of targets. The authors conclude that DIS3L2 functions as an exosome-independent, cytoplasmic mRNA degradation factor.

The scientific work is of a high technical quality and the authors' conclusions are, in general, well supported by the data presented. The authors should address the following points upon revision of the manuscript:

1.
The general conclusion that DIS3L2 functions in the degradation of selective mRNA transcripts is highly believable but the manuscript would be strengthened by including data directly showing a difference in the kinetic stability of putative target transcripts (e.g. cell cycle factor mRNAs) whose steady state levels are dramatically increased upon DIS3L2 depletion.

2.
A control for mitochondrial protein should be included in Fig. 1D.

3.
The labeling of Fig. 2 could be slightly modified to clarify that the radiolabel is only on the 3' extended strand.

4.
The pull-down of XRN1 with DIS3L2-FLAG was observed in the presence of 2mM EDTA but not in the presence of RNase A. The authors conclude that the interaction is "RNA-dependent". I would anticipate that 2mM EDTA would disrupt RNP structures. The authors should clarify whether EDTA is required for the interaction.

5.
A control for the polysome gradient analyses should be included to show that the distribution of DIS3L2 (and XRN1) through the gradient is due to association with polysomes, e.g. analysis of run-

off samples without cycloheximide treatment.

6.
The Staals et al. reference is given twice (2010a, 2010b)

Referee #2:

This manuscript describes human Dis3L2, an orthologue of the yeast 3' exonuclease Dis3. The authors provide evidence that this enzyme is localized in the cytoplasm, is not associated with the exosome (in contrast to Dis3 and Dis3L) and is active as a 3'-exonuclease. Effects of a knock-down on P body numbers and on steady-state levels suggest a role in cytoplasmic mRNA decay.

The data are interesting and, in principle, suitable for the EMBO Journal. However, a role of Dis3L2 in mRNA decay has to be demonstrated by decay time courses in knock-down versus control cells.

Detailed comments:
1. Fig. 1:
1B: I suggest not to label the figure with the localization, as this is additional, independent information that is not directly related to the experiment. Putting this information into the figure is misleading.
1D: The fraction 'nucleus + mitochondria' was generated by a 5 min 500 g spin. I wonder whether this is sufficient to pellet mitochondria. Since the authors make the point that Dis3L2 is not a mitochondrial protein, they should use a mitochondrial marker to demonstrate that their fraction in fact contains mitochondria.

2. Enzyme assays:
Please provide enzyme and substrate concentrations for all assays.
I appreciate that the authors have purified their enzyme over an additional ion exchange column after the initial Ni column, and they find no activity after introduction of an inactivating point mutation. This is very good evidence that they indeed observe activity of Dis3L2. Nevertheless, even this type of evidence has been misleading in the past. It would be nice if, as additional evidence, the authors could state that the nuclease peak coincides with the Dis3L2 protein peak in their ion exchange column.

3. MS analysis of Dis3L2-associated proteins:
In the Methods section, the paragraph on 'Sample preparation, MS analyses and data treatment' is separated by several unrelated sections from 'Protein coIP experiments'. I assume that the latter describes the experiment analyzed by MS.
State, in the main text, what the negative control was; I assume flag IP from 'empty' cells.
Table S1 has no explanation, and it is organized in a most inconvenient manner - I had the impression that columns were split over several pages, and it was hard to see what belonged to what.
The text (p. 10) states that hXRN1 was 'the only repetitively significant hit' - what about 'hornerin' highlighted in Table S1?

4. Fig. 3:
3A: I would suggest to leave out the information about unique peptides; again, this is additional information not directly related to the Western blot shown.
3B: This is not entirely convincing. A control is missing to shown that the proteins in fact sediment with polysomes, not with some other heavy structure. Either sensitivity to EDTA or a puromycin run-off should be shown.
The functional significance of the association is also uncertain; this could be just non-specific RNA affinity. How about this control: Make an extract from yeast, add recombinant Dis3L2 and run a similar gradient.

