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Cracking Pre-40S Ribosomal Subunit Structure by Systematic Analyses of RNA-Protein Cross-linking

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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 18 January 2010

Thank you for submitting your manuscript for consideration by The EMBO Journal. First, please accept my sincere apologies for the delay in getting back to you with a decision. This was caused by the limited availability of suitable and willing referees prior to and during the past Christmas holiday season.

Your manuscript has now finally been seen by three referees whose comments to the authors are shown below. As you will see all three referees are positive about publication of the paper here after a certain amount of (minor) revisions. I would thus like to invite you to prepare a revised manuscript in which you need to address (or respond to) the issues put forward by the referees. 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.

When preparing your letter of response to the referees' comments, please bear in mind 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

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REFEREE COMMENTS

Referee #1 (Remarks to the Author):

Cracking pre-40S ribosome structure by systematic analyses of RNA-protein crosslinking

Granneman et al.

In this manuscript the authors continue to use their novel CRAC method to assess association of ribosome assembly factors with pre-ribosomal subunit particles. In this work the authors have focused on six proteins that aid in 40S biosynthesis and are proposed to act at a late stage of this process (this assertion is a bit tenuous since the authors state some of the factors associate early.....minor point but should be clarified for those more peripherally associated with this field). The interaction sites of these factors are mapped by crosslinking and sequencing. The authors then use the low resolution cryo-EM models of 40S subunits to try and position the assembly proteins and thus gain a more structural understanding of their data. Overall, the data are very nice and the manuscript is well-written. This work adds details to our understanding of 40S formation and this work is of high quality.

1. The data for Tsr1 interaction with h34 is not convincing, especially given the noise in this region in the negative control. The importance and validity of this crosslink needs to be reconsidered and the statements about this interaction attenuated.
2. In Figure 2 the color choices are unfortunate. The crosslinking sites for Tsr1 and Dim1 are close to one another and the so are the color choices making this figure less easy to follow than is necessary. While it can be difficult to find "enough" colors for larger data sets, Tsr1 or Dim1 could be swapped with another protein where the crosslinks are more removed.
3. Figure 4 suffers from the lack of controls that illustrate maximal possible reactivities. One possibility would be to strip the proteins from the pre-40S particle and probe the unliganded RNA. This should aid in unfolding (especially with the appropriate conditions) and thereby allow one to judge the extent to which the region around the Dsite is single stranded. Otherwise it is very hard to establish a baseline. Some additional information is required for the conclusions that are drawn to be validated.
4. It maybe the best that can be done, but it is a bit unsatisfying to have the data for a pre-40S mapped onto a 40S structure and one that is of very low resolution. Perhaps the authors can address some of the possible caveats to their interpretations for those less initiated in these techniques.
5. There are many inconsistencies in the writing/formatting. Italics of in vitro and in vivo varies throughout (if EMBO has a policy use it or pick one and stick with it), E. coli is often presented without the space between genus and species and sometimes is not italicized, and EF-Tu needs a lower case u.
6. The title is a bit inexact since there is no pre-40S "ribosome" but simply a "ribosomal subunit".

Referee #2 (Remarks to the Author):

This is one of a series of several recent papers highlighting a new approach to map protein-RNA interactions in vivo, the so-called CRAC. ..UV crosslinking and cDNA analysis...method. In this manuscript, interactions of late-acting yeast ribosome assembly factors with pre-rRNA are identified. This is significant. To understand the functions of the more than 180 ribosome assembly factors, we must know which bind rRNA precursors in assembling ribosomes, where they bind, when in the pathway they bind, and how this changes. CRAC is the first method applied to do so. The authors make a very strategic choice of factors to test, those in late 43S precursors to the 40S ribosomal subunit. Only about 8-9 assembly factors are stably associated with these particles; thus they are much less complex than other preribosomes. These factors include one of almost every class of potential assembly factor: a methyltransferase Dim1, a putative GTPase Tsr1, a nuclease Nob1, a protein kinase Rio2, and two proteins already implicated in reorganization of RNP architecture, Enp1 and Ltv1, plus Rio1 and Dim2.

The first six proteins were found to consistently crosslink to one or several sites in rRNA. Interestingly all binding sites are in the mature 18S rRNA sequence, none in the transcribed spacer

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that is removed by the Nob1 endonuclease. The crosslinking data appear to be valid, as only those seen in multiple experiments are discussed. For the most part, the binding sites make sense. Most of the paper is devoted to discussing models for these sites. These models build upon the known phenotypes of depletion or inactivation of the six factors, the model for the structure of the mature 40S ribosomal subunit of yeast, and previous protein-protein interaction datasets.

The models make good sense and are provocative. They should fuel many future experiments. Dim1 and Tsr1 bind to sites that overlap with those for their potential bacterial equivalents, and where translation factors bind. Thus they may prevent premature association of these translation factors with preribosomes. Rio2 binds to sites near to those of ribosomal proteins S16 and S18, as well as Nob1, Ltv1, and Dim 2. These two r proteins are phosphorylated. Rio2 in humans is required for release of these three assembly factors from preribosomes. Enp1 and Ltv1 bind to or near helix 33 which undergoes significant movement during particle maturation, upon a cycle of phosphorylation and dephosphorylation of factors and r proteins.

The most initially inconsistent and surprising result was that Nob1 does not bind near the D site that it cleaves, to remove spacer sequences from the 3' end of 20S pre-rRNA to form mature 18S rRNA. This was explained by presuming that the RNA binding site of Nob1 is distinct from its active site for cleavage. Mutations in the active site that inactivate cleavage did not alter binding. Interestingly, Nob1 binds to preribosomes long before it cleaves the pre-rRNA. The cleavage site was found to be protein free and unstructured, based on accessibility to DMS and lead modification. Thus rearrangements of preribosome structure, catalyzed by factors including the helicase Prp43, were suggested to be necessary to enable cleavage.

