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Assembly of *Saccharomyces cerevisiae* 60S Ribosomal Subunits: Role of Factors Required for 27S Pre-rRNA Processing

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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 10 March 2011

Thank you for submitting your manuscript for consideration by The EMBO Journal. Three referees have now evaluated it, and their comments are shown below. As you will see, all three referees support publication of the study here after appropriate revision. We should therefore be able to consider a revised manuscript that addresses the referees' points in an adequate manner. There is one more issue. In the discussion section of the manuscript you refer extensively to a study by Granneman et al. in preparation. As this is unpublished work at this time we would need written confirmation by the authors of this work that they agree with this.

I should remind you that it is EMBO Journal policy to allow a single round of revision only and that, therefore, acceptance of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript. Please do not hesitate to get back to me at any time should you have any questions regarding the revision.

We generally allow three months as a standard revision time, and as a matter of policy, we do not consider any competing manuscripts published during this period as negatively impacting on the conceptual advance provided by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Peer Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process initiative, please visit our website:

http://www.nature.com/emboj/about/process.html

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

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1

In this manuscript, Dr. Woolford Jr. and co-workers studied in detail the events needed for yeast 27SA pre-rRNA processing at site A3. They nicely concluded that:

(1) There is a set of trans-acting factors in yeast ribosome biogenesis, named "A3 factors", involved in this processing reaction. This set is composed of seven proteins: Nop7/Yph1, Erb1, Ytm1, Rlp7, Nop15, Nsa3/Cic1 and Rrp1.

(2) All except Rrp1 are interdependent for their association with pre-ribosomal particles.

(3) Binding of Rrp17 to pre-ribosomal particles is dependent on the presence of Rlp7.

(4) Stably assembly of a set of 60S ribosomal proteins (L17, L26, L35 and L37) requires the presence of Rlp7. This is further investigated for L17, leading to the conclusion that stably assembly of L17 needed pre-rRNA processing at site A3.

(5) Very interestingly, the above ribosomal proteins act as roadblock for an exonucleolytic activity of Rat1 beyond site A3 that could promote turnover of 27SA pre-rRNAs.

The work is nice and of high quality. However, I find some critique that would be addressed to match the scopes of the prestigious EMBO Journal. My major critique concerns to the use of GAL-RLP7 construct as the sole one for the most of the analysis; I would like to encourage the authors to repeat similar experiments to those performed with the GAL-RLP7 upon depletion of genes corresponding to the remaining A3 factors (or at least a subset of them).

(a) All over the manuscript. Most northern hybridisation and primer extension analyses require quantifications of the levels of pre-rRNAs and mature rRNAs to reach properly to some conclusions.

(b) Quality of Fig. 1B must be improved;
- Site A2 must be shown for all GAL strains as a control that A3 is specifically enriched upon depletion of any of the so called "A3 proteins".
- A3 is perfectly seen in wild-type conditions, however, the exposure time used for Fig 1B is not enough to see it. Please improve.
- As mentioned above, quantification of the levels of the primer extension stops could enforced the conclusion reached.

(c) Fig 1D. needs, in addition to quantification, a control of a 90S pre-ribosomal factor to address how efficiently Rlp7, Nsa3 and Ytm1 precipitates 35S pre-rRNA.

(d) Fig 2. It is clear to me that depletion of Nop7, Erb1 or Ytm1 leads to similar profiles of pre-ribosomal proteins, but it is not obvious for the rest of A3 factors assayed. If possible, perform western blotting upon depletion of each A3 protein to clearly conclude about the interdependence of A3 factors. Moreover, the use of a GAL-RPF2 NOP7-TAP strain as a control is not appropriate.

Instead, a RPF2-TAP strain harbouring an ribosome assembly factor unrelated to the A3 factors should be used.

(e) I suggest to perform the analysis of the pre-rRNAs and rRNAs upon depletion of Rlp7 in the strain GAL-RPL7 RPF2-TAP; this could nicely complement the data of Figure 3.

(f) Figure 4.

- Authors demonstrated that upon depletion of Rlp7 in a Rpf2-TAP strain, Rrp17 is not longer recruited to the purified pre-ribosomal particles. However, Rat1 and Xrn1 are still present in the purified particles. I find that more controls are required to generalise this conclusion to the rest if A3 factors. Therefore, I suggest to repeat the experiment again with more than one strain (having Nop7, Nop15, etc factors under the control of the GAL promoter). I also suggest to perform all series of shown western blots (L5, Rat1-HA, Xrn1-GFP and Rrp17-HA) for all strains.

-Rat1-TAP co-precipitates 27SA2, 27SA3 and 27SB pre-rRNAs (Figure 4D), so maybe the binding of Rat1 and Xrn1 to the A3 site is indeed affected by the depletion of the A3 factors, however, the binding to 27SB pre-rRNAs is still taking place, explaining why these exonucleases do still associates to the Rpf2-TAP complex upon depletion of Rlp7. Please comment on this possibility at least at the discussion section.