5. Fig. 4C:
I assume that a 'no emetine' control was done in parallel and looked like in 4A. This should be stated.

6. Sequencing data:
Some of the information in the 'Methods' section is sufficiently important that it should be included in the main text, e. g. random priming and size selection to exclude small RNAs. The authors do not tell us whether the differentially expressed RNAs were exclusively mRNAs. What is 'distance closure' (p. 13)?
I do not find the GO term analysis very informative. In particular, I do not see why the results of the double knock-downs are reported only in the form of GO term analysis.
I am worried that the number of downregulated transcripts is very similar to the number of upregulated transcripts (Fig. S5B). If downregulation is an indirect effect (p. 13), how do the authors know that this is not true for upregulation?
I do not find this paper acceptable with out decay kinetics of two or three of the RNAs identified by RNAseq; knockdown of Dis3L2 and XRN1 and a double-knockdown should be compared. A Northern blot might be informative (detection of intermediates?).

6. Fig. 6 is not informative and should be deleted.

Additional minor points:

p. 4, bottom paragraph: The sentence 'Dis3p harbors a processive exonuclease activity....' is screwed up.

Second page of Discussion: I believe the term is 'deuterostomia', not 'deuterostoma'.

Methods, section on MS analyses: '...alkalized in 55 mM iodoacetamide....' - do the authors mean to say 'alkylated'?

Referee #3:

This is a potentially very interesting study characterizing the activity and cellular functions of the hDIS3L2 exonuclease. While aspects of the study are very well done, there are two major areas that require additional experimentation to provide clear support for the conclusions that are drawn:

1. Fig. 3: The important conclusion of the connections drawn between DIS3L2 and translation are not well supported by the data in Fig. 3. The separation of the sucrose gradient in the key 10-80S range for free proteins versus monosomes does not appear to be adequate. This lack of resolution in the data provided thus leads to concern whether the proteins are associating with monosomes as concluded. Second, the gradients should be probed with a control protein (that's not naturally part of a large complex like the exosome factors in the lower panel) to ensure that the smearing of hDIS3L2 throughout the gradient is indeed meaningful. Finally, the control of disrupting polysomes with EDTA and seeing a redistribution of the hDIS3L2 protein should be performed.

2. Fig. 4: The connection between hDIS3L2 and PB number is suggestive of an effect of the protein on general cytoplasmic mRNA turnover, but it is not definitive evidence. The study must assess mRNA decay rates directly for a number of model transcripts under wt and hDIS3L KD conditions to clearly establish the relationship of hDIS3L2 to general mRNA decay.

3. Other points:
a. Fig. S2: The authors conclude that the protein efficiently degraded the RNA substrate at all Mg concentrations tested. However the amount of decay shown in this entire panel is fairly negligible and unconvincing.

b. In the first paragraph of the intro, loose should be 'lose'.

1st Revision - authors' response 11 April 2013

In addition to the reviewers' requests, we decided to extend the biochemical part of the paper by repeating RNA degradation assays with additional substrates (see new Figures 2 and S2). Here, the activity of hDIS3L2 was compared to human hDIS3 and yeast Dis3p. Interestingly, we find that hDIS3L2, in contrast to other studied eukaryotic homologs of RNAse II/R, exhibits a strong activity towards structured substrates with short 2nt single stranded overhang and is even somewhat capable of degrading blunt-ended dsRNA (Figure 2C). Moreover, final degradation products of hDIS3L2 and hDIS3, compared on high-resolution polyacrylamide gels, show a size difference, i.e. 4-5nt and 3nt for hDIS3 and hDIS3L2, respectively (Figure S2F). Finally, to completely exclude the possibility of endonucleolytic hDIS3L2 activity, we performed degradation assays in the presence of divalent cations and using a circular RNA substrate (Figure S2H). These data complete our enzymatic characterization of human hDIS3L2 and are discussed in the text accordingly.

Response to reviews:

Referee #1:

The scientific work is of a high technical quality and the authors' conclusions are, in general, well supported by the data presented. The authors should address the following points upon revision of the manuscript:

A/ We thank the referee for the overall positive assessment.

1. The general conclusion that DIS3L2 functions in the degradation of selective mRNA transcripts is highly believable but the manuscript would be strengthened by including data directly showing a difference in the kinetic stability of putative target transcripts (e.g. cell cycle factor mRNAs) whose steady state levels are dramatically increased upon DIS3L2 depletion.