The paper was for the most part clear and convincing, making thorough use of existing models and data. There was one unclear section though, on page 16:
At the top of the page the authors state that the D site is unstructured because it is accessible to chemical probes, but at the bottom of the page they point out that Tsr1 and Dim1 binding sites may enclose the 3' end of 18S rRNA and might need to dissociate before Nob1 can access the D cleavage site.

It might also be helpful to clarify a little more that these binding sites might not be used simultaneously. The 43 S particles may be heterogeneous.

Referee #3 (Remarks to the Author):

In the manuscript "Cracking Pre-40S Ribosome Structure by Systematic Analyses of RNA-Protein Cross-linking", Granneman et al. analyse by a recently described in vivo protein-RNA crosslinking technique pre-rRNA interaction sites of six yeast small ribosomal subunit maturation factors, all present in "late" cytoplasmic pre-40S subunits. The observed clustering of the factors pre-rRNA interaction sites in the pre-40S head/neck area is in good agreement with previous studies showing that assembly/folding events in this region correlate with efficient nuclear export of pre-40S subunits and with their final cytoplasmic maturation. The work will for sure be an important reference for future studies aiming in understanding molecular mechanisms of eukaryotic pre-40S maturation.

Suggestions and comments:

- Abstract: "We report that the binding sites for six late-acting 40S ribosome synthesis factors cluster around the 3' end of the 18S rRNA in model 3D structures." The sentence suggests that the authors identified all ("the") binding sites of these factors in pre-ribosomes. The general "binding sites" on pre-ribosomes might well be more extended than pre-rRNA interaction sites identified in this study and probably include (pre-)ribosomal protein components as indicated by earlier observed protein-protein interactions between Nob1 and rps5/rpS14, and a salt resistant proteinacous Ltv1/Enp1/rpS3 complex. I think it would be more precise to conclude that pre-rRNA interaction sites of the examined factors were identified in this study.
The second part of the sentence ("...cluster around the 3' end of the 18S rRNA in model 3D structures") is hard for me to follow. To my knowledge, the position of the 18S rRNA 3' end is neither exactly known in eukaryotic pre-40S subunits nor in mature 40S subunits, but suspected to

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be in the head/platform cleft (see figure 3F). Several of the observed interaction sites, including the beak structure, interaction site of Enp1p, and the identified Ltv1p interaction sites seem to be rather far from there. I have the impression that the factors pre-rRNA interaction sites are largely in the 3' major and 3' minor SSU rRNA secondary structure domain (all six factors have at least one of their interaction sites there), or adjacent regions including helix 28, corresponding in 3D structure models to the small ribosomal subunit head and neck region.

Abstract: "The primary binding-sites for the 18S 3'-endonuclease Nob1 are distinct from its cleavage site and were unaltered by mutation of the catalytic PIN domain" and later " ... and we conclude that structural reorganisation is needed to bring together the catalytic PIN domain and its target" For detailed discussion of these points please see the comments on the relevant results section (p.10) and on figures 4C, 4D and S3, below.

Abstract: ".... confirmed that the cleavage site was predominately protein free and poorly structured in the pre-rRNA" I am not sure whether the structural probing experiments allow such a semi-quantitative interpretation. They provide evidence for the existence of a population of pre-rRNA which is poorly structured in this region, but do they really allow to conclude that this is the case for the large majority of pre-40S subunits?

- Introduction (p.3): "This reorganization requires phosphorylation of the pre-40S proteins Enp1 and Ltv1 by the kinase Hrr25, and subsequent dephosphorylation". Later, in Figure legend of Figure 3 it is stated that "Hrr25-dependent phosphorylation of Rps3 and subsequent dephosphorylation triggers a structural change leading to repositioning of Rps3". If I understand the work referred to correctly (Schafer et al.), it suggested that major targets of Hrr25 dependent in vitro phosphorylation include Enp1, Ltv1 and rpS3. Whether these ones are the only one in small ribosomal subunit precursors, or just major ones, and, more importantly, whether Hrr25 mediated phosphorylation of one of them or of all three is in vivo crucial for small ribosomal subunit maturation, was not addressed to my knowledge.

Introduction (p.4): "Here we report the binding sites..." see above, eventually more precise : pre-rRNA interaction sites

- Results (p. 6): "Enp1 and Ltv1 bind sequences that form the beak structure" Why Ltv1? The pre-rRNA interaction sites of Ltv1p, helix 41 and helix 16 are not part of the beak structure.

- Results (p. 6): "However, in the absence of any data on the binding sites of Ltv1 and Enp1, it was difficult to assess what, if any, direct roles they played in beak formation." It was shown that Ltv1 and Enp1 can be isolated from yeast cell extracts in a salt resistant complex with rpS3, strongly arguing that rpS3, located at the base of the beak, is a proteinacous component of the Enp1p/Ltv1p binding site in pre-ribosomes.

- Results (p. 7): "H16 and H41A are in close proximity to the beak and to Rps3"
I think the localisation of these helices is described in the discussion in a more adequate way: "Ltv1 binds sequences in H41, which are located close to the beak, but also binds H16, which is more distantly located in the shoulder region of the 40S particle "

Results (p. 7): ".. consistent with 2-hybrid interactions reported between Rps3 and Ltv1 (Ito et al, 2001; Loar et al, 2004)" If I am not wrong, Loar et al. observed two hybrid interactions between Ltv1 and Yar1, and between rpS3 and Yar1, not between rpS3 and Ltv1.