(g) Figure 5A. Clearly, the manuscript will gain strength if this western blotting analysis is similarly performed upon depletion of the rest of A3 proteins in addition to Rlp7.

(h) Figure 6. Figure 6B and 6C. It would be very worthy to use an antibody against a ribosomal protein of the small subunit to check for the specificity of purification of the Npa2 and Nog2 complexes.

(i) Figure 8. The role of Rat1 in turnover of defective 27SA pre-rrRNAs is studied. I understand that there are only evidences for this role for Rat1 (Fang et al. 2005; Fig. 7 and use of a rat1-1 mutant), however, the authors are in a perfect situation to study the putative contribution of Rrp15 and/or Xrn1 in this turnover pathway.

Minor comments:

(a) All over the manuscript is used the term "the 5' end of mature 5.8S rRNA". Authors must be more accurate in the writing to use 5.8SS when refer to as the end generated by processing at site A3 and 5.8SL when done by direct cleavage at site B1L of 27SA2 pre-rRNA.

(b) In same figures, data are commonly grouped from different experiments; I suggest to use dividing lines or an space to highlight this fact.

(c) A list of strains with all features (included tagged genes and plasmids) is strongly encouraged. Please, provide it in the Supplementary file.

(d) Figure 6A. Where does the A3 stop map? Please comment.

(e) The Material and Methods section is extremely poor. Either an extended form of this section must be provided in the text or in the Supplementary material.

Referee #2

The work presented in the submitted manuscript by Sahasranaman and colleagues enhances our understanding of the events underlying a specific step, 27SA3 processing, of eukaryotic ribosome biogenesis. Through a series of affinity purifications in strains depleted of the A3 factors, the authors demonstrate that the association of A3 factors with pre-ribosomes is concerted, their association is required for association of Rrp17, an exonuclease, and four ribosomal proteins. Additionally, they show that absence of the A3 factors or the ribosomal proteins triggers turnover of

the pre-rRNA. Taken together, their data suggests a detailed mechanism of this particular processing step.

However, there are some minor points that should be addressed.

1. There are several sentences throughout the manuscript that are long and complex. Splitting them into smaller sentences will make them more comprehensible. For example, one sentence in the abstract takes up almost 5 lines!

2. Some of the panels in Fig. 1B are not clear since their inclusion is not mentioned, their processing patterns do not align and their phenotypes are different than the A3 factors.

3. In Fig. 2A, there is another band that is consistently depleted in the A3 factor depletions, and it is marked, but it is not labeled. Similarly, there is another band under Erb1 in Fig. 2C. What are the identities of these bands? Are they possibly other A3 factors?

4. Why are Erb1 and Ytm1 not identified in iTRAQ experiments? Erb1, Ytm1 and Nop7 form a subcomplex that can be identified in the absence of pre-ribosomes. Additionally, these three factors are removed together by Rea1. It seems odd then that two are absent. Is there a better explanation for their absence?

5. Fig. 7 is not labeled correctly. In the text, the two panels are referred to as 7A and 7B, however the panels are not labeled and they are not discussed independently in the figure legend.

6. In the final paragraph of the results section, you reference lanes 7-8 of Fig. 8A. However, given the context, I believe you are referencing lanes 9-10.

7. The first sentence of the discussion says that this work has examined the roles of ALL A3 factors. Is this really true? Could the band referenced in comment 3 represent another A3 factor? Additionally, there are LSU biogenesis factors that are more significantly depleted as shown by iTRAQ than the noted A3 factors. It is possible that they could function in this step. This sentence should be modified slightly such that it is less absolute on this point.

8. In Fig. 7, one band is labeled with the asterisk. The figure legend should mention what this represents.

9. In Fig. 8A, the labels above the panels are misaligned.

10.In the figure legend, the two panels in Fig. 8C are labeled (A) and (B), but they are not labeled as such in the figure.

11. Utp6 is misspelled in Fig. 3A.

Referee #3

Many proteins required for ribosome synthesis have been referred to as "assembly factors", generally because the field has no clear idea of what they actually do. Here the authors have attempted to provide direct evidence for roles in assembly for a group of proteins implicated in the early maturation of pre-60S particles. The application of TAP purification and MS to determine the composition of pre-ribosomes (which was first achieved by the Woolford lab) brought about major changes in our understanding of ribosome synthesis. The use of quantitative MS analyses to quantify changes in pre-ribosome composition in mutant strains, as reported here, is significant development that allows the pathway to be better analyzed and will surely lead to further insights.

Overall, the data are technically good and the conclusions well supported by the data presented. The work makes a significant contribution to the understanding of pre-ribosome assembly and the functions of the ribosome synthesis factors, and is suitable for publication in the EMBO Journal.

Minor points

1: Abstract line 1: Missing word "of".

