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Immature small ribosomal subunits can engage in translation initiation in S. cerevisiae

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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 07 May 2009

Thank you for submitting your manuscript for consideration by The EMBO Journal. I apologise for the length of time it has taken to have your manuscript reviewed, but it took several reminders before we were able to obtain the comments of one of the referees, however, I have now received the final report from the three referees asked to evaluate your manuscript and I enclose their comments below.

As you will see from their reports the referees express potential interest in the findings, however, they provide mixed recommendations with respect to publication in the EMBO Journal. There is clear concern from referee #1 that the current study does not provide sufficient molecular insight into the potentially interesting model that pre-40S ribosomes are actively involved in translation. To strengthen the study this referee requests further evidence that 20S rRNA containing pre-40S ribosomes are involved in translation and that their interaction with Pab1 and mRNA is direct, to address this s/he suggests that this could be achieved by purification of cytosolic pre-40S ribosomes and identifying the mRNAs associated with these particles. Referee #2, while more supportive, also would like to see further controls for the affinity purification experiments and a quantitative description of the efficiency of pre-40S ribosomes involvement in translation. Although a number of important concerns have been raised that should be addressed, given the current interest in the potential model, if you are aable to address these concerns and convince both these referees we would be willing to consider a revised version of the manuscript.

I would like to 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 revisions included in the next, final version of the manuscript.

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 this manuscript, Soudet et al. confirm that a fraction (that, they report, varies according to strain background) of 20S pre-rRNA (the last precursor to small ribosomal subunit rRNA) comigrates with polysomal fractions in yeast. From this, and other experiments, the authors suggest that a fraction of pre-40S ribosomes is actively engaged in translation. A major argument in favor of this model is that the fraction of 20S pre-rRNA that comigrates with polysomes partially relocalizes to lighter fractions of the gradient upon omission of cycloheximide ('run off'conditions), much in a way that mature subunits do. A caviat to this is that the same polysomal fractions are shown to be highly enriched with material of nucleolar origin (presence of large pre-rRNAs and core snoRNP proteins) which obviously contains many pre-ribosomes. That the material of nucleolar origin and the pre-40S fraction under consideration do not respond similarly to 'run-off' conditions is not a sufficient argument, in my opinion, to establish a direct involvement in translation. What if the aggregation properties of these particles were simply distinctly different under the conditions tested?
Another interesting observation is that 20S pre-rRNA is detected in affinity purified material isolated with the poly-A binding protein Pab1 and several translation factors. Again, this is interesting, but the authors do not demonstrate that these interactions are mediated by mRNAs which would be necessary to strengthen the connection to translation. That mutations that primarily affect translation initiation have no effect on pre-rRNA processing is quite surprising and counter intuitive (and also unlike what was previously reported for eIF3j/Hcr1). Indeed, yeast cells are actively engaged in ribosome synthesis and known to rely on a limited pool of ribosome synthesis factors that likely requires continuous recycling and synthesis to be replenished. The connection to NGD (based on the characterization of a partly inactivated epitope-tagged allele of Rio2 defective for growth) remains quite elusive at this stage; the basis for the complementation is not clear and could be quite indirect.

In summary, this manuscript has an interesting observation at its core that led to an interesting model that remains to be validated at the molecular level. The demonstration that pre-40S ribosomes are actively engaged in translation would require extensive additional lines of experimentation. One such experiment would be to affinity select pre-40S ribosomes (ideally from cytoplasmic purified fractions) and characterize RNA pull downs by deep sequencing or tiling arrays for the presence of mRNAs.

The manuscript would benefit from some editing.

Referee #2 (Remarks to the Author):

Soudet et al. analyse in the manuscript EMBOJ-2009-70906 in several yeast mutant strains the engagement of cytoplasmic pre-40S subunits containing 3' extended 18S rRNA precursors (20S pre-rRNA) in mRNA translation. In addition, they provide evidence that factors which were suggested to be involved in the decay of mRNA associated with stalled ribosomes (NoGo decay) play an additional role in the turnover of cytoplasmic pre-18S rRNA when pre-40S ribosomal subunit maturation is delayed or blocked. I think that the manuscript raises important questions on the nature of "quality control" during cytoplasmic steps of eukaryotic ribosomal subunit maturation and that, in general, appropriate techniques are applied to answer some of these questions. I see three major issues to be adressed which should help to further clarify the significance of some of the conclusions drawn by the authors:

