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The mRNA export factor Npl3 mediates the nuclear export of large ribosomal subunits

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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 January 2011

Thank you very much for the submission of your research manuscript to our editorial office. First of all, please accept my apologies for the unusual amount of time it has taken us to get back to you with a decision on your manuscript, which was due to the holiday season during which our editorial office was closed. We have now received the three reports from the referees that were asked to assess it.

As the detailed reports are pasted below I would prefer not to repeat them here, but you will see that the reviewers, in principle, agree on the potential interest of the findings. However, they also feel that additional work is needed to substantiate the conclusions drawn.

Referee 1 points out that the defects in the export of ribosome subunits seen in the npl3delta strain could also be due to subtle defects in mRNA export and we agree that clarifying this is an important point. Both referees 1 and 2 state that stronger proof for an association of Npl3 with pre-ribosomes is needed and referee 2 suggests potential experiments on how to achieve this. Both referees also feel that the significance of Npl3's interaction with 25S rRNA needs further analysis and referees 2 and 3 point out that in some cases additional controls are needed. Reviewer 3 raises an issue with the background of the yeast strains used and states that it should be shown whether cells lacking npl3 have defects in ribosome biogenesis.

From the analysis of these comments it becomes clear that significant revision is required before the manuscript becomes suitable for publication in EMBO reports. However, given the potential interest of your study, I would like to give you the opportunity to revise your manuscript, with the

understanding that the main concerns of the referees must be addressed and their suggestions (as detailed above and in the referees' reports) taken on board. Acceptance of the manuscript will depend on a positive outcome of a second round of review and I should also remind you that it is EMBO reports 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.

I look forward to seeing a revised form of your manuscript when it is ready.

Yours sincerely

Editor
EMBO Reports

REFEREE REPORTS:

Referee #1:

In the manuscript presented here the authors investigate the possibility that the mRNA export adapter Npl3 is involved in the export of 60S ribosome subunits in yeast. The authors present data including genetic interactions, and a series of immunoprecipitations that, they claim, show Npl3 to interact with the export competent pre-60S particle and directly mediate its export via an interaction with the NPC.

Whereas the possibility of Npl3 playing a role in ribosome export is intriguing, the experiments performed and the data presented here provide only a very preliminary analysis of a potential role for Npl3 in ribosome export. Whilst I cannot exclude that Npl3 is involved in 60S export, the mechanism remains obscure. The results presented here are suggestive at best, and fail to compellingly demonstrate the claims made.

Specific comments.

1. The npl3Δ strain that is used for much of the genetic analysis may still display a subtle defect in mRNA export. Defects in ribosome subunit export could arise from such a mild defect in mRNA export. (e.g. mRNAs encoding r-proteins).

2. The physical association of Npl3 with pre-ribosomes has not been clearly demonstrated. A more comprehensive analysis of the nature of the Npl3-pre-60S interaction is required. Additionally, negative controls using other RNA binding proteins may prove helpful. The apparent association of Npl3 with the earlier Rix1 particle is unlike what was seen for the export adapters Mex67 and Nmd3. This association with earlier pre-ribosomes could give rise to an assembly defect in the absence of Npl3 (something which is not extensively investigated).

3. What is the significance of Npl3 binding to 25S rRNA? Npl3 is an RNA binding protein, and as such would be anticipated to be bind to a complex RNA species in vitro, no specificity is shown in this assay. Additionally, the association of Npl3 with components of pre-ribosomes (Rpl25 etc) does not appear to be RNase sensitive, arguing against the idea that Npl3 is interacts with pre-ribosomes via an RNA element.

4. The nature of the Npl3 - Nup60 interaction is very unclear, and many questions remain. The co-IP with Nup60 shows only that a complex containing Npl3 is capable of contacting the NPC, but not necessarily Npl3 itself. How could a Nup60-Npl3 interaction facilitate export? Is there a 60S export defect in nup60Δ? Are synergistic effects seen between Nup60 and npl3Δ?

5. A key experiment to show that Npl3 is capable of mediating export as recptor would be to show that it is capable of interacting with FG-repeats.

Referee #2:

In this manuscript, the authors have investigated the role of Npl3 in the export of the large ribosomal subunit. Apart from a few minor issues, the work is well performed and of interest. My key concerns are that a few important controls need to be provided to validate the conclusions discussed in the paper.

Points to be addressed:

1) Page 5, line 11. The authors mention that they look at the transport of both the large and small subunit in the Npl3 mutants. Only the large subunit (Rpl25-GFP) data is shown. The authors should also show the small subunit data as a negative is required to demonstrate that these defects are specific and not just due to a general export problem. In addition, while it has been established that GFP-tagged ribosomal proteins provide a good measure of ribosome localisation, it is important to also show that the pre-60S complexes, and not just the GFP-tagged protein, are accumulating in the nucleus in these mutants. FISH analysis of 18S and 25S localisation should be shown to confirm that the GFP-tagged proteins are, in this instance, providing a true reflection of ribosome localisation.

2) Page 6, last paragraph. The authors claim that the npl3 shows no obvious processing defects. Some rRNA processing defects are mild and can only be detected by Northern blotting as they hardly affect the levels of mature rRNAs. The authors need to characterise the defects fully by Northern blotting.