Results (p.10): "The region surrounding cleavage site D is predominately open and not associated with Nob1 in pre-40S ribosomes" see abstract.... (I am not sure whether the structural probing experiments allow such a semi-quantitative interpretation. They provide evidence for the existence of a population of pre-rRNA which is poorly structured in this region, but do they really allow to conclude that this is the case for the large majority of pre-40S subunits ?)

Results (p.10): "If correct, this model would predict that the active site of Nob1 would not be involved in H40 binding ...." and following paragraph. See below, discussion of Figures 4C, 4D and S3 for comments on this. The most clear argument for the Nob1 PIN domain not being involved in interactions with H40 would be to study a Nob1p variant lacking the PIN domain. The PIN domain

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mutant used here, was suggested by Pertschy et al. to be catalytically inactive, not necessarily meaning that the PIN domains capability to interact with RNA is diminished.

Results (p.11): "however, these data and recent in vitro analyses (Lamanna and Karbstein) do not support this stem structure " Didn't these authors also show in vivo analyses, similar to the ones presented here (Figure S3 in Lamanna et al.) ?

Discussion (p. 12): "A striking finding was that cross-linking sites for five of the late-acting 40S synthesis factors Rio2, Tsr1, Dim1, Nob1 (this work) and Prp43 (Bohnsack et al, 2009) are located in close proximity to functionally important sequence elements in the 3' region of the 18S rRNA." It is not completely clear what the authors mean with "3' region". Is it used in the sense of the ensemble of 3' major and minor domains in the 2D map (equivalent to head / neck regions in the 3D structure), or in the sense of the area in the 3D structure close to the 3' end of 18S rRNA? See also above.

Discussion (p. 13): "... and pre-40S ribosomes that lack Rps15 fail to efficiently incorporate Rio2 and are not exported to the cytoplasm (Leger-Silvestre et al, 2004; Zemp et al, 2009)"
More precisely, the data cited and other published data argue that pre-40S ribosomes are exported to the cytoplasm in the absence of rpS15 assembly, but that nuclear export is significantly delayed.

Discussion (p. 14) and Figure 3B: "If Ltv1 binds both sequences simultaneously ...." and following. The meaning of figure 3B, entitled "Ltv1 corresponds to the extra density near the head and the shoulder in the head domain" remains unclear to me, since, obviously, the resolution of the EM density map of Ltv1-TAP purified pre-ribosomes used in the figure is rather limited. The density attributed in the EM map to Ltv1p is relatively far from the Ltv1-p pre-rRNA interaction sites described in this work (marked yellow in Figure 3B) and does not really correspond to a prolonged tubular structure with 3nm in diameter and 9nm in length. It seems at the moment more appropriate to discuss in a more open way possible reasons for the observed Ltv1p - pre-rRNA crosslink patterns, including the option that two copies of Ltv1 interact with pre-ribosomes independently of each other at two different sites. A high flexibility in the head - body orientation in pre-40S subunits could also well explain the observed interaction sites (and could eventually also mask in part a defined beak structure in 3D reconstructions of pre-40S subunits). Clearly, more structural data, characterizing the shape of Ltv1 or further defining the architecture of defined pre-ribosomal intermediates, would be required to clarify these points.

Discussion (p.15): "Dissociation of each of these proteins from the pre-40S particles would therefore be required for translation to commence." I am not sure whether the observed pre-rRNA interaction patterns of Dim1p, Tsr1p, and Rio2p are sufficient to conclude that binding of these factors and functional interactions of translation factors/tRNA are mutually exclusive. Arguing against this conclusion is, that several of the biogenesis factors were seen to interact with the same pre-rRNA regions, for example Dim1p, Tsr1p and Nob1p with helix 28.

Figure 1: It is unclear to me how many times a certain region was finally sequenced, since some of the peaks, marked with one helix, appear broad, others as one line. In case of Ltv1p, it remains thereby obscure, how the ratio of hits in the region of helix 41 versus helix 16 is. The exact numbers should be indicated.

Figure 3: I have the impression that, instead of showing many small sub-figures, showing a reasonably large version of a recent (pseudo-)atomic resolution model of the eukaryotic small ribosomal subunit (eventually from two perspectives) in which all the main rRNA interaction sites of the analysed factors are highlighted by different colours, would be more helpful.

Figure 3B: see above. (comments on discussion section)

Fig. 3C : In the original work of Schäfer et al. it was reported that incubation of pre-ribosomes with ATP leads to dissociation of rpS3, Enp1p and Ltv1p in one complex from pre-ribosomes under certain buffer conditions, while the model In Fig. 3C suggests selective dissociation of Enp1p and Ltv1p (in a heterodimeric complex) upon kinase action. What is the argument for that? I understood the data of Schäfer et al. In the sense that ATP incubation of pre-40S subunits led in a Hrr25p dependent way to a new pre-ribosomal conformation, in which the Ltv1-Enp1-rpS3 hetero-trimer

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was loosely associated. Subsequent dephosphorylation by non-specific phosphatases induced another reorganisation in which a population of rpS3 was more stably assembled, not Ltv1p and Enp1p.
See also above for discussion of the proposed tubular structure of Ltv1p.

Figure 5: rpS14, whose C-terminus is predicted to interact with the 3' minor domain (near Tsr1/Dim1 interaction sites) is not mentioned here. Nob1p was shown to interact in vitro with both rpS14 and rpS5 (Lamanna et al., 2009), interactions which are not mentioned here. rpS0 is thought to bind in the head-body hinge (neck) region, making contact with both 3' major domain rRNA and helix 26, an interaction site of Tsr1. That might be an information worth to include. There seems to be a striking parallel in the observed interaction sites of factors associated with late cytoplasmic pre-40S subunits and the known 3D localisation of ribosomal protein components whose in vivo depletion leads to a gradual delay in pre-ribosomal nuclear export and to a significant cytoplasmic accumulation of immature subunits with 3' extended 18S rRNA (rpS2, rpS3, rpS15 and rpS20, rpS0 and the C-terminus of rpS14, for which the localisation in mature ribosomes is known). That might be worth to discuss. As indicated in case of Enp1p and Ltv1p for rpS3, some of the factors functions might be related to an event leading to stabilised incorporation/assembly of one or the other ribosomal protein into pre-40S subunits.

