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Dis3-like 1: a novel exoribonuclease associated with the human exosome

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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.)

1st Editorial Decision 12 February 2010

Thank you for submitting your manuscript for consideration by the EMBO Journal. I apologise for the length of time that it has taken to have your manuscript reviewed but this is in part to delays over the holiday period. It has now been seen by three referees whose comments are shown below.

As you will see from the reports the referees find the characterization of hDIS3L1 and its role in rRNA degradation to be interesting and are supportive of publication in the EMBO Journal pending satisfactory revision. The referees would like to see further definitive evidence for hDIS3L having 3'-5' exonuclease activity and quantification of several of the experiments. It should also be tested if they an interaction between hDIS3 and the exosome can be detected using the same approach used to validate the hDIS3L-exosome interaction. Finally, the referees request a number of control experiments.

Should you be able to address these issues, we would be happy to consider a revised manuscript. I should remind you that it is EMBO Journal policy to allow a single round of revision only and that, therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript. When 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 initiative, please visit our website: http://www.nature.com/emboj/about/process.html

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

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Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1 (Remarks to the Author):

The goal of this study is to establish the identity of the Dis3 homolog present on the cytoplasmic exosome and initial characterize it in regards to enzymatic activity, cytoplasmic localization and functional significance for 5.8S rRNA decay in vivo. The results of the study highlight important differences in the protein between yeast and human cells and thus make the study very significant as well as of broad interest given the importance of RNA decay in RNA processing and regulating gene expression.
Overall I do find the manuscript to be very interesting - however I do have some reservations about the study. Generally speaking, the authors should provide more support for a direct association of the hDis3L1 protein with the exosome core, and better describe the biochemical activities associated with the protein in terms of quantitation and depth in order to more convincingly support the key conclusions of the manuscript. These concerns are outlined in the six points below:

1. Fig. 2: This figure serves the key purpose of validating the association of hDis3L with the exosome. However since both hDis3L1 and Rrp4 are RNA binding proteins, the authors must treat the immunoprecipitate with RNAse to ensure that the interaction they are detecting in these assays is not due to an RNA bridge.
2. Fig. 3: The subcellular localization is performed with over-expressed, tagged proteins - which may or may not be consistent with the localization of the endogenous hDis3L1. Thus the authors need to at least recognize the possible limitation of their experimental approach, or more optimally generate an antibody that recognizes endogenous hDis3L1 and confirm this key conclusion of their study.
3. Fig. 4A: This set of data establishes another key conclusion of the study - exonuclease activity - and thus I would like to see it developed a bit further to make it fully convincing. First, I would recommend that the authors show their negative data for detecting endonuclease activity of hDis3L1. This is a very important distinction from the yeast protein and needs to be firmly established. Were multiple substrates used? How extensively were conditions (cation concentration, etc) altered to probe for endo activity? Second, while the authors are probably correct that hDis3L1 is showing 3'-5' exonuclease activity in the assay, since this is the first time it will be characterized for a protein that is only 29% similar to yDis3, I feel that they need to demonstrate that their assays are truly detecting 3'-5' exo activity. For example, is you use a substrate with a blocked 3' end, do the ~mononucleotide degradation products disappear?
4. Fig. 4B: How efficient was the RNAi knockdown and how reproducible was the apparent decrease in exonuclease activity in hDis3L knockdown cells? Surprisingly, when one looks at the substrate RNA, there seems to be significantly less substrate after 120 minutes in the hDis3L1 knockdown versus the control knockdowns. This discrepancy in the data must be addressed, perhaps through a quantitative presentation of enzymatic activity.
5. Fig. 4D: While these data are appealing from a qualitative perspective, a quantitative presentation of the data would be more effective in making the point, particularly if one takes into account the relative protein expression levels from panel C.
6. Fig. 5: These data are important to the study as they establish the in vivo significance of the hDis3L1 subunit. However the data as presented are extremely difficult to interpret. First, what was the extent of the knockdown of hDis3L1 and other proteins that was achieved in this panel. Second, RT-PCR assays (like the LM-PAT assay used to assess poly(A) tails on mRNAs) are notorious for being sensitive to input RNA levels and being difficult to assess quantitatively. Since a quantitative assessment of these data is vital to the authors conclusions, I believe that they must normalize there samples somehow to make these data convincing. Finally, I'm not at all clear what I am looking at in the gel. If one treats the sample with oligo dT and RNAse H, where does the unadenylated 28S rRNA band migrate? As presented, the gel is a smear of PCR products that is very difficult to connect with actual RNA species. In addition, the authors imply on pg. 12 that the details of the

