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Nol9 is a novel polynucleotide 5'-kinase involved in ribosomal RNA processing

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Transaction Report:

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

1st Editorial Decision 09 July 2010

Thank you for submitting your manuscript for consideration by The EMBO Journal. It has been evaluated by three referees and I enclose their reports below.

As you will see from their comments the referees are in general positive regarding the study but find that further experimental analysis is required before it can be further considered for the EMBO Journal. I received the reports a couple of days ago and I have tried to contact one of the referees to exactly determine what additional experiments are necessary. Unfortunately, I have not yet heard back from him/her, so I will send you the decision as it stands and I will contact you again next week regarding the additional experiments. The referees find the identification of a role for a RNA kinase in rRNA processing to be potentially very interesting, but both referee #2 and #3 raise similar concerns, one is regarding the rescue experiments, where they would like to see the effects on other processing intermediates and secondly they would like some insight into what the substrate may be. I am trying to clarify exactly what is required to address the last issue. Given the interest in the study should you be able to address these issues, we would be wiling 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 you submit a revised version to the EMBO Journal, please make sure you upload a letter of response to the referees' comments. Please note that when preparing your letter of response to the referees' comments that this will form part of the 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.

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1 (Remarks to the Author):

In the submitted manuscript, the authors identify Nol9 as a polynucleotide kinase that is involved in 60S ribosomal subunit biogenesis. The authors show that Nol9 has 5' kinase activity and that this activity is needed for 5.8S (specifically 5.8S "short") and 28S rRNA maturation. The authors claim this is the first report of a polynucleotide kinase being involved in rRNA maturation.

A few minor concerns should be addressed:

- Figures 1, 4, 5, 7 and text: The 12S rRNA is labeled in processing schemes and on gels, but bands for 12S rRNA are not clearly visible on any gel and what happens to 12S rRNA levels after Nol9 depletion is never discussed in the text. This should be included since the generation of 12S is an important processing step in LSU maturation.

- Figure 3: While it is clear that Nol9 primarily sediments with the pre-60S ribosome as indicated by the authors, some Nol9 is also present with the pre-40S. Particularly in fraction 8, the level of Nol9 is higher than that of the other two control proteins. This should be explained.

- Figure 5B: A Northern blot for 28S should be done. A lighter exposure for 5.8S would also be helpful in determining whether 5.8S levels go down when a kinase inactive Nol9 is used. A decrease in both 5.8S and 28S levels should be observed with the Nol9 mutants.
The first panel is this figure is labeled as "NB38". NB38 is not mentioned anywhere else. Is this an "NB39" blot?

- Figure 5C: Statistically significant differences should be indicated.

- When discussing levels of 5.8S short and long, the text could be more clear about whether absolute or relative levels are being analyzed. This will avoid seemingly contradictory statements such as "Nol9 knock down decreased the levels of... 5.8S long to 13% of mock transfected cells" (p.8) vs "Nol9 knock down... did not alter the levels of the long form of 5.8S" (p.11).

- A few minor punctuation errors are present.

Referee #2 (Remarks to the Author):

The data supporting the kinase activity of Nol9 and role for the protein in ribosome synthesis are clear. The idea that an RNA kinase is involved in pre-rRNA is interesting and thought provoking. From the data presented it is less certain that the kinase activity is directly required for pre-rRNA processing, and entirely unclear what is the RNA substrate. Demonstration of either of these important points would substantially strengthen the MS. I regret to write that the present version appears too preliminary for publication in EMBO J.

Specific points:

1) A major conclusion is that the kinase activity of the protein is required for pre-rRNA processing, based on the re-expression analyses (Fig. 5). This experiment also provides an important control for the specificity of the Nol9 knock down. Unfortunately, the effects shown are very modest. It would

be important to indicate the reproducibility of the differences observed. The conclusion might also have been stronger had the complemented cells been analyzed by metabolic labeling.

2) The other weakness of the MS is the lack of an apparent RNA target for Nol9 activity, since no pre-rRNA or rRNA species with a 5' hydroxyl group has been reported. The demonstration that such an RNA actually exists would greatly strengthen the MS. This might, for example, be demonstrating by labeling using Nol9.

Minor points:

The phenotype of loss of Grc3 in yeast appears to be somewhat different from that of Nol9 knock down, so some caution should be exercised when using this as supporting data for the conclusions.

P6, lines 7/8: Did the authors intend to write that spectrometry at 254nm was used to determine RNA content?

P7: For clarity, it should be made explicit that the 240min labeling was performed after siRNA treatment for 6 days.