Figure 4C, D, S3 and Materials and Methods: The experimental procedure used for structural in vitro probing by DMS of the region surrounding site D is not described. Does the section describing immuno-purification experiments refer to how HTP-Nob1 was purified? If yes, again, the exact procedure remains unclear. It is also unclear if HTP-Nob1 and HTP-Nob1 D15N were co-expressed with endogenous Nob1p in a wildtype (BY4741) strain background? Were they plasmid encoded (if yes which plasmids were used?) and under the control of which promoter? Does the N-terminal HTP-Tag lead to a pre-rRNA processing phenotype, as indicated by the apparent elevated level of 20S-pre-rRNA in the HTP-Nob1 strain as seen in figure S3 C (I assume that in lane 1 and lane 2 the input samples are loaded, the description of figure S3C is not completely clear in this regard) ? If HTP-Nob1 D15N was co-expressed with endogenous Nob1p, Nob1p-D15N hetero-oligomerisation with wildtype Nob1p might result in partially stabilised pre-rRNA interactions, making the interpretation of the described experiments more difficult. In that case, it would be preferable to do the experiments in a strain in which wildtype Nob1p can be in vivo depleted. Would one not expect dominant negative effects on pre-rRNA processing and cellular growth through co-expression of HTP-Nob1 D15N variant if its essential function in D-site cleavage, not its interaction with pre-ribosomes, is inhibited? It would be helpful to include in the experiment shown in Figure S3C the HTP-Nob1 D15N variant (in a strain in which endogenous Nob1p was depleted). Like this, quantitative differences in the stability of pre-rRNA association of HTP-Nob1 versus HTP-Nob1 D15N could be revealed.

Materials and Methods: strain construction is not clear. Oligos and template plasmid used to amplify DNA fragments with which strain BY4741 was transformed should be indicated. Did the strains show growth phenotypes? Were eventual pre-rRNA processing phenotypes of the strains analysed? These important informations should be included. A northern blot experiment with total RNA from the different strains and a wildtype loaded, similar to the one shown in Fig. S3C, would be helpful to judge these points.

1st Revision - authors' response 26 March 2010

Referee #1 (Remarks to the Author):

Cracking pre-40S ribosome structure by systematic analyses of RNA-protein crosslinking

Granneman et al.

In this manuscript the authors continue to use their novel CRAC method to assess association of ribosome assembly factors with pre-ribosomal subunit particles. In this work the

authors have focused on six proteins that aid in 40S biosynthesis and are proposed to act at a late stage of this process (this assertion is a bit tenuous since the authors state some of the factors associate early.....minor point but should be clarified for those more peripherally associated with this field).

We have reworded the text (p4) to simply state that the factors are retained in late pre-40S particles.

The interaction sites of these factors are mapped by crosslinking and sequencing. The authors then use the low resolution cryo-EM models of 40S subunits to try and position the assembly proteins and thus gain a more structural understanding of their data. Overall, the data are very nice and the manuscript is well-written. This work adds details to our understanding of 40S formation and this work is of high quality.

1. The data for Tsr1 interaction with h34 is not convincing, especially given the noise in this region in the negative control. The importance and validity of this crosslink needs to be reconsidered and the statements about this interaction attenuated.

We agree that the Tsr1 H34 hits are not numerous. However, the Tsr1 hits in this region are not the same as the negative control (see new Supplementary Tables), indicating that this interaction is specific.

2. In Figure 2 the color choices are unfortunate. The crosslinking sites for Tsr1 and Dim1 are close to one another and the so are the color choices making this figure less easy to follow than is necessary. While it can be difficult to find "enough" colors for larger data sets, Tsr1 or Dim1 could be swapped with another protein where the crosslinks are more removed.

The colors have been altered in the revised version of the Figure.

3. Figure 4 suffers from the lack of controls that illustrate maximal possible reactivities. One possibility would be to strip the proteins from the pre-40S particle and probe the unliganded RNA. This should aid in unfolding (especially with the appropriate conditions) and thereby allow one to judge the extent to which the region around the D-site is single stranded. Otherwise it is very hard to establish a baseline. Some additional information is required for the conclusions that are drawn to be validated.

Were the region protected, deproteinisation would be helpful to determine whether the protection reflects RNA secondary structure or protein binding. However, it this case the region is highly accessible, so it is not clear that this is relevant. Moreover, the treatment needed for deproteinisation may itself alter the RNA structure. In the data we can see that the region is accessible by reference to neighboring sequences that are clearly protected. In the revised figure we have included additional footprinting results in which a number of chemicals were used (1M7 and CMCT). These data strongly suggest that the D-site stem is highly flexible and likely single stranded. The data clearly demonstrate that predicted double stranded regions are protected from chemical modification. We agree with the reviewer that we cannot state that the region is always single stranded/flexible. Therefore, to substantiate the results we analyzed the structure of the D-site region in cell depleted of Nob1 (now presented as Figure 4 and discussed in the main text). This showed no alteration in protection, consistent with the conclusions that 1) the region is accessible and 2) that this does not represent the primary Nob1 binding site.

4. It maybe the best that can be done, but it is a bit unsatisfying to have the data for a pre-40S mapped onto a 40S structure and one that is of very low resolution. Perhaps the authors can address some of the possible caveats to their interpretations for those less initiated in these techniques.