3) Page 8. The authors claim that Npl3 is associated with pre-60 complexes. This is based purely on the co-IP of the protein with a GFP-tagged ribosomal protein and ribosome biogenesis factors. While the authors are probably correct, all of the data presented are indirect and it is possible that Npl3 associates with these factors independently of the pre-ribosomes. Northern blot analysis of the immunoprecipitated RNA should be performed to demonstrate the association of Npl3 with the pre-60S complexes.

4) Page 9. The authors show that Npl3 directly interacts with 25S rRNA. This protein is very likely a non-specific RNA-binding protein and therefore the authors need to use some other RNAs in this assay to show whether this is sequence/structure-specific binding or whether this is non-specific. Furthermore, since Npl3 associates with Rpl25, Arx1 and Rix1 after treatment with RNase, this would suggest that RNA-binding may not be important. Npl3 contains an RNA recognition motif (RRM), which while characterised as an RNA-binding motif, have been also shown to function as a protein-protein interaction motif as well. The authors either need to discuss these possibilities or provide stronger evidence that RNA-binding is important for Npl3 involvement in pre-60S export.

5) The figure legends were often confusing and in some cases need significant work. For example:
Figure 1A. The authors claim that GFP and DAPI staining are shown. I assume no staining was performed to reveal the GFP signal.
Figure 2. "covered by plasmid encoded NPL3 were..". The authors need to re-phrase this.
Figure 3. I found this figure legend extremely difficult to follow.
Figure 4C. What region of 25S was in vitro transcribed? It needs to be stated here and perhaps indicated in a secondary structure diagram of the 25S rRNA.

Referee #3 :

I believe that the authors should address my comment regarding strain backgrounds, and all my major points, before the paper would be suitable for publication. Assuming the authors can address the experimental issues raised in the major points, I believe that the paper would then be suitable for

publication.

In this manuscript the authors describe data suggesting that Npl3 is involved in nuclear export of the 60S ribosomal subunit. The authors show that both mutation and deletion of npl3 results in accumulation of Rpl25-GFP, a protein that forms part of the large ribosomal subunit. The accumulation of Rpl25-GFP in an npl3 mutant can be rescued by over-expression of the export factors nmd3 or mtr2. Co-immunoprecipitation experiments demonstrate that Npl3 physically interacts with various export factors, Rpl25-GFP and the nucleoporin, Nup60. The findings of this study are interesting but I believe that some more experiments, and the clarification of some key points, are required before it would be ready for publication.

In addition to the points listed below, I have a query that I would like authors to address. At several points in the manuscript, reference is made to the differences between the strain backgrounds S288c and BY, vis a vis requirement for Npl3. The authors suggest that in the S288c strain background Npl3 is essential, whereas in the BY background it is dispensable. However, it has always been my understanding that these two strain backgrounds are similar, and that Npl3 is dispensable in both. In contrast, in the W303 background, used in many mRNA export studies, Npl3 is indeed essential. Would the authors be able to confirm that they have tested an authentic S288c strain and found Npl3 to be essential in this background?

Major points
1. A key experiment currently lacking, is the demonstration that the 60S export defect observed in npl3 mutants results in a ribosome biogenesis defect (by polysome profiling). This experiment should be done. Critically, the authors should show that this ribosome biogenesis defect is rescued by over-expression of export factors Nmd3 and Mtr2 (shown to rescue the npl3 Rpl25-GFP export defect in figure 2B).
2. It should be shown that the tagged protein Rpl25-GFP is incorporated into ribosomes (or reference where this has been shown)
3. The study lacks important negative controls in a number of places:
(i) Co-immunoprecipitation experiments should include the negative control of a protein that does not physically interact with Npl3.
(ii) Treatment with RNase was not found to disrupt any physical interaction tested. A control should be included to demonstrate that the RNase treatment was successful, i.e. the authors should show that RNase treatment disrupts a physical interaction between two proteins that is known to be mediated through RNA.
(iii) An RNA that is not bound by Npl3 should be included in figure 4C.

Minor points
1. Figure S1 is confusing. Does this figure show staining for polyA RNA? If so, the legend should clearly state this. Why are DAPI stained panels in figure S1 totally blank?
2. The legend for figure S3 should include an explanation of 'E' and 'L'. The use of Hem15 as a negative control should also be explained.
3. In figure 1A it looks like some npl3 mutant cells do not express Rpl25-GFP at all, although they do stain with DAPI. The authors should comment on why there is no Rpl25-GFP expression.
4. The legend for figure 1D is wrong. The legend refers to experiments with the S22c strain, whereas the figure shows data for the 40S subunit!
5. When describing the data in figure 2A, the authors suggest that the growth of the npl3 deletion strain is further impaired by mutation of nmd3. I disagree. This double mutant strain does not appear to grow any worse than the strain where only npl3 is deleted. Additionally, the contrast is not the same on the different panels in figure 2A, and this makes it difficult to assess subtle growth differences.
6. For figure 3, can the authors confirm that the 'minus GFP' samples used in the co-immunoprecipitations are extracts with no GFP tagged protein? It is unclear from the legend what these lanes represent.
7. On page 10 the authors suggest that Npl3 directly mediates the interaction between the 60S subunit and the nuclear pore complex, but they do not show this. In order to test this, the authors should show that Rpl25-GFP interacts with Nup60 only in the presence of Npl3.