2: Published data indicate that Xrn1 is probably not a normal processing factor for 5.8S, but degrades the 5' extension when Rat1 is inhibited (El Hage et al., 2008).

3: The reduction in the recovery of Rpl17 when pre-ribosomes were purified under more stringent salt conditions is the least convincing data presented. A ~ 2-fold reduction in rpL17 was apparently seen between 50mM and 100 mM but this did not change at higher concentrations. It might have been useful to test salt concentrations above 200mM.

4: In a few places the English usage could use some attention.

1st Revision - authors' response 13 May 2011

Thank you for the efficient, thorough, and most helpful reviews. Below I describe how we have dealt with each reviewer's comments. In addition, we have requested that David Tollervey and Sander Granneman send to you confirmation that they agree with what we have written in this manuscript about their unpublished work.

Referee #1 In this manuscript, Dr. Woolford Jr. and co-workers studied in detail the events needed for yeast 27SA pre-rRNA processing at site A3. They nicely concluded that: (1) There is a set of trans-acting factors in yeast ribosome biogenesis, named "A3 factors", involved in this processing reaction. This set is composed of seven proteins: Nop7/Yph1, Erb1, Ytm1, Rlp7, Nop15, Nsa3/Cic1 and Rrp1. (2) All except Rrp1 are interdependent for their association with pre-ribosomal particles. (3) Binding of Rrp17 to pre-ribosomal particles is dependent on the presence of Rlp7. (4) Stably assembly of a set of 60S ribosomal proteins (L17, L26, L35 and L37) requires the presence of Rlp7. This is further investigated for L17, leading to the conclusion that stably assembly of L17 needed pre-rRNA processing at site A3. (5) Very interestingly, the above ribosomal proteins act as roadblock for an exonucleolytic activity of Rat1 beyond site A3 that could promote turnover of 27SA pre-rRNAs. The work is nice and of high quality. However, I find some critique that would be addressed to match the scopes of the prestigious EMBO Journal. My major critique concerns to the use of GAL-RLP7 construct as the sole one for the most of the analysis; I would like to encourage the authors to repeat similar experiments to those performed with the GAL-RLP7 upon depletion of genes corresponding to the remaining A3 factors (or at least a subset of them).

(a) All over the manuscript. Most northern hybridisation and primer extension analyses require quantifications of the levels of pre-rRNAs and mature rRNAs to reach properly to some conclusions.

We have used Image Gauge to quantify each of the primer extension assays shown in Figures 1B, 1D, 4D, 6A, and 7. (There were no northern hybridization experiments). These data support each of our conclusions and are shown in an attached excel spreadsheet *(see next page)*, which we do not plan to include with our manuscript. Figure 1B shows assays of pre-rRNA defects in strains depleted for each of the A_3 factors. Different labs have described each of these mutants before, separately. This work has been appropriately referenced in our introduction. So, this is shown as an introduction to the A_3 factors as a discrete phenotypic class of processing/assembly mutants. It is clear for each mutant that levels of $27SA_3$ pre-rRNA increase relative to unshifted wildtype controls and that levels of the Bs 5' end decrease. Figures 7 and 8A, B show primer extension assays of depletions of rpL17 or A_3 factors or the latter in concert with mutant Rat1. In each case changes from wild type are clear and reproducible. Figures 1D, 4D, and 6A show primer extension assays of pre-rRNA copurifying with A_3 factors, Rat1, or rpL17. In each case, all pre-rRNAs detected are clearly and reproducibly above background of negative controls also shown.

Figure/ Strain	Ratio (A_3/A_2)		Ratio (B_S/A_2)	
	Galactose	Glucose	Galactose	Glucose
Figure 1B				
GAL-NOP7	7.34	67.83	142.12	51.54
GAL-ERB1	7.55	56.57	147.11	55.66
GAL-YTM1	7.87	46.11	178.8	92.52
GAL-RLP7	22.33	106.44	132.11	57.46
GAL-NSA3	7.01	66.43	132.17	81.52
GAL-NOP15	25.26	285.24	84.51	78.45
GAL-RRP1	48.33	109.22	197.27	169.57
epb2-1	8.55	4.2	189.1	39.55

A_3 factor mutants show approximately 4-9 fold accumulation of $27SA_3$ pre-rRNA (when compared to levels of the control, $27SA_2$ pre-rRNA. They also show ~2-3 fold reduction in levels of $27SB_S$ pre-rRNA, upon shifting to glucose-containing medium.

Figure 1D	**Ratio of 35S in TAP Strain/ ENP1-TAP**	**Ratio of A_3/A_2 in TAP Strains/ Ratio of A_3/A_2 in whole cells**	**Ratio of B_S/A_2 in TAP Strains/ Ratio of B_S/A_2 in whole cells**
RLP7-TAP	1.1	3.5	0.8
NSA3-TAP	1.1	3	1.6
YTM1-TAP	1.5	3.6	1.2
ENP1-TAP	1		

All A_3 factors co-IP similar amounts of 35S pre-rRNA compared to the control, 90S pre-ribosomal factor Enp1. The enrichment for A_2, A_3, and B_S signals over that of whole cell RNAs indicates that these assembly factors copurify each pre-rRNA species.

