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Nmd3 is a structural mimic of eIF5A, and activates the cpGTPase Lsg1 during 60S ribosome biogenesis

Andrey G Malyutin, Sharmishtha Musalgaonkar, Stephanie Patchett, Joachim Frank and Arlen Johnson

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Transaction Report:

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

1st Editorial Decision 07 December 2016

Thank you for submitting your manuscript for consideration by the EMBO Journal. It has now been seen by three referees whose comments are shown below.

As you will see from the reports, all referees express interest in the findings reported in your manuscript although they also point to a number of technical and presentational concerns that you will have to address before they can support publication of this study in The EMBO Journal.

For the revised manuscript I would particularly ask you to focus your efforts on the following points:

-> Please elaborate on the GTPase assays as requested by refs #1 and #2. This relates both to a more detailed description of the experimental methods and to the need for additional experimental work

-> Please also provide more insight on the basis for particle sorting and classification as outlined by refs #2 and #3.

-> Finally, you will notice that ref #3 is not convinced about the proposed ability of Nmd3 to mimic the binding behaviour of eIF5a. Since this is a key observation in the manuscript I would ask you to carefully discuss this issue and - if possible - to add more experimental data in support of this model.

-> In addition to these major points all three referees list a number of minor concerns - mainly

related to text changes and presentations - that will need to be amended.

REFEREE REPORTS

Referee #1:

In this study, the authors present data regarding the mode of binding of late biogenesis factors to the 60S subunit. The structural organization of pre-ribosomal particles is still an important aspect in ribosome field, and according to my view, the structure determination of a 60S subunit with reconstituted biogenesis factors is interesting. Thus, I find this work suitable for publication in the EMBO Journal.

Specific points that need to be addressed

line 102
A recent cryo-EM study of a nucleoplasmic pre-60S particle containing the AAA+ ATPase Rea1 is should be described in the introduction and cited (Bario-Garcia C et al. 2016, NSMB).

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The authors nicely show the dependency of 60S subunits and Nmd3 for the activation of the cpGTPase Lsg1. Nevertheless, Lsg1 harbors the conserved N346 residue within its G1 motif (Ash et al., 2012, FEBS Letters) and was suggested to belong to the cation-dependent GTPases as shown for the other two cpGTPases involved in 60S ribosome biogenesis, Nug2 (Matsuo et al., 2014, Nature) and Nug1 (Manikas et al., 2016, NAR), plus RbgA (lane 144). In case of Nug1 the GTPase activity was shown to be stimulated with increasing concentrations of KCl with an optimal hydrolysis rate at about 500 mM KCl. For the GTPase assays described here a buffer containing 50 mM KOAc was used. Therefore, it should be also tested by the authors how and at which concentrations monovalent cations stimulate Lsg1 activity, both bound to 60S subunits and unbound. As different conditions (e.g. K+ conc.) were used for the GTPase assays described here for Lsg1 and for RbgA (Achila et al., 2012), the comparison of both GTPase activities should be considered with care.

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Measuring GTP hydrolysis mediated by Lsg1 might not be the appropriate assay to ensure stoichiometric binding of Nmd3 and Lsg1 to 60S subunits. Indeed, the SDS-PAGE gel provided in Figure 1D suggests sub-stoichiometric binding of MBP-TEV-(His)6-Nmd3. In general, a Supplementary Figure with an overview of the cryo-EM image processing procedure would be helpful and therefore could be provided. The presented data clearly shows stimulation of GTP hydrolysis with increasing amounts of either 60S or Nmd3 (Figure 1B, C), but the graphs shown in Figure 1B do not exhibit saturation kinetics, in contrast to what is mentioned in the text.

Minor point: Figure 1B & 1C as well as Figure legend 1: pm should be written as pM.

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Instead of 10 Lsg1 (4 Nmd3) per 60S subunit in should be written 10 Lsg1 molecules per 60S subunit.

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The formation of a stable complex between 60S-Nmd3-Lsg1 is described, but apparently the trimeric complex was not used for cryo-EM. Please explain why the 60S-Nmd3-Lsg1-Tif6 complex was instead used. Does Tif6 stabilize the interaction of Nmd3 on 60S subunits (as indicated in lane 192 and 404-405) and would it be possible that the presents of Tif6 further stimulates the GTP hydrolysis activity of Lsg1?