The 40S structure is, unfortunately, the best available. A high-resolution pre-40S structure would certainly have been preferable, but there seems little prospect of this becoming available in the near future. We have made this clearer in the revised test (page 11)

5. There are many inconsistencies in the writing/formatting. Italics of in vitro and in vivo vary throughout (if EMBO has a policy use it or pick one and stick with it), E. coli is often presented

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without the space between genus and species and sometimes is not italicized, and EF-Tu needs a lower case u.

These points have been corrected throughout the MS.

6. The title is a bit inexact since there is no pre-40S "ribosome" but simply a "ribosomal subunit".

We have modified the title of the revised MS.

Referee #2 (Remarks to the Author):

This is one of a series of several recent papers highlighting a new approach to map protein-RNA interactions in vivo, the so-called CRAC…UV crosslinking and cDNA analysis...method. In this manuscript, interactions of late-acting yeast ribosome assembly factors with pre-rRNA are identified. This is significant. To understand the functions of the more than 180 ribosome assembly factors, we must know which bind rRNA precursors in assembling ribosomes, where they bind, when in the pathway they bind, and how this changes. CRAC is the first method applied to do so. The authors make a very strategic choice of factors to test, those in late 43S precursors to the 40S ribosomal subunit. Only about 8-9 assembly factors are stably associated with these particles; thus they are much less complex than other preribosomes. These factors include one of almost every class of potential assembly factor: a methyltransferase Dim1, a putative GTPase Tsr1, a nuclease Nob1, a protein kinase Rio2, and two proteins already implicated in reorganization of RNP architecture, Enp1 and Ltv1, plus Rio1 and Dim2.

The first six proteins were found to consistently crosslink to one or several sites in rRNA. Interestingly all binding sites are in the mature 18S rRNA sequence, none in the transcribed spacer that is removed by the Nob1 endonuclease. The crosslinking data appear to be valid, as only those seen in multiple experiments are discussed. For the most part, the binding sites make sense. Most of the paper is devoted to discussing models for these sites. These models build upon the known phenotypes of depletion or inactivation of the six factors, the model for the structure of the mature 40S ribosomal subunit of yeast, and previous protein-protein interaction datasets.

The models make good sense and are provocative. They should fuel many future experiments. Dim1 and Tsr1 bind to sites that overlap with those for their potential bacterial equivalents, and where translation factors bind. Thus they may prevent premature association of these translation factors with preribosomes. Rio2, binds to sites near to those of ribosomal proteins S16 and S18, as well as Nob1, Ltv1, and Dim 2. These two r proteins are phosphorylated. Rio2 in humans is required for release of these three assembly factors from preribosomes. Enp1 and Ltv1 bind to or near helix 33, which undergoes significant movement during particle maturation, upon a cycle of phosphorylation and dephosphorylation of factors and r proteins.

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The paper was for the most part clear and convincing, making thorough use of existing models and data. There was one unclear section though, on page 16:
At the top of the page the authors state that the D site is unstructured because it is accessible to chemical probes, but at the bottom of the page they point out that Tsr1 and Dim1 binding sites may enclose the 3' end of 18S rRNA and might need to dissociate before Nob1 can access the D cleavage site.

We have altered the text to better explain this point. The degree of RNA accessibility needed for DMS modification is quite different to that needed for entry into the active site of an enzyme.

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It might also be helpful to clarify a little more that these binding sites might not be used simultaneously. The 43 S particles may be heterogeneous.

The referee raises an important point. We have altered the text in the results (page 6) and Discussion to make this clearer

Referee #3 (Remarks to the Author):

In the manuscript "Cracking Pre-40S Ribosome Structure by Systematic Analyses of RNA-Protein Cross-linking", Granneman et al. analyse by a recently described in vivo protein-RNA crosslinking technique pre-rRNA interaction sites of six yeast small ribosomal subunit maturation factors, all present in "late" cytoplasmic pre-40S subunits. The observed clustering of the factors pre-rRNA interaction sites in the pre-40S head/neck area is in good agreement with previous studies showing that assembly/folding events in this region correlate with efficient nuclear export of pre-40S subunits and with their final cytoplasmic maturation. The work will for sure be an important reference for future studies aiming in understanding molecular mechanisms of eukaryotic pre-40S maturation.

Suggestions and comments:

- Abstract: "We report that the binding sites for six late-acting 40S ribosome synthesis factors cluster around the 3' end of the 18S rRNA in model 3D structures." The sentence suggests that the authors identified all ("the") binding sites of these factors in pre-ribosomes. The general "binding sites" on pre-ribosomes might well be more extended than pre-rRNA interaction sites identified in this study and probably include (pre-)ribosomal protein components as indicated by earlier observed protein-protein interactions between Nob1 and rps5/rpS14, and a salt resistant proteinacous Ltv1/Enp1/rpS3 complex. I think it would be more precise to conclude that pre-rRNA interaction sites of the examined factors were identified in this study.

We have changed the abstract to make this clearer. We now state: “We report that rRNA binding sites…”

The second part of the sentence ("...cluster around the 3' end of the 18S rRNA in model 3D structures") is hard for me to follow. To my knowledge, the position of the 18S rRNA 3' end is neither exactly known in eukaryotic pre-40S subunits nor in mature 40S subunits, but suspected to be in the head/platform cleft (see figure 3F). Several of the observed interaction sites, including the beak structure, interaction site of Enp1p, and the identified Ltv1p interaction sites seem to be rather far from there. I have the impression that the factors pre-rRNA interaction sites are largely in the 3' major and 3' minor SSU rRNA secondary structure domain (all six factors have at least one of their interaction sites there), or adjacent regions including helix 28, corresponding in 3D structure models to the small ribosomal subunit head and neck region.