Figure 4D	**Ratio of A_2 in TAP strain/ whole cells**
RAT1-TAP	12.8

Rat1-TAP exhibits ~13-fold enrichment for $27SA_2$ pre-rRNA, indicating that it copurifies with $27SA_2$ pre-rRNA.

	Ratio of 35S in *RPL17-TAP/ENP1-TAP*	Ratio of A_3/A_2 in *RPL17-TAP*/Ratio of A_3/A_2 in whole cells	Ratio of B_S/A_2 in *RPL17-TAP*/Ratio of B_S/A_2 in whole cells
RPL17-TAP	0.4	0.8	0.9

Figure 7

	Ratio of A_2 in glucose/galactose	Ratio of B_S in glucose/galactose
GAL-RPL17	1	1.2

(b) Quality of Fig. 1B must be improved; - Site A2 must be shown for all GAL strains as a control that A3 is specifically enriched upon depletion of any of the so called "A3 proteins".

Thanks; we have now remade Fig.1B to include the A_2 site.

- A3 is perfectly seen in wild-type conditions, however, the exposure time used for Fig 1B is not enough to see it. Please improve.

In order to detect the A_3 ends in these wildtype samples (often amounts are quite low), we would have to do a longer exposure, which would grossly overexpose and obscure the changes in signals from B 5' ends in the mutant samples. These data are already published, and it is clear that our data are consistent with the published work.

- As mentioned above, quantification of the levels of the primer extension stops could enforced the conclusion reached.

See response to (a) above.

(c) Fig 1D. needs, in addition to quantification, a control of a 90S pre-ribosomal factor to address how efficiently Rlp7, Nsa3 and Ytm1 precipitates 35S pre-rRNA.

We have now added as a positive control to Fig.1D, the 90S preribosomal factor Enp1. One can see that even greater amounts of 35S pre-rRNA copurify with TAP-tagged Ytm1, Rlp7, or Nsa3, compared to Enp1, indicating that these three A_3 factors associate with 35S pre-rRNA.

(d) Fig 2. It is clear to me that depletion of Nop7, Erb1 or Ytm1 leads to similar profiles of pre-ribosomal proteins, but it is not obvious for the rest of A3 factors assayed. If possible, perform western blotting upon depletion of each A3 protein to clearly conclude about the interdependence of A3 factors.

In the strains in which Rlp7, Nop15, or Nsa3 are depleted, we can clearly see, without doing western blots, that in the SDS-PAGE gels shown in Fig.2 the silver-stained bands containing Erb1, Nop7, Ytm1, Rlp7, and Nop15 are decreasing, as shown in Figure 2A. We are confident that these specific proteins are present in these gel bands; each band was identified by mass spectrometry several times, using the Rpf2-TAP samples purified from wildtype strains. For a variety of reasons, we have no reason to suspect that these bands contain any other proteins (except for the Nsa3 band also containing rpL3), or that in these depleted strains these proteins migrate elsewhere on the gels. Nevertheless, to confirm this point, we have now shown by western blotting that amounts of Nop7 in preribosomes in each of the six A_3 factor depleted strains are decreased (See Supplementary Figure S2A). Furthermore, we have carried out additional tagging, purifications, and western blots to show that rpL17, rpL26, rpL35 and rpL37 decrease in the *GAL-NOP7* and *GAL-NOP15* depletion strains, identical to what was shown in Fig. 5 for depletion of Rlp7 (see response to comment (g) below). These new western blot data are shown in Supplementary Figure S4.

Performing western blots for ALL of these A_3 proteins would require HA-tagging Erb1, Ytm1, Rlp7, and Nop15 in each of these three strains, since there are no antibodies yet available. Likewise, time-consuming tagging would be necessary for most other proteins visible on the gels, since there are few antisera available. (In addition to being time-consuming, epitope-tagging some of these proteins is non-trivial.)

Moreover, the use of a GAL-RPF2 NOP7-TAP strain as a control is not appropriate. Instead, a RPF2-TAP strain harbouring an ribosome assembly factor unrelated to the A3 factors should be used.

Very good point! We have now included in Figure 2A an *RPF2-TAP* strain in which assembly factor Rea1 is depleted, as the negative control. Rea1 functions several steps after the A_3 step. We have omitted the *GAL-RPF2 NOP7-TAP* and *ebp2-1 RPF2-TAP* control strains.

(e) I suggest to perform the analysis of the pre-rRNAs and rRNAs upon depletion of Rlp7 in the strain GAL-RPL7 RPF2-TAP; this could nicely complement the data of Figure 3.