1st Revision - authors' response 05 April 2011

We thank all three referees for their comments on the manuscript. All suggestions were extremely helpful to improve our study and we have addressed all points that were made.

Referee #1:

In the manuscript presented here the authors investigate the possibility that the mRNA export adapter Npl3 is involved in the export of 60S ribosome subunits in yeast. The authors present data including genetic interactions, and a series of immunoprecipitations that, they claim, show Npl3 to interact with the export competent pre-60S particle and directly mediate its export via an interaction with the NPC.

Whereas the possibility of Npl3 playing a role in ribosome export is intriguing, the experiments performed and the data presented here provide only a very preliminary analysis of a potential role for Npl3 in ribosome export. Whilst I cannot exclude that Npl3 is involved in 60S export, the mechanism remains obscure. The results presented here are suggestive at best, and fail to compellingly demonstrate the claims made.

We appreciate that the referee acknowledges our study as "intriguing" and we are convinced that by adding significant new data, we corroborated our study significantly.

Specific points:

1. The npl3Δ strain that is used for much of the genetic analysis may still display a subtle defect in mRNA export. Defects in ribosome subunit export could arise from such a mild defect in mRNA export. (e.g. mRNAs encoding r-proteins).

The mRNA export assay we are using is quite sensitive and since an mRNA accumulation is not detectable in *npl3Δ* a potential remaining defect can only be slight. However, we agree that this might be possible and therefore we tested if a mutant of the nuclear porin mutant nup159 (*rat7-1*), which has severe mRNA export defects at 37°C, displays export defects of the ribosomal subunits. We clearly show that this is not the case (new Fig 1C, E and F and text on page 7, line 14). Therefore we can conclude that the export defects of the pre-60S ribosomal subunit seen in *npl3Δ* are not due to mRNA export defects.

2. The physical association of Npl3 with pre-ribosomes has not been clearly demonstrated. A more comprehensive analysis of the nature of the Npl3-pre-60S interaction is required. Additionally, negative controls using other RNA binding proteins may prove helpful. The apparent association of Npl3 with the earlier Rix1 particle is unlike what was seen for the export adapters Mex67 and Nmd3. This association with earlier pre-ribosomes could give rise to an assembly defect in the absence of Npl3 (something which is not extensively investigated).

In addition to the interaction of Npl3 with the pre-ribosomal exclusively nuclear protein Rix1, we provide now the interaction of Npl3 with the 35S rRNA (new Figure 4D and text on page 10, line 18). These results were obtained with different methods (protein coimmunoprecipitations and RNA interaction analyses) and support an early association of Npl3 with the pre-ribosomal particle.
For the requested negative control we used the RNA-binding protein Prp43, which does not show an interaction with Npl3 (new Figure 3A and text on page 9, line 10).
The referee points out that other export adapters like Mex67 and Nmd3 do not associate with the early, unprocessed pre-60S ribosomal subunit. Indeed, we do not detect an interaction of Nmd3 with the 35S rRNA (Fig. 4D). However, we think, that this does not mean that the same must be true for Npl3. We rather think that Npl3 might be recruited to the pre-rRNA (text on page 10, line 20) in analogy to its recruitment to the pre-mRNA, which occurs co-transcriptionally by RNA-polymerase II (Lei et al. 2001, Genes & Dev.) (In case of the rRNA association one could speculate a recruitment by RNA polymerase I.) However,

we think that the question of the recruitment is beyond the scope of the paper. We report on the novel function of Npl3 in transporting large ribosomal subunits.
Finally the referee fears that an early assembly of Npl3 might lead to assembly defects in an *npl3Δ* strain. This is unlikely, because we do not detect 18S and 25S rRNA maturation defects in *npl3Δ* (Fig S2B and C). Similarly, Li and colleagues found no alterations in the abundances of all types of pre-rRNA and rRNA species, measured in northern blot analyses with *npl3Δ* (Li et al, 2009, PLOS Biology). Further, overexpression of other export receptors in *npl3Δ* rescue its export defects while this is not the case for *rix1-1* (Fig 2B).
Thus, it can be concluded from our data and the data of Li et al., that no obvious maturation defects are present in *npl3Δ*. We now included the reference of Li and colleagues and added that to the text on page 7, first paragraph.

3. What is the significance of Npl3 binding to 25S rRNA? Npl3 is an RNA binding protein, and as such would be anticipated to be bind to a complex RNA species in vitro, no specificity is shown in this assay. Additionally, the association of Npl3 with components of pre-ribosomes (Rpl25 etc) does not appear to be RNase sensitive, arguing against the idea that Npl3 is interacts with pre-ribosomes via an RNA element.