The reviewer raises an important point. We now state that three of the six proteins were found to cluster at the 3’ end of the 18S rRNA in 3D model structures

Abstract: "The primary binding-sites for the 18S 3'-endonuclease Nob1 are distinct from its cleavage site and were unaltered by mutation of the catalytic PIN domain" and later " ... and we conclude that structural reorganisation is needed to bring together the catalytic PIN domain and its target" For detailed discussion of these points please see the comments on the relevant results section (p.10) and on figures 4C, 4D and S3, below.

See responses below.

Abstract: ".... confirmed that the cleavage site was predominately protein free and poorly structured in the pre-rRNA" I am not sure whether the structural probing experiments allow such a semi-quantitative interpretation. They provide evidence for the existence of a population of pre-rRNA which is poorly structured in this region, but do they really allow to conclude that this is the case for the large majority of pre-40S subunits?

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We have altered the wording of the abstract to make this statement less strong. As noted above, they at least allow us conclude that this region is less structured than are flanking regions. A local stem of the type conventionally drawn for this region would be strongly predicted to confer protection against chemical modification on the RNA. In the data we can also see that the region is accessible to three different chemicals (see revised Figure 4) by reference to neighboring sequences that are clearly protected. As the reviewer indicated, we cannot state that the region is always protected. Since submission we have performed an additional experiment that is related to this question, by analyzing the structure of the D-site region in cell depleted of Nob1 (now presented as Figure 4 and discussed in the text). This showed no alteration in protection, consistent with the conclusions that 1) the region is accessible and 2) that this does not represent the primary Nob1 binding site.

- Introduction (p.3): "This reorganization requires phosphorylation of the pre-40S proteins Enp1 and Ltv1 by the kinase Hrr25, and subsequent dephosphorylation". Later, in Figure legend of Figure 3 it is stated that "Hrr25-dependent phosphorylation of Rps3 and subsequent dephosphorylation triggers a structural change leading to repositioning of Rps3". If I understand the work referred to correctly (Schafer et al.), it suggested that major targets of Hrr25 dependent in vitro phosphorylation include Enp1, Ltv1 and rpS3. Whether these ones are the only one in small ribosomal subunit precursors, or just major ones, and, more importantly, whether Hrr25 mediated phosphorylation of one of them or of all three is in vivo crucial for small ribosomal subunit maturation, was not addressed to my knowledge.

We have altered the sentence on page 3, to which the reviewer refers, to make it clearer. We now state: “This reorganization involves phosphorylation of Enp1 and Ltv1 by the kinase Hrr25, and subsequent dephosphorylation of Rps3”.

Introduction (p.4): "Here we report the binding sites..." see above, eventually more precise : pre-rRNA interaction sites

We have corrected this on page 4 in the introduction section

- Results (p. 6): "Enp1 and Ltv1 bind sequences that form the beak structure" Why Ltv1? The pre-rRNA interaction sites of Ltv1p, helix 41 and helix 16 are not part of the beak structure.

We have rephrased this in the results section on page 6.

- Results (p. 6): "However, in the absence of any data on the binding sites of Ltv1 and Enp1, it was difficult to assess what, if any, direct roles they played in beak formation." It was shown that Ltv1 and Enp1 can be isolated from yeast cell extracts in a salt resistant complex with rpS3, strongly arguing that rpS3, located at the base of the beak, is a proteinacous component of the Enp1p/Ltv1p binding site in pre-ribosomes.

We have removed this sentence from page 6.

- Results (p. 7): "H16 and H41A are in close proximity to the beak and to Rps3"
I think the localisation of these helices is described in the discussion in a more adequate way: "Ltv1 binds sequences in H41, which are located close to the beak, but also binds H16, which is more distantly located in the shoulder region of the 40S particle "

We have rephrased this sentence in the results section on page 6

Results (p. 7): ".. consistent with 2-hybrid interactions reported between Rps3 and Ltv1 (Ito et al, 2001; Loar et al, 2004)" If I am not wrong, Loar et al. observed two hybrid interactions between Ltv1 and Yar1, and between rpS3 and Yar1, not between rpS3 and Ltv1.

The reviewer is correct and we have removed the Loar et al reference from this sentence.

Results (p.10): "The region surrounding cleavage site D is predominately open and not associated with Nob1 in pre-40S ribosomes" see abstract.... (I am not sure whether the structural probing

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experiments allow such a semi-quantitative interpretation. They provide evidence for the existence of a population of pre-rRNA which is poorly structured in this region, but do they really allow to conclude that this is the case for the large majority of pre-40S subunits ?)

We have softened our interpretation of the structural probing throughout the MS. We now state that our results demonstrate that the D-site region appears more flexible compared to other helices in the 3' minor domain. Also, as noted above, our Nob1 depletion experiment strongly suggests that the D-site region is not bound by Nob1 at steady state.

Results (p.10): "If correct, this model would predict that the active site of Nob1 would not be involved in H40 binding ...." and following paragraph. See below, discussion of Figures 4C, 4D and S3 for comments on this. The most clear argument for the Nob1 PIN domain not being involved in interactions with H40 would be to study a Nob1p variant lacking the PIN domain. The PIN domain mutant used here, was suggested by Pertschy et al. to be catalytically inactive, not necessarily meaning that the PIN domains capability to interact with RNA is diminished.

We have softened the statement in the text on page 9 (previously page 10) to make this clearer.

Results (p.11): "however, these data and recent in vitro analyses (Lamanna and Karbstein) do not support this stem structure " Didn't these authors also show in vivo analyses, similar to the ones presented here (Figure S3 in Lamanna et al.) ?