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To improve the particle orientation in ice, 0.5% glutaraldehyde was added to the sample. In lane 167, 0.5% w/w is indicated, whereas in material and methods (lane 573) 0.5% v/v is used. Please correct. Additionally, the material and methods section describing the assembly of the complexes for

cryo-EM is relatively short. For example, the molar ratios used for the final in vitro reconstitutions should be indicated. Furthermore, the described purifications of Lsg1, Nmd3 and Tif6 indicate that the proteins were stored in different buffers and in particular in Tris-based buffers, which would interfere with the cross-link reaction performed with glutaraldehyde. Did the authors perform a buffer exchange before the in vitro assembly and cross-link reaction? For how long and at which temperature the quench and the cross-link reaction were performed. It would be helpful to describe the sample preparation procedure in greater detail.

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Nmd3 was N-terminally fused to the large MBP tag (about 42 kDa). In their previous EM work (Sengupta et al. 2010, JCB), the authors suggested that the MBP tag is part of an extra density that interacts with the 60S subunit (close to helix 65). In this manuscript, the MBP tag is no longer mentioned. It would be helpful, if the authors could comment, whether the density of the MBP tag is/was distinct or delocalized? Moreover, does the MBP tag influence the interaction of Nmd3, Lsg1 or Tif6 with the mature 60S?

General comment
In a recent study, the cryo-EM structure of a late cytoplasmic pre-60S particle purified through Yvh1 was reported (Sarkar et al. 2016, NSMB), revealing two extra densities not found on the mature 60S subunit. Moreover, the L1 stalk was found in the closed conformation contacting one of the unidentified densities. As the previous particle also contains Nmd3 and Lsg1, it would be important to perform a careful comparison of both structures and show this in a figure. In this context, it is also important to comment on L10 and distorted helix 38 (Figure 2B, lane 271-278).

Referee #2:

This paper described the cryo-EM structure of the 60S ribosomal subunit in complex with protein factors Nmd3, Lsg1 and Tif6. The structure has been obtained to near atomic resolution (3.1 Å). It reveals important information about the function of these proteins in assisting the assembly of the 60S subunit as well as the functional interplays that may be existing between these protein factors. An important finding from this structure is that Nmd3 is a molecular mimic of eIF5A, which probes the L1 stalk, the E and P sites of the 60S subunit. The existence of a population of particles with clearly distorted H38 also revealed that Nmd3 may facilitate the incorporation of uL16 r-protein. A very interesting finding was that the interaction of Lsg1 with H69 cause the guanosine 2261 to flip out and it is proposed that this is a general activation mechanism for GTPases involved in ribosome biogenesis. I believe this work is of great quality and the presented structure contribute significantly to our understanding of the function and mechanisms of these protein factors. I think this manuscript deserves publication in EMBO J. However, the authors should first address these concerns before the paper is accepted for publication.

Major concerns:

1. The presentation and description of the GTPase assays in Fig. 1 should be improved. First, there is not enough information on the Materials and Methods section to know how the different GTPase assays in panels A to C were performed. What were the concentrations of the components of the reaction that were maintained fixed? What does '0.5pm, 1pm an 2pm' stands for? If the authors mean picomol, the correct abbreviation is pmol. It is also unclear from the description what the %GTP hydrolysis means. What is it considered 100%. How are the results normalized? Have the authors done any background subtraction to the measurements? It would be also desirable that the Y axis in panel B and C cover the same unit range for make comparison of these assays easier. Because of these deficiencies in the way the data and methods are presented, I found challenging to agree with the conclusion in lines 154-156 indicating that "Saturation with Lsg1 was achieved at approximately 10 Lsg1 per 60S subunit. A similar titration of Nmd3 at constant 60S subunit concentration and three concentrations of Lsg1 showed saturation with Nmd3 at a ratio of approximately 4 Nmd3 per 60S subunit." All these deficiencies should be corrected.

2. The authors should include additional figures (main text or Suppl. Material) describing the conformational classes obtained during the particle classifications of the 60S-Nmd3 and 60SNLT complexes. These new figures should provide a description of the observed conformations for the

L1 stalk described in lines 172-177.