The referee is right, that in this assay we do not show specificity for Npl3 binding. Therefore, we extended our studies and tested the binding of Npl3 to the 35S, 27S, 25S, 18S and 5,8S rRNA *in vivo*. As shown in the new Figure 4D, we find that Npl3 specifically and strongly binds to the 25S rRNA *in vivo*, whereas its affinity for the 5,8S rRNAs is rather low. We detect no binding to the 18S rRNA. Additionally we detect binding of Npl3 to the 35S and the 27S rRNA, which indicate an early recruitment of the SR-protein to the immature 25S rRNA. (new Fig 4D and text on page 10, line 18).
Additionally we now show in the new Figure 4E an *in vitro* assay, in which we investigated direct binding of the different rRNA species with recombinantly produced Npl3. Here we detect a direct interaction of Npl3 with the 25S rRNA, but no interactions with the other rRNA species. (new Fig 4E and text on page 11, line 3)
Besides its binding to the rRNA we show that Npl3 physically interacts with proteins associated with the large ribosomal subunit (Fig 3). These interactions are RNase insensitive, which means that Npl3 contacts the 60S subunit at both, the 25S rRNA and the proteins. This double interaction opens up the possibility that the binding of Npl3 to large ribosomal proteins might influence the specificity in RNA-binding. (see discussion on page 15, line 6).

4. The nature of the Npl3 - Nup60 interaction is very unclear, and many questions remain. The co-IP with Nup60 shows only that a complex containing Npl3 is capable of contacting the NPC, but not necessarily Npl3 itself. How could a Nup60-Npl3 interaction facilitate export? Is there a 60S export defect in Nup60? Are synergistic effects seen between Nup60 and Npl3

It is true that we can't exclude that the interaction between Npl3 and Nup60 is not direct. To avoid misunderstandings we re-phrased the sentence and now say: "It is currently unclear if this interaction requires other proteins, however the interaction is not sensitive to the addition of RNase, emphasizing that Npl3 physically interacts with the NPC and this interaction is not due to the presence of Npl3 on exported mRNAs." (page 11, line13).
Further, we performed the requested experiment and now show in the new Figure 4H that deletion of *NUP60* leads to export defects of the large ribosomal subunit. In fact, the double mutant *npl3Δ nup60Δ* is synthetically lethal (new Fig 4I), supporting our model in which Npl3 exports the large ribosomal subunit via Nup60. (text on page 11, line 19).

5. A key experiment to show that Npl3 is capable of mediating export as receptor would be to show that it is capable of interacting with FG-repeats.

We investigated in *in vitro* experiments if recombinantly produced Npl3 is able to bind to FG-repeats and found a clear interaction, indicating the capability of this RNA-binding protein to directly contact FG-Nups (new Fig 4G and text on page 11, line 17).

Referee #2:

We appreciate that the referee judges our work as "**well performed and of interest**" and we have addressed all specific points:

1) Page 5, line 11. The authors mention that they look at the transport of both the large and small subunit in the Npl3 mutants. Only the large subunit (Rpl25-GFP) data is shown. The authors should also show the small subunit data as a negative is required to demonstrate that these defects are specific and not just due to a general export problem. In addition, while it has been established that GFP-tagged ribosomal proteins provide a good measure of ribosome localisation, it is important to also show that the pre-60S complexes, and not just the GFP-tagged protein, are ac7 cumulating in the nucleus in these mutants. FISH analysis of 18S and 25S localisation should be shown to confirm that the GFP-tagged proteins are, in this instance, providing a true reflection of ribosome localisation.

The localization of the small ribosomal subunit is shown in Figure 1D. Moreover, we show by sucrose density gradient- and western blot-analyses that Rpl25-GFP is indeed incorporated into the large ribosomal subunit and into ribosomes (new Figure S1D and text on page 6, line 14). In addition we established a novel assay showing FISH analyses with probes against the 18S and the 25S rRNA. These novel experiments led to the same result as with Rpl25-GFP and Rps2-GFP, showing a mislocalization of the large ribosomal subunit in *npl3Δ* (new Figure 1E and F and text on page 7, line 8).

2) Page 6, last paragraph. The authors claim that the npl3Δ; shows no obvious processing defects. Some rRNA processing defects are mild and can only be detected by Northern blotting as they hardly affect the levels of mature rRNAs. The authors need to characterise the defects fully by Northern blotting.

This type of experiment was already performed fore *npl3Δ* in Li et al. 2009 (PLOS Biology, Figure 5E) and the authors have detected no rRNA processing defects. This result was cited and included into the text on page 7, line 2.

3) Page 8. The authors claim that Npl3 is associated with pre-60 complexes. This is based purely on the co-IP of the protein with a GFP-tagged ribosomal protein and ribosome biogenesis factors. While the authors are probably correct, all of the data presented are indirect and it is possible that Npl3 associates with these factors independently of the pre-ribosomes. Northern blot analysis of the immunoprecipitated RNA should be performed to demonstrate the association of Npl3 with the pre-60S complexes.

In addition to the evidence that Npl3 associates with the pre-60S particle obtained through co-immunoprecipitations with ribosomal proteins and ribosome biogenesis factors (Fig 3), we now provide novel experiments showing an association of Npl3 with the pre-90S particle that contains the 35S rRNA (new Fig 4D). In this experiment we immunoprecipitated Npl3 and performed qRT-PCRs from the co-eluted rRNA. In contrast to Npl3, we show that Nmd3 does not associate to the early 90S particle. Taken together, these results show that Npl3 is recruited early to the 90S particle, which is very similar to its recruitment to the premRNA. (see also text on page 10, line 18).