We have already shown analysis of pre-rRNAs (from whole cell extracts) in the *GAL-RLP7* strain, in Figure 1B. Maybe the reviewer is asking us to assay pre-rRNAs in preribosomes purified from the *GAL-RLP7 RPF2-TAP* depletion strain, rather than RNA from whole cell extracts. We have done so, but do not show these data, which are identical to what is seen in whole cell extracts.

(f) Figure 4. - Authors demonstrated that upon depletion of Rlp7 in a Rpf2-TAP strain, Rrp17 is not longer recruited to the purified pre-ribosomal particles. However, Rat1 and Xrn1 are still present in the purified particles. I find that more controls are required to generalise this conclusion to the rest if A3 factors. Therefore, I suggest to repeat the experiment again with more than one strain (having Nop7, Nop15, etc factors under the control of the GAL promoter). I also suggest to perform all series of shown western blots (L5, Rat1-HA, Xrn1-GFP and Rrp17-HA) for all strains.

We have shown that the other five A_3 factors are not present in preribosomes in the absence of Rlp7. Therefore, it stands to reason, and we strongly believe, that effects on most or all preribosomal proteins will be the same in all six of the A_3 factor-depleted strains, consistent with their interdependent association with preribosomes (Figure 2A). Therefore, in our opinion, epitope-tagging Rat1, Xrn1, and Rrp17 in each of the other five depletion strains and performing the fifteen purifications and western blots is unwarranted. Furthermore, epitope-tagging these exonucleases is non-trivial.

-Rat1-TAP co-precipitates 27SA2, 27SA3 and 27SB pre-rRNAs (Figure 4D), so maybe the binding of Rat1 and Xrn1 to the A3 site is indeed affected by the depletion of the A3 factors, however, the binding to 27SB pre-rRNAs is still taking place, explaining why these exonucleases do still associates to the Rpf2-TAP complex upon depletion of Rlp7. Please comment on this possibility at least at the discussion section.

This idea seems unlikely to us, since there is little 27SB pre-rRNA generated in the A_3 depleted strains. At the very least, one expects from this hypothesis to see much less Rat1 in these mutant preribosomes, which we do not. In addition, Sander Granneman and David Tollervey are using CRAC assays to study the binding of Rat1 to wildtype and mutant preribosomes.

(g) Figure 5A. Clearly, the manuscript will gain strength if this western blotting analysis is similarly performed upon depletion of the rest of A3 proteins in addition to Rlp7.

We have now HA-tagged rpL26, rpL35, and rpL37 in the Nop15 and Nop7 depletion strains, and shown by western blotting that each of these three ribosomal proteins, as well as rpL17 (for which we have an antibody), is diminished in preribosomes when Nop7 or Nop15 are depleted. These results are shown in new Supplementary Figure 4. These results are the same as for the Rlp7 depleted strain, further strengthening our idea that all six A_3 factor depleted strains should behave identically (see also our response to comments (d) and (f) above).

(h) Figure 6. Figure 6B and 6C. It would be very worthy to use an antibody against a ribosomal protein of the small subunit to check for the specificity of purification of the Npa2 and Nog2 complexes.

We are not certain how this would help determine specificity. Even if we detect some amount of a small ribosomal subunit protein, that does not tell us what fraction of the amount of a large subunit protein we detect is specific versus nonspecific. Npa2 and Nog2 have been shown by several groups to be present in and required for maturation of pre-60S subunits, not pre-40S subunits.

(i) Figure 8. The role of Rat1 in turnover of defective 27SA pre-rrRNAs is studied. I understand that there are only evidences for this role for Rat1 (Fang et al. 2005; Fig. 7 and use of a rat1-1 mutant), however, the authors are in a perfect situation to study the putative contribution of Rrp15 and/or Xrn1 in this turnover pathway.

We agree! We are trying to construct the appropriate *xrn1* mutant strain to do this experiment, but it is time-consuming and we believe beyond the scope of the work described in this manuscript. Rrp17 does not associate with preribosomes in the absence of A_3 factors. Therefore, it cannot contribute to turnover in these mutants.

Minor comments:

(a) All over the manuscript is used the term "the 5' end of mature 5.8S rRNA". Authors must be more accurate in the writing to use 5.8SS when refer to as the end generated by processing at site A3 and 5.8SL when done by direct cleavage at site B1L of 27SA2 pre-rRNA.

Excellent point! We have made all of the appropriate changes in the manuscript.

(b) In same figures, data are commonly grouped from different experiments; I suggest to use dividing lines or an space to highlight this fact.

We think that we understand what you mean for Figure 1D, and we have now done so. There we have data from two separate experiments- Rlp7 and Nsa3 pulldowns, plus a Ytm1 pulldown, immediately adjacent to each other. We have now placed a space between them. However, it is not clear to us where else this is needed. We are happy of course to make any other changes to clarify the figures. Just let us know where it is unclear.

(c) A list of strains with all features (included tagged genes and plasmids) is strongly encouraged. Please, provide it in the Supplementary file.