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Minor concerns:

a. Line 562 correct '30deg for 10 minutes' for 30 ºC
for 10 minutes.

b. A schematic figure explaining the functional relationship and hierarchy of Nmd3, Lsg1, Tif6, Sdo and Efl1 would be helpful.

c. Imidazole is spelled incorrectly (line 521, 523, 525, etc.).

Referee #3:

The paper by Malyutin and coauthors represents the cryo-EM structure of the yeast 60S ribosomal subunit in complex with nuclear export factor Nmd3, responsible for the release of pre-mature 60S into the cytoplasm, Lsg1 GTPase responsible for dissociation of Nmd3 and protein Tif6, subunits anti-association factor (yeast analog of initiation factor 6). The authors have reconstructed the complex of 60S with all three proteins simultaneously, as well as the complex of 60S and Nmd3 protein. Extensive classification of the particles revealed different conformational states of the complexes, and also helped to better interpret the regions with lower resolution. To build the atomic model of obtained complexes authors used variety of structure prediction and alignment software and tools for model building. They additionally showed the similarity of middle domain of Nmd3 to protein eL22 and the C-terminal domain of Nmd3 to initiation factor eIF5A, therefore supporting their previous hypothesis that nuclear export factors may be structurally similar to translation factors, providing a quality control of ribosome maturation. Altogether, obtained data allowed the authors to describe in details the organization of the pre-mature 60S and, moreover to hypothesize the sequence of the last steps of 60S biogenesis.

Although, an interesting observation, from the data presented in the manuscript it is hard to agree that one of the domains of Nmd3 (which authors called eIF5A-like domain) mimics structurally the protein eIF5A. Despite some shape and sequence similarities, especially between N-terminal part of eIF5A and corresponding region of Nmd3, the region 256-400 of Nmd3 does not seem to mimic eIF5A protein. Authors also report that the hypusine carrying loop is severely truncated in Nmd3 and does not contain conservative lysine residue; the interaction with ribosomal elements are differs between two proteins; and the statistics from Dali server did not show very high similarity Z=3.5, rmsd=4.0. Taking all this into account, it is suggested to replace the word "mimic" in the title.

Additionally, it would be very informative to provide detailed cryo-EM particles classification scheme, since classification was one of the major tool of the data processing.

Minor points:
Figures 4C and 4D are visually too crowded. May be depth cueing and transparency on the map can improve them.

Authors were not consistent in using the metrics (such as 1 µl or 1 ul or 1µL) in different parts of the manuscript.

Page 20. Methods. In paragraph "Purification of 60S..." last sentence. "20µl aliquots of subunits were stored at 1µm each at -80{degree sign}C". Presumably 1µm should be changed to 1µL.

Page 20. Methods. In paragraph "Protein purification" last sentence. "...buffer supplemented with 10m Immidazole and 5mM..." correct to "...10 mM Imidazole...".

In paragraphs "Protein purification" and "6His-Kemptide-Tif6" correct "Immidazole" to "Imidazole".

In paragraph "GTPase assays" correct ".5M EDTA" to 0.5M EDTA

In paragraph "GTPase assays" in the sentence "Reactions were spiked with very small amount of [gamma -32P]-GTP". Instead of "very small amount", please put units.

Page 18. "In contrast, depletion of Lsg1 traps only Nmd3 and not Tif6 (unpublished results) and Lsg1 mutants can be bypassed..." Page 12 "...between mutant alleles of LSG1 and deletion of RPL41A and RPL41B (data not shown)". Please see EMBO journal author guide: http://emboj.embopress.org/authorguide#unpublisheddata

1st Revision - authors' response 22 December 2016

Referee #1:

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The authors nicely show the dependency of 60S subunits and Nmd3 for the activation of the cpGTPase Lsg1. Nevertheless, Lsg1 harbors the conserved N346 residue within its G1 motif (Ash et al., 2012, FEBS Letters) and was suggested to belong to the cation-dependent GTPases as shown for the other two cpGTPases involved in 60S ribosome biogenesis, Nug2 (Matsuo et al., 2014, Nature) and Nug1 (Manikas et al., 2016, NAR), plus RbgA (lane 144). In case of Nug1 the GTPase activity was shown to be stimulated with increasing concentrations of KCl with an optimal hydrolysis rate at about 500 mM KCl. For the GTPase assays described here a buffer containing 50 mM KOAc was used. Therefore, it should be also tested by the authors how and at which concentrations monovalent cations stimulate Lsg1 activity, both bound to 60S subunits and unbound. As different conditions

(e.g. K+ conc.) were used for the GTPase assays described here for Lsg1 and for RbgA (Achila et al., 2012), the comparison of both GTPase activities should be considered with care.