1) Cosedimentation experiments shown in Fig. 2+3.
It would be important to quantify in a "standard" wildtype yeast and in the tetO7::RIO1 (+/-

Doxycycline) strain used in the experiments shown in Fig. 2 the relative distribution of both 18S rRNA and 20S rRNA in the fractions of sucrose gradients containing 40S ribosomal subunits, 80S ribosomes and polysomes. That would help to clarify how efficient 18S rRNA (=mature) in comparison to 20S pre-rRNA (=immature) containing ribosomal subunits are recruted in translational active ribosomal particles in different strains or, in other words, how efficient immature subunits are kept away from beeing active in translation. This issue is in part discussed in the discussion section of the current version of the manuscript, but should be clearly adressed directly in a quantitative way in the result section.

2) Affinity purification experiments shown in Fig.4+5.
The affinity purification experiments shown in Fig. 4+5 provide the crucial arguments to conclude that the cosedimentation of immature 20S pre-rRNAs with 80S ribosomes and polysomes is due to their incorporation into these translational active ribosomal particles.
To strengthen these arguments, the authors could
a) show as additional internal controls in the experiments shown in Fig. 4A tRNA (see Inada et al.) and, if possible 23S rRNA, a 5' and 3' extended nuclear form of 18S rRNA
b) show for the experiment reported in Fig 5A at least one internal control. 27SB pre-rRNA, contained in nuclear precursors of the large ribosomal subunit, would be appropriate. A quantitation of the results of the affinity purification experiments would be helpfull.
c) explain in the result section why they use different affinity purification conditions in the experiments shown in Fig. 4A and in Fig. 5A.

3) Genetic interactions between NoGo decay components and the pre-40S ribosomal subunit maturation factor Rio1 shown in Fig. 6.
The authors suggest that inactivation of NoGo component Hbs1 delays degradation of non processed pre-18S rRNAs in a rio1-tap allele and thereby gives more time for their maturation under these conditions (page 14 and 18, bottom). If inactivation of Hbs1 would just delay 20S pre-rRNA degradation, not accelerate its maturation, we would necessarily expect a relative accumulation of 20S pre-rRNA in the rio1-tap/hbs1 strain when compared with the rio1-tap/HBS1 strain, the opposite of which is observed in Fig. 6D. Accordingly, other explanations of how hbs1-delta suppresses the rio1-tap phenotype have to be considered and discussed. In addition, the authors could at least compare easily wether expression levels of rio1-tap, due to modulation of translation efficiencies, are changed in strains rio1-tap, rio1-tap/dom34-delta and rio1-tap/hbs1-delta.

Referee #3 (Remarks to the Author):

In this work, Soudet et al. demonstrate that, in the yeast Sacharomyces cerevisiae, the cytoplasmic precursor, 20S pre-rRNA, of 18S rRNA of the small (40S) ribosomal subunit (SSU) participates in the formation of a pre-initiation 40S complex. This complex can, furthermore, recruit the large (60S) ribosomal subunit (LSU) in the formation of an 80S ribosomal initiation complex. The experiments suggest that the pre-SSU containing 80S ribosomes are elongation deficient, that 20S pre-rRNA is unstable due to an involvement of the No Go Decay (NGD) pathway. Finally, the authors speculate that the Nonfunctional rRNA Decay (NRD) and NGD pathways are fundamentally linked.
I think that they have a good set of experiments and that they argue their points well.

Minor suggestions:
1. The ITS1 probe brought into Results on page 6, may be explained at this point.
2. The significance of the elongation inhibitor cycloheximide is briefly explained in the legend of Fig. 3. Since, however, the inhibitor is used already in Fig. 2, it is advised to introduce it in the legend of Fig. 2 and also in the main text on page 6 describing the experiments in Fig. 2. Now, the inhibitor is introduced on page 7, but one needs it earlier, I think.
3. At the beginning of page 10, the significance of ACT1 mRNA may briefly be indicated for non-yeast specialists.
4. On page 11 experiments at high and low salt concentrations are discussed. It is stated that naked ribosomes dissociate at high salt, while mRNA co

1st Revision - authors' response 03 August 2009

Answers to Referee's #1 comments and suggestions.