4) Page 9. The authors show that Npl3 directly interacts with 25S rRNA. This protein is very likely a non-specific RNA-binding protein and therefore the authors need to use some other RNAs in this assay to show whether this is sequence/structurespecific binding or whether this is non-specific. Furthermore, since Npl3 associates with Rpl25, Arx1 and Rix1 after treatment with RNase, this would suggest that RNAbinding may not be important. Npl3 contains an RNA recognition motif (RRM), which while characterised as an RNA-binding motif, have been also shown to function as a protein-protein interaction motif as well. The authors either need to discuss these possibilities or provide stronger evidence that RNA-binding is important for Npl3 involvement in pre-60S export.

To address this issue, we performed novel *in vitro* rRNA binding studies with recombinant Npl3 protein and found a direct and strong interaction of Npl3 with the 25S rRNA and very slightly interaction with the 5.8S rRNA, both part of the large ribosomal subunit. In contrast we don't detect binding of Npl3 to the 18S rRNA (new Fig 4E and text on page 11, line 3). This clearly demonstrates a direct specific physical interaction of Npl3 with the 25S rRNA. Its physical RNase insensitive interaction with large ribosomal proteins might be important for recruitment, positioning and/or specificity of the rRNA-Npl3 interaction. This is now also discussed in the text on page 15, line 6.

5) The figure legends were often confusing and in some cases need significant work.

We apologize for the in parts confusing figure legends and included all suggestions.

For example:
Figure 1A. The authors claim that GFP and DAPI staining are shown. I assume no staining was performed to reveal the GFP signal.

There was indeed no GFP staining performed and we have changed that accordingly.

Figure 2. "covered by plasmid encoded NPL3 were..". The authors need to re-phrase this.

We have change the sentence to: "carrying plasmid encoded *NPL3 „*

Figure 3. I found this figure legend extremely difficult to follow.

We tried our best to rephrased and restructure the text.

Figure 4C. What region of 25S was in vitro transcribed? It needs to be stated here and perhaps indicated in a secondary structure diagram of the 25S rRNA.

We included the new Fig 4C to the main figures and explained the experiment in detail in supplemental material.

Referee #3:

I believe that the authors should address my comment regarding strain backgrounds, and all my major points, before the paper would be suitable for publication. Assuming the authors can address the experimental issues raised in the major points, I believe that the paper would then be suitable for publication.

We thank the referee for evaluating our paper as "suitable for publication" upon revision of several issues. We have addressed all points that were made and answered the raised questions below:

In this manuscript the authors describe data suggesting that Npl3 is involved in nuclear export of the 60S ribosomal subunit. The authors show that both mutation and deletion of npl3 results in accumulation of Rpl25-GFP, a protein that forms part of the large ribosomal subunit. The accumulation of Rpl25-GFP in an npl3 mutant can be rescued by over-expression of the export factors nmd3 or mtr2. Coimmunoprecipitation experiments demonstrate that Npl3 physically interacts with various export factors, Rpl25-GFP and the nucleoporin, Nup60. The findings of this study are interesting but I believe that some more experiments, and the clarification of some key points, are required before it would be ready for publication.

In addition to the points listed below, I have a query that I would like authors to address. At several points in the manuscript, reference is made to the differences between the strain backgrounds S288c and BY, vis a vis requirement for Npl3. The authors suggest

that in the S288c strain background Npl3 is essential, whereas in the BY background it is dispensable. However, it has always been my understanding that these two strain backgrounds are similar, and that Npl3 is dispensable in both. In contrast, in the W303 background, used in many mRNA export studies, Npl3 is indeed essential. Would the authors be able to confirm that they have tested an authentic S288c strain and found Npl3 to be essential in this background?

We can assure that we used the indicated strains. The viability of *npl3Δ* in *BY* is shown in the new Figure S1B and we show that *S288C* can't survive without *NPL3* in the new Figure S1A. Although the *BY*-strain historically arose from *S288C*, several differences have already been detected. An informative overview is given on the SGD website: http://wiki.yeastgenome.org/index.php/Commonly_used_strains
Besides other differences, the main differences between the three strains are that *S288C* is mutated in the *HAP1*, *MIP1* and *GAL2* genes. In contrast to that is the *BY*-strain only mutated in *MIP1*. *W303* is mutated in *MIP1* and in *RAD5*. These genetic differences might explain the essentiality of certain genes in one or the other background. *HAP1* encodes for a transcription factor and it is well conceivable that its expression might change the requirement for Npl3. While the exact reason for the essentiality of Npl3 is currently unknown, several publications have been made with Npl3 in the essential background (e.g. Lee et al. 1996, Genes & Dev.) and some with the inessential background (e.g. Kress et al. 2008, Mol. Cell). In none of these papers this issue was discussed. In contrast we openly tackle this issue and moreover make use of the situation in which Npl3 is not essential for the mRNA export out of the nucleus to study its novel function in ribosomal export. Finally, however, we were able to demonstrate that this function is present in both strain backgrounds and one function does not influence the other.