Referee #3 (Remarks to the Author):

In this article, the authors demonstrate that the human Nol9 protein functions as a 5'end specific RNA and DNA kinase in vitro. They further show by Northern blot experiments that depletion of Nol9 in HeLa cells leads to an increase in the steady-state levels of the 45S, 41S and 32S pre-rRNAs and to a strong decrease in the levels of the mature 28S and 5.8S rRNAs (the effect being more severe for the short form of 5.8S rRNA compared to the long form). Consistent with this, the authors show that Nol9 depletion results in a dramatic drop in the levels of the 60S ribosomal subunits and hence 80S ribosomes. Pulse-chase analyses suggest that this is in part the result of defective processing of the 45S and 41S pre-rRNAs. Mutation of the Walker A motif of Nol9 inhibits its kinase activity in vitro and its ability to correct the over-accumulation of the 32S pre-rRNA in HeLa cells depleted of endogenous wild-type Nol9. This suggests that Nol9 kinase activity as such is important for pre-rRNA processing.
This is an interesting report demonstrating for the first time that a polynucleotide kinase plays a crucial role in eukaryotic ribosome biogenesis.

Specific comments:

- I would recommend showing a coomassie blue-stained gel (or equivalent) containing the recombinant Nol9 proteins (both wild-type and mutants). In some of the assays presented in the Figures 2A and 5A, why is there a decrease in band intensity after incubation with the recombinant protein (contaminating nuclease?).

- The authors claim having demonstrated that Nol9 is part of pre-60S RNPs. In fact, they only show that Nol9 co-sediments with pre-60S RNPs and that this sedimentation behaviour is lost after RNase treatment of the extract. To definitely demonstrate that Nol9 is part of pre-60S RNPs, the authors should analyze the pre-rRNAs co-precipitated with Nol9 following its immunoprecipitation with specific antibodies (which the authors have at their disposal). This may clarify with which pre-rRNAs Nol9 associates and may help the identification of the Nol9 substrates. If possible, the authors may also analyze if RNAse MRP RNA is co-precipitated with these antibodies. (An alternative approach to study Nol9/pre-rRNA interactions would be to generate dominant negative versions of Nol9 that display increased affinity for the substrate; I understand this cannot be achieved in the 3 months time allowed for the revision, unless the authors already have dominant negative mutants of Nol9 at their disposal).

- In their rescue experiment of pre-rRNA processing defects due to endogenous Nol9 depletion by over-expression of wild-type or mutant Nol9, the authors only analyze the effects on 32S pre-rRNA accumulation. Their argument that the kinase activity of Nol9 as such is required for pre-rRNA processing would be greatly strengthened if they could provide data for the accumulation of the 45S

and 41S pre-rRNAs as well.

-There seems to be some contradiction between the middle and the end of the results section concerning the effects of Nol9 depletion on the accumulation of the long form of 5.8S. Based on quantification of Northern results, the authors state on page 8 that "Nol9 knock down decreased the levels of... 5.8SL to 13% of mock transfected cells". Yet, analysis of the primer extension results of Figure 6C leads them to conclude on page 11 that "Nol9 knock down ... did not alter the levels of the long form of 5.8S". Some explanation/harmonization is needed.

Minor point:

The 18SE precursor to 18S is missing in Figure 1

1st Revision - authors' response 04 October 2010

Referee 1

12S rRNA: We have added a panel to Figure 4 showing 12S rRNA levels and discuss the impact of Nol9 depletion on 12S rRNA levels in the manuscript. We apologize for previously omitting these data, since 12S is an important intermediate in the maturation of LSU rRNAs.

Figure 3: We discuss the co-sedimentation of Nol9 with pre-40S fractions in the manuscript and adapted the text concerning the association of Nol9 with pre-60S.

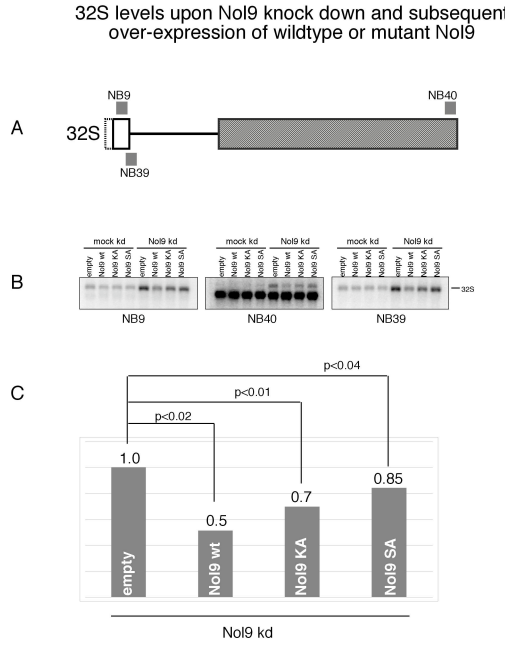
Figure 5C: We have conducted Northern blots for 28S and 5.8S rRNA levels, however, we could never observe striking differences. This is due to the low knock down efficiency achieved with the short hairpin RNA targeting the 3' UTR of Nol9. As displayed in Figure 4C, panel NB40, only a Nol9 knock down efficiency of 97% leads to a significant decrease of 28S rRNA levels. This knock down efficiency could only be achieved using Nol9 smart pools (lane 5). Short hairpin RNAs against the 3' UTR led to a maximum knock down efficiency of 83% and did not affect 28S levels (lane 4). Since we want to rescue Nol9 knock down by over-expression of wildtype or mutant Nol9, we cannot use Nol9 smart pools, which consist of four siRNAs targeting within the ORF of Nol9. Therefore, we are restricted by the lower knock down efficiency of the short hairpin RNA. However, we hope to satisfy the referee's request by the addition of a Northern blot displaying the 45/41S rRNAs.
We have corrected the typing mistake and changed NB38 into NB39.