Referee#1 stresses essentially 4 points:

1/ Pre-40S aggregation properties: *"that the material of nucleolar origin and the pre-40S fraction do not respond similarly to "run-off" conditions is not a sufficient argument, to establish a direct involvement in translation. What if the aggregations properties of these particles were simply distinctly different under the conditions tested?"*

2/ 20S pre-rRNA association to Pab1p and translation initiation factors: *"...20S pre-rRNA is detected in affinity purified material isolated with the polyA-binding protein Pab1 and several translation initiation factors...This is interesting but does not demonstrate that these interactions are mediated by mRNAs which would be necessary to strengthen the connection to translation".*

Points 1 and 2 both deal with the same aspect of our work, namely that pre-40S SSUs can engage and complete translation initiation. Referee #1 suggests that this could be addressed by *"affinity selecting pre-40S ribosomes (ideally from cytoplasmic purified fractions) and characterize RNA pull down by deep sequencing or tiling arrays".*

Such an approach looks quite seductive indeed. But, to achieve this we would have to first define experimental conditions allowing purification of polysomes devoid of nuclear contamination, secondly to optimize conditions allowing purification of 20S pre-rRNA-containing ribosomes, again without the contamination of the overwhelmingly more abundant mature ribosomes, third run and analyze either the deep sequencing or array results. All of this can't be reasonably achieved in as short a period as the 3 months allowed before resubmission.

Rather, in order to answer these remarks, we used the following two step approach.

1/ 20S pre-rRNA cellular distribution (Supplementary figure 2B). In order to demonstrate that part of 20S pre-rRNA sedimenting in ribosome- polysome- fractions is cytoplasmic, we prepared cytoplasmic and nuclear fractions from TetO7-RIO1 cells expressing Rio1p, and determined the distribution of 20S pre-rRNA in these fractions: mature rRNAs are used as indicators of cytoplasmic spill over into the nuclear fraction, and 27S pre-rRNA (a nuclear specific RNA) as a measure of nuclear contamination into the "cytoplasmic" fraction. Considering the observed cellular distributions (Supplementary figure 2B), even if all nuclear 20S pre-rRNA sediments in the ribosome- polysome- fractions, at least 50% of 20S pre-rRNA sedimenting in these fractions would still be cytoplasmic 20S. The distribution observed is quite similar to previously reported data (Léger-Silvestre et al., 2004). This point is developed on page 8: "This was further confirmed by fractionating a TetO7-RIO1 cellular extract..."

Using this method we also attempted to purify polysomes from cytoplasmic fractions. Unfortunately, polysome complexes are destabilized during the cell fractionation procedure.

2/ Analysis of RNAs coimmunoprecipitated with pre-40S (Figure 4B). In order to investigate the association of 20S pre-rRNA with mRNAs, we determined if mRNAs are co-immunoprecipitated with late pre-SSUs using Nob1p as a bait. Nob1p was chosen because it has been shown that this protein associates with late pre-SSUs, and is considered as a likely candidate for the final endonucleolytic processing of 20S pre-rRNA. We show that indeed Nob1p associates with 3 different mRNAs. This result is included in the text (page 11) and in figure 4 (Figure 4B).

To summarize, we show (1) that at the very least 50% of 20S pre-rRNA found in the ribosome-polysome fractions of the gradient is cytoplasmic 20S pre-rRNA, (2) that not only does 20S pre-rRNA coimmunoprecipitate with Pab1p, translation-initiation factors and rpL proteins, but also that mRNAs associate with Nob1p. We think that altogether our results bring convincing evidence that pre-40S ribosomes can complete translation initiation.

3/ Absence of pre-rRNA maturation defects upon translation-initiation factor depletion: *"That mutations that primarily affect translation initiation have no effect on pre-rRNA processing is quite surprising and counter intuitive (and also unlike what was previously reported for eIF3j/Hcr1)".*

So far depletion of most translation initiation factors analysed does not lead to significant pre-rRNA processing or export defects: for instance eIF3c/Rip1 (Schäfer, unpublished cited in Yarunin et al. 2005) or subunits of eIF2B or eIF2B (Senger et al., 2001). As far as we know eIF3j/Hcr1, a non essential factor, is quite exceptional in this respect. This point is now discussed in the text (page 13-14): "Other authors have already reported that depletion for translation initiation factors does not significantly affect pre-rRNA processing (Yarunin et al. 2005, Senger et al. 2001). However this is not an absolute rule since depletion or inactivation of eIF3j/Hcr1, a non essential factor, leads to a delay in the maturation process of 25S and 18S rRNAs, a small reduction in SSU steady state level and ribosomal subunit nuclear export defect (Valašek et al. 2001b, Yarunin et al 2005)."