We had tested higher potassium concentrations with the expectation that Lsg1 would be more highly activated but we found that it was not. We have repeated those assays now with free Lsg1, Lsg1+60S and Lsg1+60S+Nmd3 and have included the results as supplemental figure S1B. We show that the 60S and Nmd3-dependent GTPase activity of Lsg1 is inhibited above 150 mM KCl and speculate that this is due to loss of binding of Lsg1.

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Measuring GTP hydrolysis mediated by Lsg1 might not be the appropriate assay to ensure stoichiometric binding of Nmd3 and Lsg1 to 60S subunits. Indeed, the SDS-PAGE gel provided in Figure 1D suggests sub-stoichiometric binding of MBP-TEV-(His)6-Nmd3. The presented data clearly shows stimulation of GTP hydrolysis with increasing amounts of either 60S or Nmd3 (Figure 1B, C), but the graphs shown in Figure 1B do not exhibit saturation kinetics, in contrast to what is mentioned in the text.

We have rewritten this section to say that GTPase activity was used as a proxy for occupancy and included a sentence about the rationale for this. Because activation of the GTPase likely requires Lsg1 to bind to a specific site, GTPase activity can be used as a proxy for occupancy of this site and will avoid problems that could arise if Lsg1 binds non-specifically to the ribosome. Indeed, we have observed superstoichiometric binding of Lsg1 to 60S subunits as measured by cosedimentation in sucrose gradients whereas Cryo-EM resolved Lsg1 bound only to the joining face at helix69. We replaced the graphs in Figure 1C with a new panel that titrates Nmd3 against 60S rather than against Lsg1, as we realized the previous plots did. The graphs for Nmd3 titrations in Fig 1C are close to saturating but we agree with the reviewers that the graphs in 1B only approach saturation. We also removed the plots for the highest concentrations of 60S as these were further from saturation than the plots shown. We have amended the text accordingly.

The gel in Fig 1D was meant only to show that Nmd3 and Lsg1 co-sediment with 60S but was not intended to show saturation. However, we have replaced the gel image with a new image that is closer to stoichiometry. It should be noted that Nmd3 runs as a doublet on SDS-PAGE. (This is not a gel artifact nor degradation.) Consequently, at stoichiometry, Nmd3 will appear to be under-loaded.

In general, a Supplementary Figure with an overview of the cryo-EM image processing procedure would be helpful and therefore could be provided.

We agree and have now provided figures describing our classification and refinement strategies. These are added as Supplemental Figures S3-8

Minor point: Figure 1B & 1C as well as Figure legend 1: pm should be written as pM.

We have corrected this.

line 154 & 156
Instead of 10 Lsg1 (4 Nmd3) per 60S subunit in should be written 10 Lsg1 molecules per 60S subunit.

We have corrected this.

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The formation of a stable complex between 60S-Nmd3-Lsg1 is described, but apparently the trimeric complex was not used for cryo-EM. Please explain why the 60S-Nmd3-Lsg1-Tif6 complex was instead used. Does Tif6 stabilize the interaction of Nmd3 on 60S subunits (as indicated in lane 192 and 404-405) and would it be possible that the presents of Tif6 further stimulates the GTP hydrolysis activity of Lsg1?