Major points
1. A key experiment currently lacking, is the demonstration that the 60S export defect observed in npl3 mutants results in a ribosome biogenesis defect (by polysome profiling). This experiment should be done. Critically, the authors should show that this ribosome biogenesis defect is rescued by over-expression of export factors Nmd3 and Mtr2 (shown to rescue the Δnpl3 **Rpl25-GFP export defect in figure 2B).**

A ribosome profile of *npl3*Δ has been published in Li et al. 2009, PLOS Biology. It shows a high 80S peak, which was also detected for Mtr2 and Mex67 downregulated strains. The authors classify this type of defects as translation defective. It is currently unclear if Npl3 has an additional function in translation and since this can't be excluded we turned the suggested experiment around and tested if the ribosomal subunit receptor function of Npl3 in high copy would suppress the receptor function defects in *nmd3-2*. Mutations in *NMD3* lead to the production of halfmers that were suggested to reflect 60S biogenesis defects (in case of *nmd3-2* originated by the nuclear export defects of pre-60S ribosomal subunits). We now show in the new Fig 2 that overexpression of Npl3 leads to the suppression of the defective growth rate (new Fig 2C), the pre-60S export defects (new Fig 2D) and a significantly reduced halfmer profile (new Fig 2E). These experiments clearly indicate that Npl3 can compensate for the defective export receptor function in *nmd3-2*. (see also text on page 8, last paragraph).

2. It should be shown that the tagged protein Rpl25-GFP is incorporated into ribosomes (or reference where this has been shown)

We include this experiment in the supplemental section as new Fig S1D. Sucrose density gradients and western blot analyses show that Rpl25-GFP is incorporated into the 60S ribosomal subunit, the mono- and polysomes. RNase treatment, which destroys polysomes also shifts Rpl25-GFP into the low molecular weight fractions.

3. The study lacks important negative controls in a number of places:
(i) Co-immunoprecipitation experiments should include the negative control of a protein that does not physically interact with Npl3.

In all experiments, the mitochondrial proteins Hem15 or Aco1 served as negative controls. In addition we now show in the new Fig 3A that Npl3 does not interact with the RNAbinding protein Prp43.

(ii) Treatment with RNase was not found to disrupt any physical interaction tested. A control should be included to demonstrate that the RNase treatment was successful, i.e. the authors should show that RNase treatment disrupts a physical interaction between two proteins that is known to be mediated through RNA.

We routinely perform co-immunoprecipitation studies with RNase treatment and published that the interaction between Dbp5 and Pab1 is RNase sensitive (Gross et al. 2007, Science). The same experimental set up was used in the case of Npl3.

(iii) An RNA that is not bound by Npl3 should be included in figure 4C.

We extended our study and investigated the binding of the 35S, 27S, 25S, 5.8S and the 18S rRNA and show now in the new Fig 4D and E that Npl3 strongly and specifically binds to the 25S rRNA (see also text on page 10, line 18).

Minor points
1. Figure S1 is confusing. Does this figure show staining for polyA RNA? If so, the legend should clearly state this. Why are DAPI stained panels in figure S1 totally blank?

Figure S1 was extended and this part is now depicted in the new Fig S1C. Here we show the GFP-tagged Npl3 protein, which contains a mutation that leads to a slow import (GFPNpl3c). Thus it can be used to monitor the export requirements of this protein. We hope that the figure legend is clearer now. Additionally we provide lighter versions of the DAPIstaining to solve the problem.

2. The legend for figure S3 should include an explanation of 'E' and 'L'. The use of Hem15 as a negative control should also be explained.

We included the explanation of “L and E” and explained our negative controls as mitochondrial proteins.

3. In figure 1A it looks like some npl3 mutant cells do not express Rpl25-GFP at all, although they do stain with DAPI. The authors should comment on why there is no Rpl25-GFP expression.

Not all cells show similarly strong Rpl25-GFP expression as it is a) expressed from a plasmid and b) expressed in addition to the wildtype protein. A certain variance in the expression level is thus normal.

4. The legend for figure 1D is wrong. The legend refers to experiments with the S22c strain, whereas the figure shows data for the 40S subunit!

We are terribly sorry for that. Now we provide the correct figure legend.

5. When describing the data in figure 2A, the authors suggest that the growth of the npl3 deletion strain is further impaired by mutation of nmd3. I disagree. This double mutant strain does not appear to grow any worse than the strain where only npl3 is deleted. Additionally, the contrast is not the same on the different panels in figure 2A, and this makes it difficult to assess subtle growth differences.

Maybe the impression was due to the differences in the contrast. We repeated the experiment and show now a clearer version of the experiment.

6. For figure 3, can the authors confirm that the 'minus GFP' samples used in the co-immunoprecipitations are extracts with no GFP tagged protein? It is unclear from the legend what these lanes represent.

Yes we can confirm that and stated that more clearly now in the figure legend.

7. On page 10 the authors suggest that Npl3 directly mediates the interaction between the 60S subunit and the nuclear pore complex, but they do not show this. In order to test this, the authors should show that Rpl25-GFP interacts with Nup60 only in the presence of Npl3.

The requested experiment can't be done, because the pre-60S ribosomal subunit is exported by several export receptors. Even if Npl3 is missing in the cell, another export receptor will lead to the interaction of Rpl25-GFP with the NPC and thus potentially a Nup60 interaction.
However, we now provide direct evidence for the binding of Npl3 with the NPC by interaction of recombinantly expressed Npl3 and the FG-repeats of Nup116 (new Fig 4G and text on page 11, lane 17). These novel findings strongly support a model in which Npl3 is capable of functioning as a receptor and directly interacting with the NPC to mediate the export of the large ribosomal subunit to the cytoplasm.