4/ Suppression of 20S pre-rRNA processing defect in NGD deficient cells: *"the connection to NGD ...remains quite elusive at this stage, the basis for the complementation is not clear and could be quite indirect".*

We agree with this remark and indeed the effect of NGD inactivation could be due, for instance, to indirect increase of the level or activity of pre-rRNA processing factors. Since, in the strains analysed, Rio1p is the only rRNA processing factor altered, we assessed whether in the relevant strains Rio1p-TAP levels are modified (see text page 16 and Supplementary figure 6), which is clearly not the case. Thus suppression of the 20S pre-rRNA maturation defect is not due to an increased amount of Rio1p-TAP, but this does not exclude a more indirect origin for the restoration of 18S rRNA production.

As mentioned in the note added at the end of the manuscript, while this work was under revision, another paper implicating components of the NGD pathway in 18S rRNA quality control was published (Cole et al., 2009). Although this paper deals with the degradation of mature translation-defective 18S rRNA and not with the fate of 20S pre-rRNA, the demonstration that NGD components are involved in 18S non-functional decay (NRD) supports our proposal that NGD factors Hbs1 and Dom34 are involved in small ribosomal subunit quality control. It also comforts our suggestion that 20S pre-rRNA containing SSUs, while able to complete translation initiation, are not active in elongation and as such sensed by the NGD machinery.

Answers to Referee's #3 remarks and suggestions.

Point # 1 *"ITS1 probe... on page 6, may be explained at this point", and point # 3 "significance of ACT1 mRNA may briefly be indicated",* have been dealt with by shortly defining them as required by the Referee.

(1) ITS1 page 6: "...in situ *hybridization of an ITS1 probe..."* has been changed to "...*in situ* hybridization of an 18S rRNA-precursor specific probe (D-A2 fragment in Figure 1)..."

(3) Act1 mRNA page 10: *"...Moreover ACT1 mRNA was immunoprecipitated..."* has been modified to "...Moreover the relatively abundant actin encoding ACT1 mRNA was immunoprecipitated..."

Point # 2. As suggested by the Referee "cycloheximide" is now introduced in the legend of Figure 2 instead of introducing it in the legend of Figure 3. Likewise, in the main text the inhibitor its uses and effects are inserted earlier (page 6): "Cycloheximide an inhibitor of translation-elongation was added shortly before cell harvest and during cell extract preparation in order to stabilize the polysome complexes."

Point #4: Since we did not get a complete sentence, it was not possible to edit the text relating to the high /low salt experiment.

Answers to Referee's #2 remarks and suggestions

1/ Cosedimentation experiments (Figures 2 and 3)

As Referee #2 suggested, we provide (Supplementary Figure 1) a quantification of the relative distribution of 18S rRNA and 20S pre-rRNA in 80S ribosome- and polysome-fractions. This clearly shows that 80S ribosome fractions are enriched in 20S pre-rRNA relatively to polysome fractions, suggesting that 20S containing ribosomes translate inefficiently, if at all. The distribution of free 40S subunits is not included because (1) as shown in Figures 2 and 3 the fraction of 18S contained in free subunit fractions is very low, (2) nuclear 20S pre-rRNA constitutes probably a large proportion of the 20S found in these fractions.

We also provide as "Referee only" supplementary material the distribution observed in the wild type reference strain S288C and in strain R1158 (BY 4741 background) which carries a wild type RIO1 locus and the Tet transactivator. In both strains, and particularly in S288C, the amount of 20S pre-rRNA observed is lower than in the TetO7-RIO1 strain.

2/ Affinity purification experiments (Figures 4 and 5)

"A quantification of the results of the affinity purification experiments would be helpful."