We have rewritten the results section describing the complexes we analyzed. As described in the text, in the absence of Tif6, we observed density attributed to Nmd3 projecting toward the binding site for Tif6. This prompted us to add Tif6 into our complexes. The addition of Tif6 did appear to stabilize the N-terminal domain of Nmd3, as it was only in the presence of Tif6 that we could resolve the N-terminus. However, we have not assayed the stabilizing effect of Tif6 explicitly. We have, however, tested if Tif6 alters the Nmd3-dependent activation of Lsg1 and found only slight stimulation on Lsg1 GTPase activity with Tif6. This is now included in line 228-229 and as Supplemental Figure 1C.

line 167 & line 570-575
To improve the particle orientation in ice, 0.5% glutaraldehyde was added to the sample. In lane 167, 0.5% w/w is indicated, whereas in material and methods (lane 573) 0.5% v/v is used. Please correct. Additionally, the material and methods section describing the assembly of the complexes for cryo-EM is relatively short. For example, the molar ratios used for the final in vitro reconstitutions should be indicated. Furthermore, the described purifications of Lsg1, Nmd3 and Tif6 indicate that the proteins were stored in different buffers and in particular in Tris-based buffers, which would interfere with the cross-link reaction performed with glutaraldehyde. Did the authors perform a buffer exchange before the in vitro assembly and cross-link reaction? For how long and at which temperature the quench and the cross-link reaction were performed. It would be helpful to describe the sample preparation procedure in greater detail.

We have changed this to 0.5% w/v, which is what was consistently used. This reviewer is correct in pointing out that our proteins were stored in Tris buffers. However, the final concentration of Tris in the reactions for assembly of complexes was quite low due to dilution of the proteins and was at least an order of magnitude below the concentration of glutaraldehyde. The procedure that we used is now more completely described in Materials.

line 223 & line 402
Nmd3 was N-terminally fused to the large MBP tag (about 42 kDa). In their previous EM work (Sengupta et al. 2010, JCB), the authors suggested that the MBP tag is part of an extra density that interacts with the 60S subunit (close to helix 65). In this manuscript, the MBP tag is no longer mentioned. It would be helpful, if the authors could comment, whether the density of the MBP tag is/was distinct or delocalized? Moreover, does the MBP tag influence the interaction of Nmd3, Lsg1 or Tif6 with the mature 60S?

We have added a sentence in the text noting that we did not detect MBP in our reconstructions of Nmd3-containing particles. (see lines 200-201) Presumably MBP does not adopt a discrete position on the 60S subunit. We speculate that the tag may partially destabilize the interaction between Nmd3 and Tif6, accounting for the poorer resolution of the extreme N-terminus of Nmd3.

General comment
In a recent study, the cryo-EM structure of a late cytoplasmic pre-60S particle purified through Yvh1 was reported (Sarkar et al. 2016, NSMB), revealing two extra densities not found on the mature 60S subunit. Moreover, the L1 stalk was found in the closed conformation contacting one of the unidentified densities. As the previous particle also contains Nmd3 and Lsg1, it would be important to perform a careful comparison of both structures and show this in a figure. In this context, it is also important to comment on L10 and distorted helix 38 (Figure 2B, lane 271-278).

We appreciate this comment. The Yvh1-60S particle from Ed Hurt's lab was published after we initially submitted this work. In the Yvh1-particle, unassigned densities that are attributed to Nmd3 and Lsg1 match perfectly the density we have assigned to Nmd3. This nicely validates our reconstructed particle as reflecting a native position of Nmd3. We cite and discuss this work in our revised manuscript (lines 216-220 and 425-427).

Referee #2:

This paper described the cryo-EM structure of the 60S ribosomal subunit in complex with protein factors Nmd3, Lsg1 and Tif6. The structure has been obtained to near atomic resolution (3.1 Å). It

reveals important information about the function of these proteins in assisting the assembly of the 60S subunit as well as the functional interplays that may be existing between these protein factors. An important finding from this structure is that Nmd3 is a molecular mimic of eIF5A, which probes the L1 stalk, the E and P sites of the 60S subunit. The existence of a population of particles with clearly distorted H38 also revealed that Nmd3 may facilitate the incorporation of uL16 r-protein. A very interesting finding was that the interaction of Lsg1 with H69 cause the guanosine 2261 to flip out and it is proposed that this is a general activation mechanism for GTPases involved in ribosome biogenesis. I believe this work is of great quality and the presented structure contribute significantly to our understanding of the function and mechanisms of these protein factors. I think this manuscript deserves publication in EMBO J. However, the authors should first address these concerns before the paper is accepted for publication.