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assay are apparently is being submitted for publication elsewhere. It might be very helpful to present the controls for the assay in a separate panel in Fig. 5.

Referee #2 (Remarks to the Author):

In this paper the authors describe three DIS3 homologs in humans. In yeast, DIS3 is the catalytic component of the RNA processing and degrading exosome complex. One human homolog (hDIS3) has previously been described, but its association with the exosome was not detected. In this paper the authors show that a second homolog (hDIS3L1) does associate with the exosome. They also conclude that hDIS3 is mainly nuclear, and hDIS3L exclusively cytoplasmic. These significant conclusions are generally well-supported by the data and merit publication in EMBO. However, there are a number of relatively minor issues that should be addressed.

1. On p 12 the authors show that knock down of hDIS3L results in the accumulation of polyadenylated 28S rRNA fragments. The experiment needs a control rt-PCR of some polyadenylated RNA that is not affected to show that similar amounts of RNA and cDNA were used.

2. The interpretation of this experiment is also problematic. The authors conclude that hDIS3L plays a role in cytoplasmic rRNA degradation. They have not included any controls that their fractionation only contains cytoplasmic RNA. Even if they only analyzed cytoplasmic RNA, have they considered the possibility that the 28S rRNA fragments are normally degraded in the nucleus, and only enter the cytoplasm in the hDIS3L knock down cells? Generation of polyadenylated 28S rRNA fragments in the cytoplasm would require a cytoplasmic TRAMP-like complex. Is there any evidence for this? Have the authors considered the possibility that accumulation of A+ 28S fragments is a secondary effect?
I think the important conclusion of this paper is that knock down of hDIS3L results in an RNA metabolism defect (but see comment 1). Whether cytoplasmic 28S rRNA degradation is a significant pathway is not central to this paper and that conclusion should be deleted from the paper

3. The authors conclude that hDIS3L is exclusively located in the cytoplasm, but I do not think their analysis is thorough enough to exclude the possibility of a cytoplasmic pool, especially given that they report 4 isoforms and the accompanying manuscript by Tomecki et al. shows a western blots with a slightly smaller protein in the cytoplasmic fraction.

4. The authors come to the firm conclusion that hDIS3 does not interact with the exosome. Presuming they have seen the manuscript by Tomecki et al that was submitted for joint consideration, can the authors comment on the conclusion by Tomecki et al that hDIS3 does interact with the exosome? On pages 5 and 10 the authors state they have a manuscript in preparation showing that hDIS3 does not associate with the exosome. The current manuscript would have been much stronger if they had chosen to include such data.

5. On page 11 the authors describe analyzing a D166N mutation in the PIN domain, but figure S1 shows that E167 is the appropriate residue to target. The authors need to clarify this discrepancy.

minor comments:

6. On p 16 (and continuing on p17) the authors write "The depletion of one of the exosome core components had a less pronounced effect on the accumulation of these molecules, which may indicate that the association of hDis3L1 with the exosome core is not necessary for this activity." an equally likely explanation is that the knock down of PM-SCL100 and RRP40 was not efficient enough to get the effect.

7. The heading on p9 "hDis3L1 displays exoribonuclease activity" should be changed to "hDis3L1 displays ribonuclease activity" as the authors never test whether it acts as an endo or exonuclease. The same change needs to be made in other places.