2nd Editorial Decision 06 May 2011

Many thanks for submitting the revised version of your manuscript to our editorial office. First of all, I would like to apologize for the delay in getting back to you. Your manuscript was sent back to the original referees and we have now received their feedback.

As you will see, referees 2 and 3 now, in principle, support publication of the study in our journal. Referee 3, however, still feels that the addition of a positive control for the RNase experiments is crucial, and I tend to agree with his/her argumentation.

Referee 1 is still not convinced that there is no ribosome assembly defect (due to defects in rRNA processing), as s/he feels that the analysis conducted in the present paper is not sufficient and because s/he is of the opinion that there is indeed a processing defect in the 2009 PloS Biology paper you cited. I have discussed this concern further with both referees 1 and 2 and, on balance, would suggest that you could briefly discuss this possibility in your paper, also stating why you think that this is unlikely to be the case.

What in my opinion is more important is to improve the data on the interaction of Npl3 with the FG repeats of the nuclear pore proteins, as this would lend further support to a direct role of Npl3 in mediating export of the large ribosomal subunit. Referee 1 states that additional controls would strengthen this experiment and I would kindly ask you to incorporate this in your manuscript.

Please do not hesitate to contact me if you have any further questions.

Yours sincerely,

Editor
EMBO Reports

REFEREE REPORTS:

Referee #1:

The authors present data clearly showing that deletion of Npl3 results in the nuclear retention of pre-60S ribosomal particles. Further, they propose that this accumulation is due to the direct involvement of Npl3 in export. However, a great difficulty when studying the export of ribosomal subunits is to distinguish a bona fide export defect from a general defect or delay in subunit assembly. It is for this reason that I, along with reviewer 2, requested a more detailed analysis of the processing and assembly or ribosomal subunits in the Npl3 delta strain. The analysis that the authors perform themselves is minimal, and only the levels of mature 25S and 18S rRNA are analyzed. Analysis of only the mature RNAs does not give a comprehensive view of the processing pathway.

For a more complete analysis of pre-rRNA processing, the authors site an earlier work (Li et al 2009), which they state shows "no obvious maturation defects". In point of fact there does appear to be a substantial defect in the steady state levels of some of the pre-rRNA species, namely that of the 27S precursors which are significantly under-represented compared to the wild-type(li et al 2009, fig.5 e and f). This is indicative of a defect or at least a change in the kinetics of the assembly of the large subunit, which could itself result in the nuclear accumulation of pre-60S subunits seen. Therefore it would appear unclear whether the nuclear accumulation results from a delay/defect in the assembly or, as the authors state due to Npl3 mediating export.

That there is an apparent defect in ribosome assembly does not preclude the possibility that Npl3 is involved directly in the export of the large subunit to the cytoplasm. An experiment that was suggested by this reviewer which would lend support to a direct role for Npl3 in the export process was a binding assay between Npl3 and FG containing NUPs. Whilst the authors have performed this experiment, the specificity is unclear, as the assay has been performed using purified proteins and lacks the presence of competitor proteins (eg E coli whole cell extract) that would act to block non-specific interactions, and lacks important positive and negative controls. Additionally, binding of an export receptor to the FG Nups should be robust enough to visualize via coomassie staining, and not only by Western analysis which is presented.

That Npl3 associates in the nucleus with pre-ribosomal particles appears convincing, however the major conclusion of this study, that Npl3 mediates the nuclear export of the large ribosomal subunit, is overstated as the presented data does not justify the authors claims.

Referee #2:

The authors have addressed all of the points I have raised.

Referee #3:

Firstly, I would like to thank the authors for their informative response regarding strain differences in Npl3 essentiality. In general the authors have dealt with all my concerns, with one exception. I am still concerned by the lack of a positive control for RNase digestion in the IP experiments. I take the authors point that they have successfully disrupted protein-protein interactions through RNase treatment previously (as shown in Gross et al., 2007) but my concern is not so much with the validity of the approach, but rather with the demonstration of successful RNase treatment in this instance. As Npl3 is known to bind RNA, and the authors state that the interaction of Npl3 with Rpl25 is RNase insensitive, I think that demonstration of successful RNase treatment in this instance is key. This could be achieved either by repeating the Npl3/Rpl25-GFP co-IP in the presence and absence of RNase, with the Dbp5/Pab1 co-IP shown alongside (using the same batch of RNase), or by directly detecting RNA and demonstrating that it has been successfully degraded.

2nd Revision - authors' response 21 June 2011

Once again we would like to thank the referees for their valuable comments on the manuscript.