A/ In the revised manuscript, we have applied 4-thiouridine (4sU) labelling of newly synthetized RNA (Dolken, 2008) coupled to qRT-PCR analysis to measure relative changes in transcript half-lives upon hDIS3L2-depletion. Target mRNAs were chosen based on p-values derived from RNAseq analysis and confirm that up regulated transcripts have prolonged half-lives upon depletion of hDIS3L2 (new Figure 6D). We have also examined by pulse-chase analysis an AU-rich element (ARE)-containing reporter transcript and shown by northern blotting analysis that its half-life is significantly prolonged upon depletion of hDIS3L2 (new Figure 5). ARE-mRNA levels are known to also be controlled by both XRN1 and the exosome, which is in agreement with our RNAseq data, indicating an overlap between these decay pathways.

2. A control for mitochondrial protein should be included in Fig. 1D.

A/ We have included the mitochondrial matrix protein - SOD2 – as a control (Figure 1D).

3. The labelling of Fig. 2 could be slightly modified to clarify that the radiolabel is only on the 3' extended strand.

A/ We have now clarified this issue (Figure 2).

4. The pull-down of XRN1 with DIS3L2-FLAG was observed in the presence of 2mM EDTA but not in the presence of RNase A. The authors conclude that the interaction is "RNA-dependent". I would anticipate that 2mM EDTA would disrupt RNP structures. The authors should clarify whether EDTA is required for the interaction.

A/ The presence of 2mM EDTA, in contrast to Mg2+ ions and RNAse A, was used to diminish the activity of cellular RNAses. We observe this interaction consistently using such experimental setup. This is also routinely used by others to capture mRNP complexes (e.g. Damgaard and Lykke-Andersen, G&D, 2011), and is comprised in the widely used 'RIPA buffer' (http://cshprotocols.cshlp.org/). Also, note that if EDTA would disrupt these RNPs, the observed interaction would be even less likely.

5. A control for the polysome gradient analyses should be included to show that the distribution of DIS3L2 (and XRN1) through the gradient is due to association with polysomes, e.g. analysis of run-off samples without cycloheximide treatment.

A/ An additional polysome gradient analysis has been included using buffer without cycloheximide and with a high EDTA concentration (12.5mM) to efficiently disrupt polysomes. The presence of both hDIS3L2 and hXRN1 is sensitive to EDTA treatment (Figure 3B).

6. The Staals et al. reference is given twice (2010a, 2010b)

A/ This has now been corrected.

Referee #2:
The data are interesting and, in principle, suitable for the EMBO Journal. However, a role of Dis3L2 in mRNA decay has to be demonstrated by decay time courses in knock-down versus control cells.

A/ We thank the referee for the overall positive assessment. Regarding decay assays, as described above ('referee 1, point 1') we now include analyses showing that hDIS3L2 knockdown indeed affects mRNA half-lives as shown for an ARE-containing reporter (Figure 5) and several targets identified by RNAseq (Figure 6D).

Detailed comments:
1. Fig. 1:
1B: I suggest not to label the figure with the localization, as this is additional, independent information that is not directly related to the experiment. Putting this information into the figure is misleading.

A/ We agree with the referee and have removed this information.

1D: The fraction 'nucleus + mitochondria' was generated by a 5 min 500 g spin. I wonder whether this is sufficient to pellet mitochondria. Since the authors make the point that Dis3L2 is not a mitochondrial protein, they should use a mitochondrial marker to demonstrate that their fraction in fact contains mitochondria.

A/ We have now included the mitochondrial matrix protein - SOD2 – as a control (Figure 1D). The digitonin fractionation protocol used in this study allows for the solubilisation of the plasma membrane with only minor permabilization of intracellular membranes (including the mitochondria).

2. Enzyme assays: Please provide enzyme and substrate concentrations for all assays.

A/ This information has been included in the Materials and Methods section.

3. I appreciate that the authors have purified their enzyme over an additional ion exchange column after the initial Ni column, and they find no activity after introduction of an inactivating point mutation. This is very good evidence that they indeed observe activity of Dis3L2. Nevertheless, even this type of evidence has been misleading in the past. It would be nice if, as additional evidence, the authors could state that the nuclease peak coincides with the Dis3L2 protein peak in their ion exchange column.

A/ Subsequent fractions from the ion exchange chromatography were tested and showed that the activity coincides with the hDIS3L2 peak. Data are now included in Figure S2B.