Thank you!!!! An outstanding and much needed suggestion. Our strain list is now Supplementary Table 2.

(d) Figure 6A. Where does the A3 stop map? Please comment.

We have now added a label indicating the A_3 stop.

(e) The Material and Methods section is extremely poor. Either an extended form of this section must be provided in the text or in the Supplementary material.

We very carefully crafted the Materials and Methods to be as short as possible to save valuable space. We checked that every single experiment that we did could be reproduced using the information that we supplied. Almost every method is identical to those we have used previously; therefore we have referenced those papers. We have now added a more detailed explanation for the single-step purification protocol using Dynabeads. Additionally, we have included details of immunoprecipitations done under conditions of increasing salt concentration.

Referee #2 The work presented in the submitted manuscript by Sahasranaman and colleagues enhances our understanding of the events underlying a specific step, 27SA3 processing, of eukaryotic ribosome biogenesis. Through a series of affinity purifications in strains depleted of the A3 factors, the authors demonstrate that the association of A3 factors with pre-ribosomes is concerted, their association is required for association of Rrp17, an exonuclease, and four ribosomal proteins. Additionally, they show that absence of the A3 factors or the ribosomal proteins triggers turnover of the pre-rRNA. Taken together, their data suggests a detailed mechanism of this particular processing step.

However, there are some minor points that should be addressed.

1. There are several sentences throughout the manuscript that are long and complex. Splitting them into smaller sentences will make them more comprehensible. For example, one sentence in the abstract takes up almost 5 lines!

Thank you! We have systematically evaluated every sentence in the manuscript and tried to shorten and clarify whenever it appeared necessary and possible.

2. Some of the panels in Fig. 1B are not clear since their inclusion is not mentioned, their processing patterns do not align and their phenotypes are different than the A3 factors.

Thank you! These panels in Fig. 1B are two negative controls, primer extension assays of pre-rRNA from mutants that are not A_3 factor mutants. We have now made this clear in the Figure Legend**.**

3. In Fig. 2A, there is another band that is consistently depleted in the A3 factor depletions, and it is marked, but it is not labeled. Similarly, there is another band under Erb1 in Fig. 2C. What are the identities of these bands? Are they possibly other A3 factors?

Outstanding points! We do not know what protein is in the other (low molecular weight, fast migrating) band labeled with a dot in Figure 2A. We have shown by mass spectrometry and western blotting (using HA tags) that the band under Erb1 contains a shorter, faster-migrating form of Erb1. This explains its decrease in the mutants. Most importantly, we agree that there may be other A_3 factors. In the Introduction of the manuscript, we state that there are ~80 assembly factors routinely found in one or more precursors to 60S subunits. Mutant strains for ~ 70 of these factors have been constructed, to deplete or inactivate these proteins, and have been assayed for pre-rRNA processing. Of these 70 mutants, only the seven strains that we describe in this manuscript have been found to accumulate significant amounts of $27SA_3$ pre-rRNA and contain decreased 27SBs pre-rRNA. Of course, this does not mean that there might not be other A_3 factors among the ten proteins for which we do not have mutants. Thus, we have tried to clarify this in the fourth paragraph of the Introduction, where we introduce our definition and description of A_3 factors. We also mention this important point in the results section, p9.

4. Why are Erb1 and Ytm1 not identified in iTRAQ experiments? Erb1, Ytm1 and Nop7 form a subcomplex that can be identified in the absence of pre-ribosomes. Additionally, these three factors are removed together by Rea1. It seems odd then that two are absent. Is there a better explanation for their absence?

We mentioned in the manuscript that we occasionally fail to detect Erb1 or Ytm1 by mass spectrometry in cases where we suspect strongly or show by western blots, that they are indeed present. Others have observed the same for these two proteins. Some proteins simply fail to be consistently solubilized or "fly" in the mass spectrometer. We have now explained this with an additional sentence.

5. Fig. 7 is not labeled correctly. In the text, the two panels are referred to as 7A and 7B, however the panels are not labeled and they are not discussed independently in the figure legend.

Thank you! We have changed the labels and references in the text, now to state: Fig.7, left panel and Fig.7, right panel.

6. In the final paragraph of the results section, you reference lanes 7-8 of Fig. 8A. However, given the context, I believe you are referencing lanes 9-10.

Yes, you are absolutely correct! Thanks! We have corrected this.

7. The first sentence of the discussion says that this work has examined the roles of ALL A3 factors. Is this really true? Could the band referenced in comment 3 represent another A3 factor?

It seems unlikely that there are many more A_3 factors among known assembly factors. See response to comment 3 above.

Additionally, there are LSU biogenesis factors that are more significantly depleted as shown by iTRAQ than the noted A3 factors. It is possible that they could function in this step. This sentence should be modified slightly such that it is less absolute on this point.