Figure 5D: We have attached a figure to the referees where we show three different Northern blots for 32S levels in the rescue experiment, their quantification and p-values as determined by the Student's t-test. Figure 5D solely depicts the quantification of the Northern blot above in Figure 5C and serves representative means.

P11, 5.8S relative and absolute levels: We have amended the text accordingly and thank the referee for the suggestion.

Referee 2

1) We have conducted several metabolic labeling experiments to emphasize our statement in Figure 5. However, for the rescue experiment we are restricted to the use of shRNAs targeting the 3'UTR of Nol9, which are not very efficient. This is the reason for the modest effects on rRNA processing in Figure 5 as compared to Figure 4. In metabolic labeling experiments, we could not observe significant differences between wildtype cells and cells expressing shRNAs against the 3'UTR of Nol9, and therefore a rescue experiment was not possible.
However, we hope to convince the referee of the reproducibility of our data with the attached "referee's figure".



Wildtype or mutant Nol9 was overexpressed in HeLa cells previously transfected with a mock control or siRNAs targeting Nol9. RNA was extracted an analysed by Northern blot using different probes targeting within the 32S intermediate (A). The 32S accumulation observed after Nol9 depletion could be partially rescued with the overexpression of wildtype but not mutant Nol9 (B). Signal intensities of the individual 32S bands were quantified, the mean differences calculated relative to the Nol9 kd / empty vector control and depicted in a graph (C). p-values were calculated using the Student's t-test.

2) We have not been able to reveal the distinct substrate for Nol9 in the rRNA processing pathway, however, we hope the referee appreciates the addition of Figure 7C. There we show that over-expression of wildtype but not kinase-inactive Nol9 efficiently counteracts the defect in 5' end formation of 32S by previous Nol9 depletion.

Minor points:
We absolutely agree with the referee, that our results for Nol9 do not overlap fully with the data by Peng et al for Grc3. However, we want to stress, that the role of Grc3 in rRNA processing has never been studied by itself, but was part of a large scale screen. As suggested, we do not mention the publication by Peng et al to undermine our hypothesis, but solely for the sake of completeness.

P6, lines 7/8: we have corrected the text

P7: we have amended the text accordingly

Referee 3

Specific Comments

-We have added Coomassie blue-stained gels showing the purification of wildtype and mutant Nol9 to Figures 2 and 5.
We have repeated the kinase assays with the recombinant Nol9 proteins several times and frequently observe degradation of the substrate to a certain extent. As suggested by the referee, this could be due to a contaminating nuclease in the purifications, attacking especially DNA substrates which are not protected by the RNase inhibitors added to the reactions.

-We have amended the text from "Nol9 is a component of the pre-60S RNP" to "Nol9 co-sediments with the pre-60S RNP" and adapted our statements accordingly throughout the manuscript. We apologize for the mis-leading overstatement.

We indeed have antisera against Nol9 in our hands, yet their poor efficiency in immunoprecipitations was only sufficient for the highly sensitive kinase assays, but were not suitable for co-immunoprecipitation experiments.

-We have added Northern blot analyses for 41S and 45S to Figure 5 and thank the referee for this suggestion. We agree, that these data strengthen our conclusion.

- We have amended the text to clarify our statements on the distinct requirement for Nol9 in the generation of the long and short forms of 5.8S and apologize for the previous imprecision.

Minor point:
- We have added 18S-E to the rRNA processing schemes in Figure 1 and 7 and apologize for the previous omission.

2nd Editorial Decision 15 October 2010

Thank you for submitting a revised version of your manuscript to the EMBO Journal, I have received comments from two referees that have looked at the new version and both recommend publication. Therefore, I am happy to accept your manuscript for publication in The EMBO Journal, you will receive the official acceptance letter early next week.

Sincerely yours,

Editor
EMBO Journal

REFEREE COMMENTS

Referee #2
The revised MS has been strengthened and I would now support publication.

Referee #3
I feel that the authors have answered the referee's concerns in an appropriate manner