8. On p 12 the authors write "Western blotting or RT-PCR quantification, in case appropriate antibodies were lacking, showed significant reduction of the silenced proteins (results not shown)"

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What do the authors consider "significant reduction"? do they mean statistically significant or biologically significant? Why is the data not shown?

9. In figure 2 the authors use GFP as a control to show that the exosome interacts with Rrp4. They need to show a larger part of the western blot to show that the control GFP was expressed and successfully precipitated to make this control meaningful

10. In several places (including the abstract) the authors use the phrase "the human Dis3 homologue" but the over all message of the paper is that there are three homologs.

11. on p18 "washbuffercontaining" should be "wash buffer containing"

12.on p19 buffer A and buffer B should be changed to solution A and B

13. The materials and method section is incomplete. How was the DIS3L1 cDNA subcloned into pEGFP-C3, pCI-neo-5'VSV and pEGFP-N2. how where PM-SCL100 and RRP40 knocked down?

Referee #3 (Remarks to the Author):

This paper was submitted with the publication (Tomecki at al) that provied an independent finding of similar results. The two manuscripts could be eventually be published back to back in the same issue. The results obtained in this mauscript in general support the results showed in the other work. Here authors also discovered novel yeast RRP44 homologue in human Dis3L and characterized its association with exosome and localization. Additionally authors give the stronger evidence for cytoplasm function of Dis3L protein, and identified another gene coding potential protein involved in RNA metabolism Dis3L2.
Except this point the paper gives less information than that the one introduced by Tomecki at al. Authors focused mostly on localization and function of Dis3L protein, they proved its association with exosome, exonucleolytic activity and subcellular distribution. It should be mentioned that authors show localization of only overexpressed proteins and in case of Tomecki at al they show also localization of endogenous ones.

One think is worth to mention comparing these papers is the results concerning cytoplasm degradation of rRNA. Authors have shown that depletion of Dis3L protein causes stabilization of transient polyadenylated substrates and depletion of the exosome component RRP40 does not have the same effect. This is evidence that at least in this case Dis3L do not works or together with exosome or can work independently. In the paper of Tomecki at al authors suggest rather different hypothesis in which Dis3 and Dis3L proteins are rather always linked to the exosome core. And they show evidences for such a association of nuclear located Dis3 with exosome, depletion of exosome core subunits gives the same effect as depletion of Dis3 which suggests that this protein works only as the component of the complex and independently is not able to serve its functions.
Together these results do not exactly exclude each other but they lead to two different hypothetical models and probably the future work will clarify this part of our knowledge.
We can also have the situation where cytoplasm Dis3L is able to work independently and nuclear Dis3 not.

Below I state some comments that should be addressed:

First you tested the presence of proteins that came associated with the core of the human exosome and you detected only hDIS3L1, and then you cloned it to confirm its interaction with the exosome. The paper that was submitted back to back with yours shows that hDIS3 also associates with the exosome.
Therefore it is imperative that you also clone hDIS3 and perform the same procedures that lead to your confirmation of hDIS3L1. With this approach it is quite possible that you confirm that hDIS3 also interacts.

And what about hDIS3L2? What is its localization? You mention it in the paper but then it is unfortunate that it was "forgotten"...

You do not say what is the size of the end-product of the enzyme. They only mention that it can be mononucleotides. It would be good to compare with the end product of yeast Dis3/Rrp44.

When you localize the protein it would be much better if you also localize the nucleus with a dye and then you merge the images,

If the author have seen that the protein does not have endonucleolytic activity I do not see the reason why they have constructed the mutants D62N e D166N,

Figure 5B is of low quality and we can not distinguish the fragments, Besides there are no indication regarding what are the fragments that are "visible".

Page 16: You say that the link to the exosome could be performed by the PIN domain, similar to what happens in years. You could test a truncated protein without the PIN.