4. MS analysis of Dis3L2-associated proteins: In the Methods section, the paragraph on 'Sample preparation, MS analyses and data treatment' is separated by several unrelated sections from 'Protein coIP experiments'. I assume that the latter describes the experiment analysed by MS.

A/ Yes. This has now been clarified.

5. State, in the main text, what the negative control was; I assume flag IP from 'empty' cells.

A/ Yes. This information is now included.

6. Table S1 has no explanation, and it is organized in a most inconvenient manner - I had the impression that columns were split over several pages, and it was hard to see what belonged to what.

A/ The labeling of the table has been changed to make it easier to read.

7. The text (p. 10) states that hXRN1 was 'the only repetitively significant hit' - what about 'hornerin' highlighted in Table S1?

A/ hXRN1 was the only repetitive and significant hit detected in coIP/MS analyses, and also confirmed by western blotting analysis. This is not the case for hornein, which is a previously reported MS contaminant, resulting from handling of the samples.

8. Fig. 3:
3A: I would suggest to leave out the information about unique peptides; again, this is additional information not directly related to the Western blot shown.

A/ We have decided to keep the peptide information, since our primary finding came from the MS analysis.

3B: This is not entirely convincing. A control is missing to shown that the proteins in fact sediment with polysomes, not with some other heavy structure. Either sensitivity to EDTA or a puromycin run-off should be shown.

A/ We agree with the referee's comments and have now included an additional control experiment using EDTA to collapse polysomes along with a higher number of fractions to better resolve these ribosomal complexes. Indeed, both hXRN1 and hDIS3L2 shift to lighter fractions upon treatment with EDTA (Figure 3B).

9. The functional significance of the association is also uncertain; this could be just non-specific RNA affinity. How about this control: Make an extract from yeast, add recombinant Dis3L2 and run a similar gradient.

A/ We agree that the functional significance of this observation is not entirely clear. Still, we find it interesting and worth reporting, that the other 3'-5' degradation machinery (the exosome) is absent from the polysome fractions.

10. Fig. 4C: I assume that a 'no emetine' control was done in parallel and looked like in 4A. This should be stated.

A/ Yes, it was done and is now stated in the text.

11. Sequencing data: Some of the information in the 'Methods' section is sufficiently important that it should be included in the main text, e.g. random priming and size selection to exclude small RNAs. The authors do not tell us whether the differentially expressed RNAs were exclusively mRNAs.

A/ We have modified the main text according to the referee's comment. We have chosen to focus our study on mRNAs due to the observed effects on P-bodies. The supplementary tables include all differentially expressed RNAs provided by the most recent gene annotations from iGenomes (Illumina).

12. What is 'distance closure' (p. 13)?

A/ It refers to the 'difference' between the relative abundances of transcripts in two different RNAi depletion experiments. This has now been clarified.

13. I do not find the GO term analysis very informative. In particular, I do not see why the results of the double knock-downs are reported only in the form of GO term analysis. I am worried that the number of down regulated transcripts is very similar to the number of up regulated transcripts (Fig. S5B). If down regulation is an indirect effect (p. 13), how do the authors know that this is not true for up regulation?

A/ We find that the GO analysis is helpful to 'sort' the transcripts. However, following the referee's worry about the large pool of down regulated transcripts, we have now compared various RNAseq normalization approaches and found that the previously used internal DESeq normalization does not handle optimally a generally up regulated transcriptome. Consequently, we have re-analysed the RNAseq data using normalization calculated based on the upper quartile of the libraries (see updated Figures in the RNAseq section, corresponding supplementary data and according text changes). The obtained results are now in better agreement with our qPCR validation and show that the majority of affected mRNAs are up regulated upon decay enzyme depletion. We thank the referee for this important note. Moreover, this aided in conducting meaningful half-life analyses (see 'referee 1, point 1') to conclude that many transcripts are indeed up regulated directly due to stability changes.

14. I do not find this paper acceptable with out decay kinetics of two or three of the RNAs identified by RNAseq; knockdown of Dis3L2 and XRN1 and a double-knockdown should be compared. A Northern blot might be informative (detection of intermediates?).

A/ As described above ('referee 1, point 1'), we have now included analyses showing that hDIS3L2 knockdown indeed affects the half-lives of several mRNA targets identified by RNAseq (Figure S6D). Moreover, we have carried out a pulse-chase decay assay of an ARE-containing reporter, revealing a significantly prolonged half-life upon depletion of hDIS3L2 (Figure 5).