We have reworded the text to state that: "these data and recent *in vivo* and *in vitro* data". Our data extend on the Lamana and Karbstein data in that we also demonstrate the flexibility of the D-site stem in purified pre-40S particles. The results of our *in vivo* analysis differ from those reported by Lamana and Karbstein, since were unable to reproduce the (minor) difference in site D protection that they reported in the presence and absence of Nob1. We now briefly discuss these points in the text.

Discussion (p. 12): "A striking finding was that cross-linking sites for five of the late-acting 40S synthesis factors Rio2, Tsr1, Dim1, Nob1 (this work) and Prp43 (Bohnsack et al, 2009) are located in close proximity to functionally important sequence elements in the 3' region of the 18S rRNA." It is not completely clear what the authors mean with "3' region". Is it used in the sense of the ensemble of 3' major and minor domains in the 2D map (equivalent to head / neck regions in the 3D structure), or in the sense of the area in the 3D structure close to the 3' end of 18S rRNA? See also above.

We have rephrased this in the results section on page 11 (previously page 12). We now state that these factors are located in close proximity to functionally important regions.

Discussion (p. 13): "... and pre-40S ribosomes that lack Rps15 fail to efficiently incorporate Rio2 and are not exported to the cytoplasm (Leger-Silvestre et al, 2004; Zemp et al, 2009)"
More precisely, the data cited and other published data argue that pre-40S ribosomes are exported to the cytoplasm in the absence of rpS15 assembly, but that nuclear export is significantly delayed.

We have rephrased this in the discussion section (p12).

Discussion (p. 14) and Figure 3B: "If Ltv1 binds both sequences simultaneously...." and following. The meaning of figure 3B, entitled "Ltv1 corresponds to the extra density near the head and the shoulder in the head domain" remains unclear to me, since, obviously, the resolution of the EM density map of Ltv1-TAP purified pre-ribosomes used in the figure is rather limited. The density attributed in the EM map to Ltv1p is relatively far from the Ltv1-p pre-rRNA interaction sites described in this work (marked yellow in Figure 3B) and does not really correspond to a prolonged tubular structure with 3nm in diameter and 9nm in length. It seems at the moment more appropriate to discuss in a more open way possible reasons for the observed Ltv1p - pre-rRNA crosslink patterns, including the option that two copies of Ltv1 interact with pre-ribosomes independently of each other at two different sites. A high flexibility in the head - body orientation in pre-40S subunits could also well explain the observed interaction sites (and could eventually also mask in part a defined beak structure in 3D reconstructions of pre-40S subunits). Clearly, more structural data, characterizing the shape of Ltv1 or further defining the architecture of defined pre-ribosomal

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intermediates, would be required to clarify these points.

We have discussed these conclusions at length with Bettina Böttcher, who performed the cryoEM analyses referred to and confirmed our estimate of the dimensions of the extra density observed. We have made more explicit the possibility of alternative explanations but we believe this to be the most likely interpretation.

Discussion (p.15): "Dissociation of each of these proteins from the pre-40S particles would therefore be required for translation to commence." I am not sure whether the observed pre-rRNA interaction patterns of Dim1p, Tsr1p, and Rio2p are sufficient to conclude that binding of these factors and functional interactions of translation factors/tRNA are mutually exclusive. Arguing against this conclusion is, that several of the biogenesis factors were seen to interact with the same pre-rRNA regions, for example Dim1p, Tsr1p and Nob1p with helix 28.

In the translating ribosome, the tRNAs and translation factors occupy very precisely constrained 3-dimensional spaces, with little freedom of movement. The binding sites of these ribosome synthesis factors appear incompatible with translation.

Figure 1: It is unclear to me how many times a certain region was finally sequenced, since some of the peaks, marked with one helix, appear broad, others as one line. In case of Ltv1p, it remains thereby obscure, how the ratio of hits in the region of helix 41 versus helix 16 is. The exact numbers should be indicated.

We were unclear exactly how the referee envisaged that the exact numbers would be presented. We could divide the pre-rRNA into a series of bins and display total hit numbers within each bin, but this would make the figure very busy. Helix 41 is much longer than helix 16 and this region appears to be better protected from RNase digestion, explaining the broader peak. To allow the reader to see the exact number, we have now included the multiple sequence alignments of our CRAC data as Supplementary Tables 4 – 9. These tables also show secondary structure information and should make our interpretation of the results easier to follow.

Figure 3: I have the impression that, instead of showing many small sub-figures, showing a reasonably large version of a recent (pseudo-)atomic resolution model of the eukaryotic small ribosomal subunit (eventually from two perspectives) in which all the main rRNA interaction sites of the analysed factors are highlighted by different colours, would be more helpful.

The problem is that a (pseudo-)atomic resolution model of the eukaryotic small ribosomal subunit could only show a surface projection with little possibility of seeing any molecular interactions or distances. The complexity of the 40S subunit means that details cannot be seen within the whole structure at the molecular level – there are always too many layers of structure behind and/or in front of any region of interest for this to been presented clearly. In addition, the actual picture will become very busy.

Figure 3B: see above. (comments on discussion section)

See above.

Fig. 3C : In the original work of Schäfer et al. it was reported that incubation of pre-ribosomes with ATP leads to dissociation of rpS3, Enp1p and Ltv1p in one complex from pre-ribosomes under certain buffer conditions, while the model In Fig. 3C suggests selective dissociation of Enp1p and Ltv1p (in a heterodimeric complex) upon kinase action. What is the argument for that? I understood the data Schäfer et al. In the sense that ATP incubation of pre-40S subunits led in a Hrr25p dependent way to a new pre-ribosomal conformation, in which the Ltv1-Enp1-rpS3 hetero-trimer was loosely associated. Subsequent dephosphorylation by non-specific phosphatases induced another reorganisation in which a population of rpS3 was more stably assembled, not Ltv1p and Enp1p.
See also above for discussion of the proposed tubular structure of Ltv1p.