Yes! You are referring to Spb1, Cgr1, and Ybl028C from the iTRAQ data. Kressler et al. showed by northern and primer extension assays that depletion of Spb1 causes accumulation primarily of 27SB pre-rRNA. Thus, Spb1 is unlikely to be an A_3 factor. Moy et al. showed by pulse-chase assays that 27S pre-rRNA accumulates upon depeletion of Cgr1. $27SA_2$, $27SA_3$, and 27SB pre-rRNAs were not

distinguished from each other by their assay. This phenotype is unlike that of A_3 factors, but probably needs to be studied in more detail, e.g., by primer extension assays that distinguish the three different 27S pre-rRNAs. Ybl028C has not yet been studied in sufficient detail to know. We have added to the results (p9) to clarify this point. In addition, it seems reasonable that factors other than A_3 factors might be affected by depletion of A_3 factors.

8. In Fig. 7, one band is labeled with the asterisk. The figure legend should mention what this represents.

Thanks; we have now done so to indicate that this band is a 5' truncated pre-RNA seen only upon depletion of rpL17.

9. In Fig. 8A, the labels above the panels are misaligned.

Sorry, that happened during loading of the figures into the *EMBO J.* website. We have corrected this.

10. In the figure legend, the two panels in Fig. 8C are labeled (A) and (B), but they are not labeled as such in the figure.
11. Utp6 is misspelled in Fig. 3A.

Thank you; we have made these corrections.

Referee #3 Many proteins required for ribosome synthesis have been referred to as "assembly factors", generally because the field has no clear idea of what they actually do. Here the authors have attempted to provide direct evidence for roles in assembly for a group of proteins implicated in the early maturation of pre-60S particles. The application of TAP purification and MS to determine the composition of pre-ribosomes (which was first achieved by the Woolford lab) brought about major changes in our understanding of ribosome synthesis. The use of quantitative MS analyses to quantify changes in pre-ribosome composition in mutant strains, as reported here, is significant development that allows the pathway to be better analyzed and will surely lead to further insights. Overall, the data are technically good and the conclusions well supported by the data presented. The work makes a significant contribution to the understanding of pre-ribosome assembly and the functions of the ribosome synthesis factors, and is suitable for publication in the EMBO Journal.

Minor points

1: Abstract line 1: Missing word "of".

Thank you! We have corrected this error.

2: Published data indicate that Xrn1 is probably not a normal processing factor for 5.8S, but degrades the 5' extension when Rat1 is inhibited (El Hage et al., 2008).

Yes, there is some confusion about whether or not Xrn1 normally processes $27SA_3$ pre-rRNA because of its localization primarily to the cytoplasm. However, we and others find that deletion of Xrn1 causes accumulation of $27SA_3$ pre-rRNA. Furthermore, we and Marlene Oeffinger and Mike Rout (Oeffinger et al, 2007), find Xrn1 in preribosomes.

3: The reduction in the recovery of Rpl17 when pre-ribosomes were purified under more stringent salt conditions is the least convincing data presented. A ~ 2-fold reduction in rpL17 was apparently seen between 50mM and 100 mM but this did not change at higher concentrations. It might have been useful to test salt concentrations above 200mM.

In concentrations above 200mM salt, Npa2-TAP preribosomes eluted off the beads, preventing us from assessing this possibility.

4: In a few places the English usage could use some attention.

We have tried to systematically evaluate this problem and improve the writing. Thank you!

2nd Editorial Decision 01 June 2011

Thank you for sending us your revised manuscript. Our original referee 1 has now seen it again, and you will be pleased to learn that in his/her view you have addressed the criticisms in a satisfactory manner. He/she has only two minor remaining issues (please see below) that should be addressed.

Furthermore, there are two editorial issues that need further attention:

First, please specify the number of independent experiments in the legend of figure 3A, and 3B.

Second, prior to acceptance of every paper we perform a final check for figures containing lanes of gels that are assembled from cropped lanes. While cropping and pasting may be considered acceptable practices in some cases (please see Rossner and Yamada, JCB 166, 11-15, 2004) there needs to be a proper indication as well as an explanation in the figure legend in all cases where such processing has been performed according to our editorial policies. Please note that single lanes should not be presented as single panels, even if they come from the same gel. I should add that it is our standard procedure when images appear like they have been pasted together without proper indication (like a white space or a black line between) or without an explanation to ask for the original scans.

In the case of the present submission there are a number of panels that do not meet these requirements: figure 1B (GAL-ERB1 and GAL-YTM1 panels), figure 1D (all panels), figure 6A and 6B; figure 7, figure 8A (GAL-ERB1 and GAL-YTM1 panel), figure 8B (middle panel GAL-RLP7 in Glu)

I therefore like to kindly ask you to send us a new version of the manuscript that contains suitably amended versions of these figures. Please clarify and indicate in all cases whether/that all lanes come from the same gel in the figure legends. Please be reminded that according to our editorial policies we also need to see the original scans for the figures in question. Furthermore, could you clarify the nature of the circles seen in figure 4B, please?