15. Fig. 6 is not informative and should be deleted.

A/ We simply wanted to present a scheme, which summarizes our results and therefore decided to keep the Figure. This was also advised by the editor.

Additional minor points:

- p. 4, bottom paragraph: The sentence 'Dis3p harbors a processive exonuclease activity....' is screwed up.
- Second page of Discussion: I believe the term is 'deuterostomia', not 'deuterostoma'.
- Methods, section on MS analyses: '...alkalized in 55 mM iodoacetamide....' - do the authors mean to say 'alkylated'?
A/ All above points have been corrected.

Referee #3:
This is a potentially very interesting study characterizing the activity and cellular functions of the hDIS3L2 exonuclease. While aspects of the study are very well done, there are two major areas that require additional experimentation to provide clear support for the conclusions that are drawn:
A/ We thank the referee for the overall positive assessment.

1. Fig. 3: The important conclusion of the connections drawn between DIS3L2 and translation are not well supported by the data in Fig. 3. The separation of the sucrose gradient in the key 10-80S range for free proteins versus monosomes does not appear to be adequate. This lack of resolution in the data provided thus leads to concern whether the proteins are associating with monosomes as concluded. Second, the gradients should be probed with a control protein (that's not naturally part of a large complex like the exosome factors in the lower panel) to ensure that the smearing of hDIS3L2 throughout the gradient is indeed meaningful. Finally, the control of disrupting polysomes with EDTA and seeing a redistribution of the hDIS3L2 protein should be performed.

A/ We agree with the referee's comments and have now included an additional control experiment using EDTA to collapse polysomes along with a higher number of fractions to better resolve these ribosomal complexes. Indeed, both hXRN1 and hDIS3L2 shift to lighter fractions upon treatment with EDTA, suggesting that hDISL2 associates with polysomes (Figure 3B). We have also included an exosome component as a control as suggested. Some of these issues have also been addressed above (see 'referee 1, point 5').

2. Fig. 4: The connection between hDIS3L2 and PB number is suggestive of an effect of the protein on general cytoplasmic mRNA turnover, but it is not definitive evidence. The study must assess mRNA decay rates directly for a number of model transcripts under wt and hDIS3L KD conditions to clearly establish the relationship of hDIS3L2 to general mRNA decay.

A/ As described above ('referee 1, point 1') we have now included analyses showing that depletion of hDIS3L2 affects mRNA half-lives of several targets identified by RNAseq as well as an ARE-containing reporter (Figure 5 and S5E).

3. Other points:
a. Fig. S2: The authors conclude that the protein efficiently degraded the RNA substrate at all Mg concentrations tested. However the amount of decay shown in this entire panel is fairly negligible and unconvincing.

A/ Using a more active protein prep., we find that hDIS3L2 is active over a broad range of Mg2+ ion concentrations. Interestingly, this contrasts hDIS3, hDIS3L and yeast Dis3p (Fig. 3B), which is now discussed in the text.

b. In the first paragraph of the intro, loose should be 'lose'.

A/ This has now been corrected.

2nd Editorial Decision 19 April 2013

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

As you will see, while referee #3 finds that all criticisms have been sufficiently addressed, referee #2 still raises a few minor concerns. We therefore have to ask you to provide data to exclude off-target effects using a second Dis3L2-targeting siRNA or an RNAi-resistant Dis3L2 clone if available. You also need to comment on the processivity and catalytic rates for Dis3 and Dis3L2 as suggested by the referee.

Thank you again for giving us the chance to consider your manuscript for The EMBO Journal, I look forward to your revision.

Please feel free to contact me with any questions regarding this final revision

REFEREE REPORTS:

Referee #2:

In this revised version, the authors have answered my criticism in a satisfying manner. This is a good paper and should be published provided that one additional modification is made:
As far as I could tell, knock-down of hDis3L2 was done with only one single siRNA. It is essential that off-target effects be excluded either by use of a second siRNA or complementaton with an RNAi-resistant clone.