A quantification of the immunoprecipitation results in figures 4 and 5 has been added as Supplementary figures 4A and 4B respectively.

a) *"additional internal controls in the experiments shown in Fig.4A tRNA and if possible 23S rRNA..."* As shown in Figure 4A, 23S pre-rRNA has been added as an internal control. No immunoprecipitation of this nuclear pre-rRNA, neither with Pab1p nor with rpL proteins is observed. This comforts the specificity of 20S pre-rRNA association with Pab1p and rpL proteins that we observed. We also provide as "Referee only" supplementary figure, the Ethidium bromide pattern of the coimmunoprecipitated RNAs showing that in these experiments, and as described in Inada et al.2002, tRNAs do not coimmunoprecipitate with rpL proteins. This latter point is mentioned in the text as "data not shown" (page 10).

b) *"show for the experiment reported in Fig.5A at least one internal control. 27SB...would be appropriate..."* 35S and 27S pre-rRNAs, both nuclear precursors of 25S rRNA, have been added as internal controls in Fig.5A. No immunoprecipitation of these pre-rRNAs with the tagged translation-initiation factors we analysed is observed, which supports the specificity of the interaction of the translation-initiation factors with 20S pre-rRNA.

c) *"Explain in the result section why they used different affinity purification conditions in the experiments shown in Fig.4A and Fig.5A."* In this experiment, since we investigated the possible interaction of translation initiation factors with 20S pre-rRNA, we used the method reported in an earlier work describing the affinity purification of 20S pre-rRNA with TAP-tagged Rli1p and Ded1p, two proteins which participate in pre-rRNA processing and in translation initiation (Yarunin et al., 2005). This is added at the appropriate location in the result section (page 13): "In these experiments immunoprecipitations were performed according..."

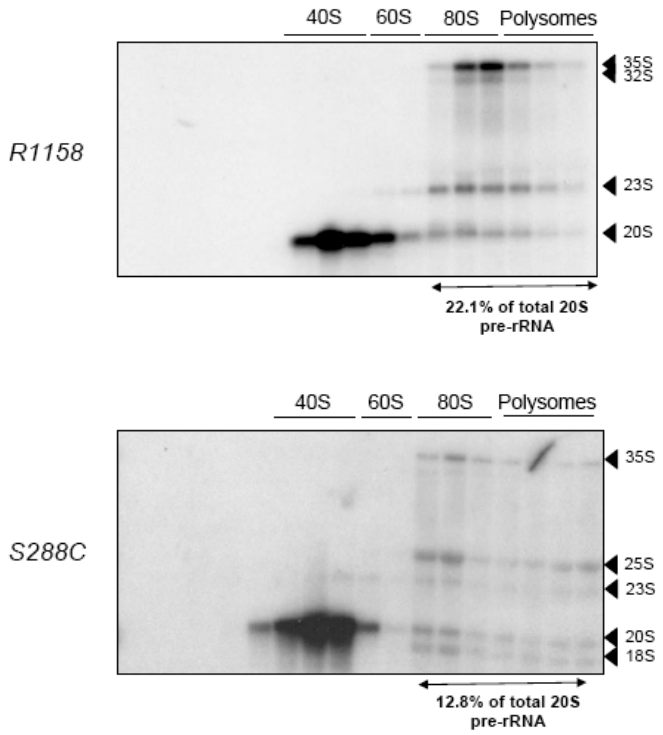
3/ Genetic interaction between No Go Decay components and pre-40S ribosomal subunit maturation
Referee #2 raises two points concerning this part of our work. (1): *"If inactivation of Hbs1 would just delay 20S pre-rRNA degradation, not accelerate its maturation, we would necessarily expect a relative accumulation of 20S pre-rRNA in the RIO1-TAP hbs1 strain...the opposite of which is observed inFig.6D."* (2): *"Other explanations have to be considered",* in other words, suppression of 20S pre-rRNA maturation defect by NGD inactivation, as observed in the RIO1-TAP hbs1 strain, could be an indirect effect of NGD pathway inactivation.

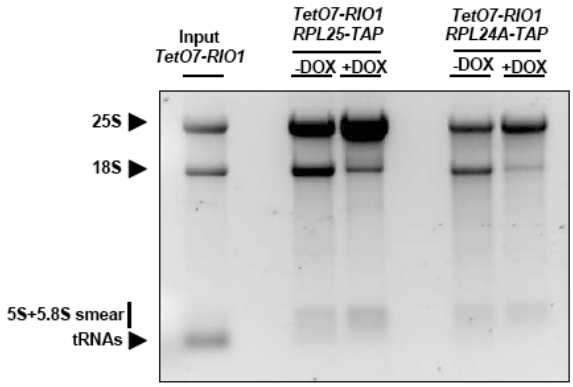
(1) Hbs1 inactivation does not lead to 20S pre-rRNA accumulation.
We agree with Referee #2's comment that, in this particular genetic context, NGD inactivation is