1st Revision - authors' response 09 May 2010

Reviewer #1:

1. We agree with the reviewer that the data presented in Figure 2 do not exclude the possibility that the interaction between hDis3L1 and the exosome is due to an RNA bridge. To investigate this issue we have performed two additional experiments, one in which the immunoprecipitate was treated with nucleases and one in which the cell lysates were treated with nucleases prior to the immunoprecipitations. In both cases the co-precipitation of hRrp4 with EGFP-hDis3L1 was not abolished, which indeed supports the direct association of hDis3L1 with the exosome. The results of these experiments are now described in the revised manuscript (Page 8 and 9).

2. As requested by the reviewer we have now analyzed the subcellular localization of the endogenous hDis3L1 protein in addition to the overexpressed, tagged proteins. The results of the new experiments, in which besides an anti-hDis3L1 antibody an antibody to exosome core component hRrp40 was used to visualize the localization of these proteins by confocal microscopy, are now added to Figure 3 of the manuscript (panels E-J). In addition, the possible limitations of localization studies using overexpressed proteins are now mentioned in the revised manuscript (Page 9).

3. The reviewer would like to see more details for the experiments in which the potential endonuclease activity of hDis3L1 was investigated. In addition to the magnesium concentration series already shown in Figure 4A, similar experiments with increasing concentrations of manganese were performed, the results of which are now added (Supplementary Figure S2A). In addition, the results of an experiment in which the effects of the hDis3L1 mutants on the putative endonuclease activity (at 1 mM manganese) were investigated are now also included (Supplementary Figure S2B). Replacement of the Tris-based buffer by a HEPES-based buffer gave rise to similar results (not shown).
To confirm that hDis3L1 is showing 3’-5’ exonuclease activity we have performed activity assays similar to those presented in Figure 4A, but with pre-incubation of the substrate RNA with oligonucleotides complementary to either with the 5’- or the 3’-end of the substrate. The results indicate that degradation of the substrate RNA was impaired when pre-incubated with an oligonucleotide hybridizing to the 3’-end, in contrast to pre-incubation with the other oligonucleotide. These observations are now described in the manuscript on page 10.

4. To provide insight into the efficiency of RNAi knockdown we have added the results of a western blot probed with an anti-hDis3L1 antibody, as well as with exosome core and loading control antibodies in the Supplementary data (Figure S3).
We agree with the reviewer that reproducibility information is needed to interpret the data shown in Figure 4B. This experiment has been repeated 5 times with identical results. We have now replaced the results shown in the original Figure 4B by those obtained in the replication experiment from which the cell lysates were used to prepare the western blot of Figure S3.

5. In contrast to the reviewer's suggestion, there is no direct quantitative correlation between the data in Figures 4C and 4D. The results in Figure 4C show that the expression levels of the wild-type and mutant EGFP-hDis3L1 proteins are very similar, as determined by western blotting of total cell lysates. The activity assay of Figure 4D, on the other hand, was performed with immunoprecipitates from these lysates. The immunoprecipitates were obtained with equal amounts of immobilized anti-GFP antibodies and as a result the relative amounts of precipitated fusion proteins may be determined by these antibodies and not by their levels in the lysates.

6. The paper in which the details of this type of experiment are described has recently been published and a reference to this article has been added (Slomovic et al., 2010; Ref. 30). To facilitate the interpretation of the data presented in Figures 5A and 5B, the figure has been modified as described below.
a) The schematic illustration of the PCR labeling process has been changed to make it easier to understand and to allow the viewer to make a connection between the signals in the gel and the degradation intermediates in the scheme.
b) The efficiency of the knockdown of the different proteins is now illustrated in Supplementary Figure S4, panel B, containing the results of RT-PCR analyses for the hDis3L1, hRrp40, and PM/Scl-100 mRNAs.
c) We agree with the reviewer that in view of the sensitivity to input RNA levels the RT-PCR assays should be normalized. Therefore, we've added a panel showing the RT-PCR results for the B2M gene. This confirms that indeed identical levels of RNA were used as template for the reverse transcription of each of the samples.
d) We hope that the changes in the layout of the figure and the rearrangement of the schematic illustration now make it easier to interpret the results of the gel. Data obtained after treatment of the samples with RNase H and oligo(dT) have been added as well (Figure 5B). A sample of cells treated with an siRNA for hXrn1 was included in these control experiments (lane 5), because this corresponds to the original negative control in which oligo(dT) was omitted (Figure 5A, lane 5). Lane 6 in Figure 5B shows that if there is no oligo(dT) in the RNase H reaction, labeled products derived from polyadenylated RNA accumulate. When oligo(dT) and RNase H are both in the reaction, all poly(A) tails are removed and the following reverse transcription reaction, primed with oligo(dT), does not yield any cDNA and hence no accumulation of such products is observed.
The text of the manuscript has been modified in accordance with the changes made in Figure 5. (Page 13-14).