Referee #1:

The authors present data clearly showing that deletion of Npl3 results in the nuclear retention of pre-60S ribosomal particles. Further, they propose that this accumulation is due to the direct involvement of Npl3 in export. However, a great difficulty when studying the export of ribosomal subunits is to distinguish a bona fide export defect from a general defect or delay in subunit assembly. It is for this reason that I, along with reviewer 2, requested a more detailed analysis of the processing and assembly or ribosomal subunits in the Npl3 delta strain. The analysis that the authors perform themselves is minimal, and only the levels of mature 25S and 18S rRNA are analyzed. Analysis of only the mature RNAs does not give a comprehensive view of

the processing pathway. For a more complete analysis of pre-rRNA processing, the authors site an earlier work (Li et al 2009), which they state shows "no obvious maturation defects". In point of fact there does appear to be a substantial defect in the steady state levels of some of the pre-rRNA species, namely that of the 27S precursors which are significantly under-represented compared to the wild-type(li et al 2009, fig.5 e and f). This is indicative of a defect or at least a change in the kinetics of the assembly of the large subunit, which could itself result in the nuclear accumulation of pre-60S subunits seen. Therefore it would appear unclear whether the nuclear accumulation results from a delay/defect in the assembly or, as the authors state due to Npl3 mediating export.

In our study we present several pieces of evidence for a role of Npl3 in transporting pre-60S ribosomal particles. The mislocalization of Rpl25-GFP is only one aspect. In addition we present interaction studies with the actively transported, e.g. Nmd3-containing pre-60S-particle (Fig. 3C), interactions of Npl3 with the NPC (Fig. 4F,G) and most importantly high copy suppression studies either with *MTR2* and *NMD3* in *npl3*Δ (contrary to the processing mutant *rix1-1*) or with high copy *NPL3* in *nmd3-2* mutants (Fig. 2). All of this clearly argues for a novel role of Npl3 in export of the large ribosomal subunit, which is novel and as we think of high importance for the research community. However, the referee is right that we can't exclude that Npl3 might still also have an additional function in the maturation of the pre-60S ribosomal subunit. The conclusion that was drawn from Li and colleagues (PLOS Biology 2009) that *npl3Δ* has no remarkable rRNA maturation defects, was simply cited. To solve this issue, we have now changed our text and say that …, *npl3*Δ seems unlikely to possess *significant* pre-60S maturation defects,… (page 6, 3rd last line). And we have changed the sentence in which we cite Li et al. to: This was also concluded by Li and colleagues showing northern blot analyses that demonstrate only little differences in the production of different rRNA species between *npl3Δ* and wildtype (Li et al, 2009). (page 7, line 1)

That there is an apparent defect in ribosome assembly does not preclude the possibility that Npl3 is involved directly in the export of the large subunit to the cytoplasm. An experiment that was suggested by this reviewer which would lend support to a direct role for Npl3 in the export process was a binding assay between Npl3 and FG containing NUPs. Whilst the authors have performed this experiment, the specificity is unclear, as the assay has been performed using purified proteins and lacks the presence of competitor proteins (eg E coli whole cell extract) that would act to block non-specific interactions, and lacks important positive and negative controls. Additionally, binding of an export receptor to the FG Nups should be robust enough to visualize via coomassie staining, and not only by Western analysis which is presented.

We thank this reviewer for the suggestions to improve the binding experiment and we present now the novel Fig 4G that includes whole bacterial lysate as a competitor. The result from this experiment is the same: Npl3 interacts with the FG-repeats of Nup116. We also did a coomassie gel that we are now showing in the new Fig S5, however, since we have several unspecific bands from the bacterial lysate and degradation bands from the GLFGrepeates of Nup116-GST, we preferred to document the interaction by western blot in the main figures.

That Npl3 associates in the nucleus with pre-ribosomal particles appears convincing, however the major conclusion of this study, that Npl3 mediates the nuclear ex5
port of the large ribosomal subunit, is overstated as the presented data does not justify the authors claims.

We disagree with the reviewers opinion that we have overstated the novel function of Npl3 in transporting large ribosomal subunits, because we present several evidences that support this novel function. Clearly, we do not exclude other potential functions of this multifunctional protein e.g. in biogenesis of the pre-60S, however, we present a multitude of different experiments that support its novel export function. The large size of the pre-60S particle necessitates the cooperation of several export factors for efficient export and Npl3 as we show is one of them.

Referee #2:

The authors have addressed all of the points I have raised.

Referee #3:

Firstly, I would like to thank the authors for their informative response regarding strain differences in Npl3 essentiality. In general the authors have dealt with all my concerns, with one exception. I am still concerned by the lack of a positive control for RNase digestion in the IP experiments. I take the authors point that they have successfully disrupted protein-protein interactions through RNase treatment previously (as shown in Gross et al., 2007) but my concern is not so much with the validity of the approach, but rather with the demonstration of successful RNase treatment in this instance. As Npl3 is known to bind RNA, and the authors state that the interaction of Npl3 with Rpl25 is RNase insensitive, I think that demonstration of successful RNase treatment in this instance is key. This could be achieved either by repeating the Npl3/Rpl25-GFP co-IP in the presence and absence of RNase, with the Dbp5/Pab1 co-IP shown alongside (using the same batch of RNase), or by directly detecting RNA and demonstrating that it has been successfully degraded.

As requested we provide now a novel Fig 3A that was performed together with the RNase sensitive control IP shown in the new Fig S4A. By using the same batch of the RNase, we are now certain, that the treatment was effective. Moreover, we show that there is no intact rRNA present in the lysates (Fig S4B). This clearly shows that the RNase treatment was successful.

3rd Editorial Decision 28 June 2011

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports.

Thank you for your contribution to EMBO reports and congratulations on a successful publication. Please consider us again in the future for your most exciting work.

Yours sincerely,

Editor
EMBO Reports