Major concerns:

1. The presentation and description of the GTPase assays in Fig. 1 should be improved. First, there is not enough information on the Materials and Methods section to know how the different GTPase assays in panels A to C were performed. What were the concentrations of the components of the reaction that were maintained fixed? What does '0.5pm, 1pm an 2pm' stands for? If the authors mean picomol, the correct abbreviation is pmol. It is also unclear from the description what the %GTP hydrolysis means. What is it considered 100%. How are the results normalized? Have the authors done any background subtraction to the measurements? It would be also desirable that the Y axis in panel B and C cover the same unit range for make comparison of these assays easier. Because of these deficiencies in the way the data and methods are presented, I found challenging to agree with the conclusion in lines 154-156 indicating that "Saturation with Lsg1 was achieved at approximately 10 Lsg1 per 60S subunit. A similar titration of Nmd3 at constant 60S subunit concentration and three concentrations of Lsg1 showed saturation with Nmd3 at a ratio of approximately 4 Nmd3 per 60S subunit." All these deficiencies should be corrected.

We have revised the description of our GTPase assays to provide the necessary details. A more complete description of the assay is given in the Materials section and specific molarities of components of the reactions are given in each figure's respective figure legend. We have now explained that percent GTP hydrolysis was calculated as (free phosphate/$_{total}$phosphate)*100. 100% hydrolysis would result when all GTP was hydrolyzed to free phosphate. We have now explicitly stated that non-enzymatic background signals were subtracted from all data points.

The curves in our new version of Figure 1C show saturation of GTPase activity with increasing Nmd3 concentration at 60S:Nmd3 =1:4 for both the curves (reactions containing 25nM and 50nM 60S). We agree that in Figure 1B, curves only approach saturation and we have corrected this in the manuscript. We used this nearly saturating stoichiometry of components from these assays to prepare complexes with maximum occupancy of 60S for cryo-EM imaging.

2. The authors should include additional figures (main text or Suppl. Material) describing the conformational classes obtained during the particle classifications of the 60S-Nmd3 and 60SNLT complexes. These new figures should provide a description of the observed conformations for the L1 stalk described in lines 172-177.

As noted above, we have rewritten the results section that described classification and refinement. We have also added in Supplemental Figures describing these strategies (see Figures S3-S8 and Table S1).

3. I am concerned with the practice of the authors to combine multiple datasets of the 60S-Nmd3 and 60S-Nmd3-Lsg1-Tif6 complexes into a single data set to further increase the quality of the density for Nmd3. The authors are obtaining these structures at ~ 3 Å resolution. Most likely at this resolution there are conformational differences between the two complexes. Analysis of these conformational differences should be informative on the function of these factors and the distinct conformational differences that each one of them induce in the ribosomal particle. Did the authors do this analysis? Were they able to conclude anything? It would be useful if the authors could provide additional explanation/justification for the approach taken.

The decision to combine the data sets was made due to lack of any homology models for Nmd3, poor existing in silico generated models, and inability to assign the sequence based on the density at the time. The thought was that combining the datasets and focusing only on Nmd3 would potentially boost the resolution of Nmd3 density sufficiently enough to allow us to start building the atomic model. It was obvious that the particles in the combined dataset would lack Lsg1 and/or Tif6 and so the reconstruction would not be suitable for conformational analysis.

Regarding the conformational differences within each complex, these were examined during processing of the individual complexes. The major conformational changes that we observed and described in the manuscript are of L1 stalk/Nmd3 movement and helix 38 movement. We observed additional changes in the positions of Lsg1 and the N-terminal domain of Nmd3. However, most of these classes were sparsely populated, producing relatively poor densities and consequently were not pursued in the current work. Please see supporting Figures S3 through S8 and table S3 for additional information on processing and conformational states.

4. Lsg1 has a typical GTPase domain that has all the essential motifs. In these enzymes switch1 (G1 motif) typically acquires a different conformation in presence and absence of GTP and upon interaction with effectors. In the presented structure, switch1 probably senses the conformation of H69 via base G2261. The activation of GTP hydrolysis require orientation of the catalytic residue, which can then activate by attacking the catalytic water. This water molecule must come from the gamma phosphate in the GTP. The proposed mechanism of GTPase activation does not fulfill this condition. H69 may acquire a specific conformation that might help in catalysis but its role in activation of GTPase activity is not properly justified by the structure. The RNA part that is involve in catalysis should be at a position close to the analogous catalytic Histidine residue proposed for RbgA [(Nucleic Acids Res. 2013 Mar 1;41(5):3217-27)] since Lsg1 and RbgA are close homologues. The authors should discuss this concern.