I am sorry to have to be insistent on this at this late stage. However, we feel that it is in your as well as in the interest of our readers to present high quality figures in the final version of the paper.

Thank you for your cooperation.

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1

The revised version of this nice manuscript is significantly much better that the original submission. Congratulations !

Most points I did raise have properly been addressed or discussed. Some experiments I requested have not been done but I totally agree that epitope-tagging proteins is non-trivial, and time consuming just simply to confirm the clear results they have already obtained.

I only find two typos must be corrected:

- Figure 2. I guess that the GAL-REA1 strain is RPF2-TAP instead of NOP7-TAP

- In many figures and legends the authors write BL and BS. I guess the correct terminology is B1L and B1S.

2nd Revision - authors' response 22 August 2011

Thank you for handling the many details with this manuscript EMBOJ-2011-77249R1.

First, in response to the reviewer's comments, we have corrected Figure 2 and the corresponding Figure legend to read *GAL-REA1 RPF2-TAP*. Also wherever appropriate in the text, the Figure Legends, and the Figures, we have changed B_S, B_L, $27SB_S$ or $27SB_L$ to B_{1S}, $B_{1L,}$ $27SB_{1S}$ or $27SB_{1L}$.

Second, we have tried to address all of the editing issues that you described, as follows:

(1) We did two independent biological replicates of the iTRAQ experiments (two mutant and two wild type). The data were collected as a four-plex after a single LCLC MALDI TOFTOF run. We added this information to the legend of Figure 3A.

(2) Regarding cropped lanes of gels:

Fig.1B: we repeated this experiment and now show all results from one gel, except for *GAL-ERB1* for which there was not room. This is from a separate gel, on which we ran a second sample of *GAL-RLP7* as a control for separate gels. Also, we added to Figure1B samples from *GAL-TIF6* as well as wildtype cells, grown in galactose and shifted to glucose, as additional controls (strains that are mutant for factors other than A_3 factors, or that are not mutant). We replaced the *ebp2-1* control with the more comparable *GAL-EBP2* strain (a depletion strain, rather than a temperature-sensitive strain). These additional controls and that two gels were used now are stated in the Figure legend. The data from these primer extension experiments were quantified and are now in Supplementary Table 3.

Fig.1D: we rewrote the Figure legend to state that results are from three separate gels, each with identical negative controls (although we show only one control). We placed a space in the Figure between lanes from different gels.

Fig.6A: we have added to the legend that the two samples from the left are from one gel and the two samples on the right are from a second gel.

Fig.6B: We have added to the Figure legend that all samples are from the same gel and blot.

Fig.7: we have added to the Figure legend that all samples are from the same gel.

Fig. 8A: as in Fig. 1B, we repeated this experiment and state now in the Figure legend that all samples are from the same gel, except *GAL-ERB1* which was run on a separate gel together with *GAL-RLP7*.

Fig.8B: we have redone this figure to now place a space between lanes 3 and 4, indicating that they come from nonadjacent lanes of the same gel.

When we upload the final version of the manuscript, we will include pdf files of the raw data for each of the figures above.

Third, in response to your question, the circles in Figure 4B are bubbles that were underneath the gel when it was photographed.

Fourth, we have added one author, Jill Dembowski from my lab, who repeated the experiments in Figures 1B and 8A. What Jill did is included in the descriptions of who did what, in the Author Contributions section.

Fifth, to make the manuscript less redundant and more clear, and to make room for additional text requested from you (in figure legends), we have deleted or rearranged sentences from the Results and Discussion that we think are unclear, unnecessary, or redundant. We do not think that these changes in any way alter our points or interpretations of what was written in the previous version of the manuscript. They just make the manuscript more clear and concise. I have attached in this email the edited version for you to see these exact changes. (I did not include in THIS version all of the requested changes of $27SB_S$ or $27SB_L$ to $27SB_{1S}$ or $27SB_{1L}$ etc.). However, we will download to the EMBO J. website only the final version, without edits being marked.

These changes are:

Results:
page 7, lines 17-19
page 13, lines 2-3, 7-9, and 10-11
page 16, lines 6-11

Discussion:
page 18, lines 16-18 to page 19, lines 1-2
page 21, last 8 lines
page 22, lines 1-4 and 18-21
page 23 these changes were made before the resubmission
page 24, lines 1-5 and 16-23
page 25, lines 1-7 and 10-15

Sixth, as an update, we have replaced the Pymol figure in Figures 5B and C with an updated figure based on the new crystal structure of the yeast ribosome (Ben-Shem et al. 2010). This in no way changes any interpretation of what we have done or written.

Finally, we have changed references for Sander Granneman's paper on CRAC of Rat1 and the A_3 factors, from (Granneman et al submitted) to (Granneman et al in press) and added this to the reference list. I presume that this would change again in proofs.

I hope that all is in order, and thanks again for all of your attention.