Reviewer #2:

1. The controls requested by the reviewer have now been added. See also response to point 6 of Reviewer #1.

2. The results of control analyses to demonstrate that the cytoplasmic fractions only contain cytoplasmic RNA have now been added (Supplementary Figure S4, panel A). The possibility that polyadenylated 28S rRNA fragments originate from the nucleus and enter the cytoplasm in the hDis3L1 knock-down cells can indeed not be excluded completely, and therefore is now discussed in the manuscript (page 18).
We agree with the reviewer that the cytoplasmic polyadenylation of 28S rRNA fragments would require a cytoplasmic TRAMP-like complex. We would like to refer the reviewer to a discussion on this topic in our recently published article in PNAS (Slomovic et al., 2010; Ref. 30).
Indeed, the question whether cytoplasmic 28S rRNA degradation is a significant pathway is not central to our paper. To our knowledge, we did not draw this conclusion in the original manuscript.

3. As outlined above, we have added additional data (Figure 3E-J) to elucidate the subcellular localization of hDis3L1, including immunofluorescence microscopy data obtained with an anti-hDis3L1 antibody to detect the endogenous protein. Importantly, this polyclonal antibody is expected to be reactive with all four isoforms and the results confirm the cytoplasmic accumulation of hDis3L1 in the HEp-2 cells. Potential differences between the different hDis3L1 isoforms are now discussed (page 16).

4. Indeed, we have seen the manuscript by Tomecki et al. In their experiments they had to use less stringent conditions (lower NaCl concentrations) to be able to detect exosome core components in

the material co-purified with hDis3-FLAG. These observations indicate that if hDis3 does interact with the exosome, this interaction appears to be very instable. As suggested by the reviewer, we have now included the results of one of our experiments in which the interaction of hDis3 with the exosome core was studied (Supplementary Figure S5). These results confirm the very salt-sensitive interaction of hDis3 with the exosome core. In this respect it is interesting to stress that the association of hDis3L1 with the exosome core apparently is, at least in part, resistant to 1 M salt, as indicated by the results of our mass spectrometry analyses. In the Discussion of the original version of our manuscript we already stated ëIn the nucleus, hDis3 seems to act independently of the core of the exosome, although an instable association that is not resistant to cell lysis and fractionation can not be excluded.í (page 17 of revised manuscript).

5. The original sequence alignment (Supplementary figure S1) was generated with the ClustalW algorithm, which unfortunately resulted in a suboptimal alignment of D166 (see also the sequence alignment in the Tomecki paper). We have now generated the alignment using the MUSCLE algorithm (and replaced Supplementary Figure 1), which indeed indicates that D166 and not E167 is the appropriate residue to target.

6. Although the RT-PCR analyses shown in the new Supplementary Figure S4 indicate that the knock-down of PM/Scl-100 and Rrp40 is quite efficient, we cannot completely exclude the possibility the depletion at the protein level is less pronounced and have therefore added this explanation to the manuscript (page 18).

7. Because several new data in the revised manuscript in our view more convincingly show that hDis3L1 acts as an exoribonuclease (mononucleotide products; no detectable endoribonuclease activity; inhibition of the activity by blocking the 3' end of the substrate RNA), we have not introduced the changes suggested by the reviewer.