In our suggestion for how G2261 stimulates the GTPase activity of Lsg1 we did not mean to imply that G2261 contributes directly to the catalytic site. Rather, we suggested that G2261 interacts with amino acids that are adjacent to what is classically considered Switch I. We suggested that this interaction indirectly stabilizes Switch I. We have rewritten this section to clarify this proposed mechanism. To avoid misinterpretation, we also removed the sentence saying that G2261 acts in a way that is loosely analogous to how an adenosine of the SRL activates translational GTPases.

Minor concerns:

a. Line 562 correct '30deg for 10 minutes' for 30 ºC
for 10 minutes.

This has been corrected.

b. A schematic figure explaining the functional relationship and hierarchy of Nmd3, Lsg1, Tif6, Sdo and Efl1 would be helpful.

We have now included a model figure (Fig 6) showing how we envision the binding of Nmd3 and Sdo1 to be coordinated.

c. Imidazole is spelled incorrectly (line 521, 523, 525, etc.).

This has been corrected.

Referee #3:

The paper by Malyutin and coauthors represents the cryo-EM structure of the yeast 60S ribosomal subunit in complex with nuclear export factor Nmd3, responsible for the release of pre-mature 60S into the cytoplasm, Lsg1 GTPase responsible for dissociation of Nmd3 and protein Tif6, subunits

anti-association factor (yeast analog of initiation factor 6). The authors have reconstructed the complex of 60S with all three proteins simultaneously, as well as the complex of 60S and Nmd3 protein. Extensive classification of the particles revealed different conformational states of the complexes, and also helped to better interpret the regions with lower resolution. To build the atomic model of obtained complexes authors used variety of structure prediction and alignment software and tools for model building. They additionally showed the similarity of middle domain of Nmd3 to protein eL22 and the C-terminal domain of Nmd3 to initiation factor eIF5A, therefore supporting their previous hypothesis that nuclear export factors may be structurally similar to translation factors, providing a quality control of ribosome maturation. Altogether, obtained data allowed the authors to describe in details the organization of the pre-mature 60S and, moreover to hypothesize the sequence of the last steps of 60S biogenesis.

Although, an interesting observation, from the data presented in the manuscript it is hard to agree that one of the domains of Nmd3 (which authors called eIF5A-like domain) mimics structurally the protein eIF5A. Despite some shape and sequence similarities, especially between N-terminal part of eIF5A and corresponding region of Nmd3, the region 256-400 of Nmd3 does not seem to mimic eIF5A protein. Authors also report that the hypusine carrying loop is severely truncated in Nmd3 and does not contain conservative lysine residue; the interaction with ribosomal elements are differs between two proteins; and the statistics from Dali server did not show very high similarity Z=3.5, rmsd=4.0. Taking all this into account, it is suggested to replace the word "mimic" in the title.

We feel that the case for a domain of Nmd3 being an eIF5A mimic is quite strong and our preference is to retain this word in the title. The two proteins are homologous in structure: topology of the eIF5A domain matches eIF5A and we describe limited sequence identity. In addition, the way in which the two proteins bind to the ribosome is analogous: the position of eIF5A and the eIF5A-domain of Nmd3 occupy the same position in the 60S subunit, they both induce closure of the L1 stalk and they both interact with the same surfaces of uL1 and eL42. We have revised Fig 3C, showing side-by-side comparison of Nmd3 and eIF5A bound to the ribosome. In addition, we have included Supplemental Movie1 to better illustrate their similarity.

Additionally, it would be very informative to provide detailed cryo-EM particles classification scheme, since classification was one of the major tool of the data processing.

We agree and, as noted above, we have now provided this in Supplemental data.

Minor points:
Figures 4C and 4D are visually too crowded. May be depth cueing and transparency on the map can improve them.

We have reworked these panels, as suggested.