See above for response to discussion of the proposed tubular structure of Ltv1p.

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Figure 5: rpS14, whose C-terminus is predicted to interact with the 3' minor domain (near Tsr1/Dim1 interaction sites) is not mentioned here. Nob1p was shown to interact in vitro with both rpS14 and rpS5 (Lamanna et al., 2009), interactions which are not mentioned here. rpS0 is thought to bind in the head-body hinge (neck) region, making contact with both 3' major domain rRNA and helix 26, an interaction site of Tsr1. That might be an information worth to include. There seems to be a striking parallel in the observed interaction sites of factors associated with late cytoplasmic pre-40S subunits and the known 3D localisation of ribosomal protein components whose in vivo depletion leads to a gradual delay in pre-ribosomal nuclear export and to a significant cytoplasmic accumulation of immature subunits with 3' extended 18S rRNA (rpS2, rpS3, rpS15 and rpS20, rpS0 and the C-terminus of rpS14, for which the localisation in mature ribosomes is known). That might be worth to discuss.
As indicated in case of Enp1p and Ltv1p for rpS3, some of the factors functions might be related to an event leading to stabilised incorporation/assembly of one or the other ribosomal protein into pre-40S subunits.

We have now included rpS14 and rpS5 in the revised Figure 5. We also noticed that the cross-linking sites appear to be in close proximity to ribosomal proteins. We now mention this in the Discussion

Figure 4C, D, S3 and Materials and Methods: The experimental procedure used for structural in vitro probing by DMS of the region surrounding site D is not described. Does the section describing immuno-purification experiments refer to how HTP-Nob1 was purified? If yes, again, the exact procedure remains unclear. It is also unclear if HTP-Nob1 and HTP-Nob1 D15N were co-expressed with endogenous Nob1p in a wildtype (BY4741) strain background? Were they plasmid encoded (if yes which plasmids were used?) and under the control of which promoter? Does the N-terminal HTP-Tag lead to a pre-rRNA processing phenotype, as indicated by the apparent elevated level of 20S-pre-rRNA in the HTP-Nob1 strain as seen in figure S3 C (I assume that in lane 1 and lane 2 the input samples are loaded, the description of figure S3C is not completely clear in this regard) ? If HTP-Nob1 D15N was co-expressed with endogenous Nob1p, Nob1p-D15N hetero-oligomerisation with wildtype Nob1p might result in
partially stabilised pre-rRNA interactions, making the interpretation of the described experiments more difficult. In that case, it would be preferable to do the experiments in a strain in which wildtype Nob1p can be in vivo depleted. Would one not expect dominant negative effects on pre-rRNA processing and cellular growth through co-expression of HTP-Nob1 D15N variant if its essential function in D-site cleavage, not its interaction with pre-ribosomes, is inhibited? It would be helpful to include in the experiment shown in Figure S3C the HTP-Nob1 D15N variant (in a strain in which endogenous Nob1p was depleted). Like this, quantitative differences in the stability of pre-rRNA association of HTP-Nob1 versus HTP-Nob1 D15N could be revealed.

We have included further details of the experimental procedures in the revised supplementary materials. We tested the Nob1 mutant in a strain with conditional expression of endogenous Nob1, but the quality of the cross-linking data were poor, possibly due to the impaired growth and ribosome synthesis under non-permissive growth conditions. The Nob1D15N mutation is indeed dominant negative, even when wild-type Nob1 is expressed at high levels (GAL-Nob1 strain). These results are consistent with our conclusions, and we now mention this in the revised text (p9) and in Supplementary Figure 4.

Materials and Methods: strain construction is not clear. Oligos and template plasmid used to amplify DNA fragments with which strain BY4741 was transformed should be indicated. Did the strains show growth phenotypes? Were eventual pre-rRNA processing phenotypes of the strains analysed? These important informations should be included. A northern blot experiment with total RNA from the different strains and a wildtype loaded, similar to the one shown in Fig. S3C, would be helpful to judge these points.

We have included further details of the experimental procedures in the revised supplementary materials. We added two supplementary tables, one containing a list of the oligonucleotides used and one containing a strain list. None of the tagged strains showed any growth defects, however, the C-terminally tagged Nob1 showed a modest 20S pre-rRNA processing defect. The N-terminally tagged Nob1 does efficiently process 20S and cross-linked to the same site as Nob1-HTP. We now

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mention this explicitly and show the data in the new Supplementary Figure 3.

2nd Editorial Decision 12 April 2010

Thank you for sending us your revised manuscript. Our original referees have now seen it again, and you will be pleased to learn that in their view you have addressed their criticisms in a satisfactory manner, and that the paper will therefore be publishable in The EMBO Journal.

Before this will happen, however, I was wondering whether you would like to consider addressing the minor issue suggested by referee 1 (modification of the title for figure 3B; see below). Please let us have a suitably amended manuscript via e-mail as soon as possible. We will upload it for you, and I will then formally accept the manuscript.

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1 (Remarks to the Author):

The authors have adequately addressed my concerns.

Referee #2 (Remarks to the Author):

My concerns have been adequately addressed in this revision.

Referee #3 (Remarks to the Author):

In the revised version of manuscript EMBOJ-2009-73291R several issues were adressed to further improve the manuscripts quality. Apart from this, the statement in the title of Figure 3B: "Ltv1 corresponds to the extra density near the head and the shoulder in the head domain" still lacks accurate experimental proof. Therefore, rephrasing of the title of Fig. 3B seems recommendable.

2nd Revision - authors' response 14 April 2010

The comment is fair enough. I have inserted "may" into the title, which I think should satisfy the referee.

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