expected to result in an increased 20S pre-rRNA steady state level. Indeed, assuming that part of incoming 20S pre-rRNA is degraded via NGD, and part of it is matured to 18S rRNA, assuming that in NGD deficient cells everything but NGD remains equal, one should expect an increase in the amount of steady state 20S pre-rRNA. But (1) when NGD is inactivated the instantaneous concentration of 20S pre-rRNA available to the maturation process is increased and thus an activation of the maturation process might occur (V= k[S]). Since the actual steady state amount of 20S pre-rRNA observed is decreased, this might suggest that the increase of the Vmat (maturation speed) is high enough to more than compensate the lack of NGD degradation. (2) Another possibility is that two (at least two) forms of cytoplasmic 20S pre-RNA are present at equilibrium, one competent for processing to 18S rRNA, one engaged in NGD pathway and no longer competent for maturation. NGD inactivation would displace the equilibrium towards an increased concentration of the "maturable" form and thus lead to activation of the processing pathway. Clearly testing these hypotheses would be far beyond the scope of the present work. This is briefly discussed on page 16 ("...NGD inactivation allows more 20S pre-rRNA molecules to be processed...and thus to an increased processing speed") and in the Discussion (page 20).

(2) NGD inactivation suppresses 20S pre-rRNA processing defect imparted by the *RIO1-TAP* allele. As suggested by Referee #2 the effect of NGD inactivation could be due to the indirect increase of the level of pre-rRNA processing factors. Since in the strains analysed, Rio1p is the only rRNA processing factor altered, we assessed whether in the relevant strains Rio1p-TAP levels are modified (see text page 16 and Supplementary figure 6), which is clearly not the case. Thus suppression of the 20S pre-rRNA maturation defect is not due to an increased amount of Rio1p-TAP, but this does not exclude a more indirect origin for the restoration of 18S rRNA production.

As mentioned in the note added at the end of the manuscript, while this work was under revision, another paper implicating components of the NGD pathway in 18S rRNA quality control was published (Cole et al. 2009). Although this paper deals with translation-defective 18S rRNA and not with the fate of 20S pre-rRNA, the demonstration that NGD components are involved in 18S non-functional decay (NRD) supports our proposal that NGD factors Hbs1 and Dom34 are involved in small ribosomal subunit quality control. It also comforts our suggestion that 20S pre-rRNA containing SSUs, while able to complete translation initiation, are not active in elongation and as such sensed by the NGD machinery.





2nd Editorial Decision 03 September 2009

I would like to let you know that I have received the referee comments regarding your revised manuscript and I enclose them below. Your study has been accepted for publication and you will receive the official acceptance letter in the next couple of days.

Sincerely,

Editor
The EMBO Journal

Referee #1

Soudet et al. Revised manuscript.
The authors have argued their points well. They clearly have not performed all the experiments suggested (but I'll admit that some were quite challenging though of the kind needed for a definitive contribution).

As far as I can see it, there are three novel lines of evidences here:
First, the authors have shown that a late pre-40S synthesis factor (Nob1p) interacts with three (abundant) mRNAs in conditions where the protein is bound to 20S pre-rRNA and not to 18S rRNA (Fig 4B). This is good news.

Second, the authors have performed a nuclear-cytoplasmic preparation in a tet::rio strain to address how much of the 20S pre-rRNA accumulated in this strain is cytoplasmic (Fig S2). In the conditions of nuclear contamination used here (about 50%) the authors conclude that about 50% of 20S pre-rRNA is cytoplasmic (Leger Sylvestre et al. previously reported that 90% of 20S pre-rRNA is cytoplasmic in wild-type cells; this leaves me wonder if the analysis shown here is of any use at all?). I think that this analysis is quite redundant [the FISH analysis in Fig 2B in the original submission nearly made a better case that the 20S pre-rRNA accumulated in rio-deplete is cytoplasmic].

Third, the authors have provided further useful quantitations (e.g. coprecipitation analysis) and additional loading controls, as requested by the other referees.

For the NRD/NGD section, a simple pulse-chase analysis could have easily answered the question raised by referee 2; i.e. showed that pre-rRNA processing is

likely restored owing to the increased half-life of 20S pre-rRNA in the absence of functional NRD/NGD.

Referee #2

In my opinion, the revisions made in manuscript EMBOJ-2009-70906R by Soudet et al. improved its general quality and further strengthen certain conclusions made by the authors. I would recommend to publish this work even if some aspects tackled in it remain a bit obscure and will need future clarification.