8. The data of several experiments, in which the reduction of expression levels after siRNA-mediated knock-down was monitored, are now included in the revised manuscript (Supplementary Figures S3 and S4). We meant biologically significant reduction and have changed 'significant reduction' into 'a strong reduction' to prevent confusion.

9. Unfortunately, the region of the blot containing free GFP was not probed with the anti-GFP antibody but instead with the anti-hRrp4 antibody (hRrp4 migrates at approximately the same position in SDS-PAGE as GFP). The expression of GFP is, however, illustrated in Figure 4C, which contains in lane 1 similar material from a separate experiment.

10. We have changed "the human Dis3 homologue" at several places in the manuscript to clarify which homologue is meant there.

11. The text was changed as suggested by the reviewer.

12. The text was changed as suggested by the reviewer.

13. The Materials & Methods section now includes details on the subcloning of the hDis3L1 coding sequence to the pEGFP and pCI-neo-5'VSV vectors (Page 22). In addition, the siRNA-mediated knock-down of PM/Scl-100 and Rrp40 is now described (Page 25).

Reviewer #3:

1. As described on page 6 of the original manuscript, the experiments that led to the identification of hDis3L1 (identification of proteins co-purifying with the exosome by mass spectrometry) failed to detect hDis3 as an exosome-associated protein. The results of Tomecki and collaborators show that the interaction of hDis3 with the exosome core is only found at very low salt concentrations. We have performed experiments very similar to those that confirmed the association of hDis3L1 to investigate the association of hDis3. The results of one of these experiments are now added (Supplementary Figure S5) and confirm the salt sensitivity of this interaction. See also response to point 4 of Reviewer #2.

2. Like the reviewer, we are also very interested to learn more about hDis3L2. In fact we performed a series of experiments to investigate the properties of this protein as well. A cDNA encoding this protein was cloned and inserted into several transfection constructs. Unfortunately, the results of all experiments with these constructs suggest that the expression of tiny amounts of this protein is already lethal for the cells. As a consequence, we did not succeed to monitor its expression by western blotting or immunofluorescence microscopy. We hope we will manage to develop procedures in the near future to characterize this protein as well.

3. Using radiolabeled mononucleotides as a marker in denaturing polyacrylamide gel electrophoresis, we have confirmed that the end-products of the hDis3L1 activity assays are indeed mononucleotides. The text has been changed accordingly (page 10).

4. In the new experiments performed to localize hDis3L1 (Figure 3 E-G), Hoechst staining was included to visualize the nucleus, as recommended by the reviewer. In addition, the merged images are included.

5. We agree with the reviewer that it might not make much sense to generate mutants which are mutated in a domain that does not seem to be catalytically active. However, when the mutagenesis of the cDNA was started, no data for endo- or exo-nuclease activities were available yet. On the other hand, mutations in one domain may serve as controls for mutations in another domain (see for example the results in Figure 4D). Moreover, in vitro endoribonuclease assays are very sensitive to trace contaminations of other endoribonucleases and therefore the availability of both catalytically active and inactive mutants of the protein of interest may facilitate the interpretation of the data (see e.g. Supplementary Figure S2B).

6. As already pointed out above (Reviewer 2, point 6), Figure 5 has been revised to increase its clarity.

7. We hope to be able to study the way in which hDis3L1 interacts with the exosome in the near future. The suggestion of the reviewer to delete the PIN domain to investigate the requirement of this domain indeed seems to be a good experiment to start with.

2nd Editorial Decision 17 May 2010

I have looked through your revised manuscript, your responses to the original reports and I have also discussed them with one of the referees, we find that you have satisfactorily addressed all the concerns raised (see below). I am happy to accept the manuscript for publication in The EMBO Journal. You will receive the original acceptance letter in the next day or so.

REFEREE REPORT

Referee #1

The authors have done a good job at addressing the criticisms/suggestions that were raised in the initial reviews.

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