I also have some comments regarding the nuclease assays:

Apparently, some assays were performed with pCp-labeled RNAs (e. g. Fig. S2C). It is not common that a 3' exonuclease will accept a substrate containing a 3'phosphate. The authors might wish to mention this in the Results section.
The authors' claim that Dis3L2 is processive is probably correct. However, in order to judge processivity one has to observe a mixture of untouched substrate and end products. This condition is not met very well, since, at most time points, the substrates are already completely degraded. In some cases, it seems like there is both substrate and product present at the 0 min time point. Thus, this does not seem to be really 0 min. The assays could be improved by including a real 0 min time point (i. e. sample taken before addition of enzyme), and a short time point to really prove the point of processivity. Mixtures of substrate and product are visible with dsRNA substrates (Fig. 2C), but here the molar excess of substrate was not sufficient for statements concerning processivity.
The authors point out (bottom of p. 9) that hDis3L2 and hDis3 release products of different lengths. It would be better to call these 'end products' to distinguish them from the nucleotides released during shortening.
They also claim that the activities of hDis3L2 and hDis3 are similar (Fig. S2C; bottom of p. 9). This is not justified as all substrate is already degraded at the first time point with either enzyme; thus rates cannot be judged.
Why are substrates with two 3' terminal thymidine residues resistant? Are these deoxythymidines?

Fig. S5A is not well explained.

The results of 4sU labeling are not presented in Fig. S5E but in Fig. 6D (p. 16).

Referee #3:

The authors have adequately addressed the points raised in the original critique in this revised version.

2nd Revision - authors' response 15 May 2013

Response to Referee #2:

Referee #2

In this revised version, the authors have answered my criticism in a satisfying manner. This is a good paper and should be published provided that one additional modification is made:
As far as I could tell, knock-down of hDis3L2 was done with only one single siRNA. It is essential that off-target effects be excluded either by use of a second siRNA or complementation with an RNAi-resistant clone.

A/ We have employed a second siRNA against hDIS3L2 and performed RT-qPCR validations, demonstrating similar effects of the two siRNAs used (Figure S5E). Moreover, in agreement with our GO analysis, cell cycle mRNAs are consistently up regulated upon hDIS3L2 depletion. We have also performed additional experiments and revised the half-life analysis of Figure 6D to include an additional AU-rich mRNA (ZWINT).

I also have some comments regarding the nuclease assays:
Apparently, some assays were performed with pCp-labelled RNAs (e.g. Fig. S2C). It is not common that a 3' exonuclease will accept a substrate containing a 3'phosphate. The authors might wish to mention this in the Results section.

A/ pCp-labelled RNAs are substrates of exoribonucleases from the RNAseR family, which accept the 3'phosphate (Vincent et al. 2006, JBC). This is now mentioned in the relevant figure legend.

The authors' claim that Dis3L2 is processive is probably correct. However, in order to judge processivity one has to observe a mixture of untouched substrate and end products. This condition is not met very well, since, at most time points, the substrates are already completely degraded. In some cases, it seems like there is both substrate and product present at the 0 min time point. Thus, this does not seem to be really 0 min. The assays could be improved by including a real 0 min time point (i. e. sample taken before addition of enzyme), and a short time point to really prove the point of processivity. Mixtures of substrate and product are visible with dsRNA substrates (Fig. 2C), but here the molar excess of substrate was not sufficient for statements concerning processivity.

A/ We now include short time-point decay analysis for a single-stranded RNA substrate (ssRNA-5A), showing both undigested substrate and degradation products (Figure S2G).

The authors point out (bottom of p. 9) that hDis3L2 and hDis3 release products of different lengths. It would be better to call these 'end products' to distinguish them from the nucleotides released during shortening.

A/ Done.

They also claim that the activities of hDis3L2 and hDis3 are similar (Fig. S2C; bottom of p. 9). This is not justified as all substrate is already degraded at the first time point with either enzyme; thus rates cannot be judged.

A/ Agree. We now use Figure S2G to compare the activities of hDIS3 and hDIS3L2.

Why are substrates with two 3' terminal thymidine residues resistant? Are these deoxythymidines?

A/ Yes, the substrate contains deoxythymidines, which is now mentioned in the figure legend.

Fig. S5A is not well explained. The results of 4sU labelling are not presented in Fig. S5E but in Fig. 6D (p. 16).

A/ We decided to keep the current description of Figure S5A. We corrected the figure order.