Authors were not consistent in using the metrics (such as 1 µl or 1 ul or 1µL) in different parts of the manuscript.
Corrected

Page 20. Methods. In paragraph "Purification of 60S..." last sentence. "20µl aliquots of subunits were stored at 1µm each at -80{degree sign}C". Presumably 1µm should be changed to 1µL.
Corrected to read 1uM.

Page 20. Methods. In paragraph "Protein purification" last sentence. "...buffer supplemented with 10m Immidazole and 5mM..." correct to "...10 mM Imidazole...".
Corrected

In paragraphs "Protein purification" and "6His-Kemptide-Tif6" correct "Immidazole" to "Imidazole".
Corrected

In paragraph "GTPase assays" correct ".5M EDTA" to 0.5M EDTA
Corrected

In paragraph "GTPase assays" in the sentence "Reactions were spiked with very small amount of [gamma -32P]-GTP". Instead of "very small amount", please put units.
We have modified the text to read "we spiked the reactions with approximately 10^5 cpm of [gamma -32P]-GTP."

Page 18. "In contrast, depletion of Lsg1 traps only Nmd3 and not Tif6 (unpublished results) and Lsg1 mutants can be bypassed..." Page 12 "...between mutant alleles of LSG1 and deletion of RPL41A and RPL41B (data not shown)". Please see EMBO journal author guide: http://emboj.embopress.org/authorguide#unpublisheddata

We have now added additional experiments previously referred to as data not shown as Supplemental data:
Fig S11, showing the lack of activation of Lsg1 by Rpl41 and lack of genetic interaction between RPL41 and LSG1.
Fig S12, showing that depletion of Lsg1 inhibits the nuclear recycling of Nmd3 but not Tif6.

2nd Editorial Decision 16 January 2017

Thank you for submitting a revised version of your manuscript. It has now been seen by two of the original referees whose comments are shown below. As you will see they both find that all criticisms have been sufficiently addressed and recommend the manuscript for publication. However, before we can go on to officially accept the manuscript there are a few editorial issues concerning text and figures that I need you to address in a final revision:

-> Please include a brief conflict of interest statement.

-> Please change the label 'online materials' in the manuscript to 'Materials and Methods'

-> Please provide a brief legend for the movie EV1 (this can be uploaded as a separate text file). In addition, could I ask you to change the labeling and call-outs for this to movie EV1 throughout the manuscript (in accordance with our author guidelines)?

3rd Editorial Decision 18 January 2017

Thank you for submitting the final revision, I am pleased to inform you that your manuscript has now been officially accepted for publication in the EMBO Journal.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Arlen Johnson
Journal Submitted to: EMBO Journal
Manuscript Number: EMBOJ-2016-96012

Reporting Checklist For Life Sciences Articles (Rev. July 2015)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the information can be located. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable).

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	All GTPase assays in Figures 1 and S1 were done in triplicate and mean and standard deviation are shown. The assays in Figure S10A were from a single repicate but at multiple concentrations and the negative results showed a common trend.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	Not applicable
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	Not applicable
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	Not applicable
For animal studies, include a statement about randomization even if no randomization was used.	Not applicable
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	Not applicable
4.b. For animal studies, include a statement about blinding even if no blinding was done	Not applicable
5. For every figure, are statistical tests justified as appropriate?	Yes. Two-tailed T-test was used in Fig S1C to establish P-value.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Not applicable
Is there an estimate of variation within each group of data?	Not applicable
Is the variance similar between the groups that are being statistically compared?	Not applicable

C- Reagents

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
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http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
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http://www.ebi.ac.uk/ega

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http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
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6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	Not applicable
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Not applicable

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	No animal models were used
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	No animal models were used
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	No animal models were used

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	No human subjects were used
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	No human subjects were used
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	No human subjects were used
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	No human subjects were used
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	No human subjects were used
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	No human subjects were used
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	No human subjects were used

F- Data Accessibility

18. Provide accession codes for deposited data. See author guidelines, under 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	Structures were deposited under codes EMDB-8368 and EMDB-8362 and PDB 5T6R and 5T62, respectively.
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	Not applicable
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21. As far as possible, primary and referenced data should be formally cited in a Data Availability section. Please state whether you have included this section. Examples: **Primary Data** Wetmore KM, Deutschbauer AM, Price MN, Arkin AP (2012). Comparison of gene expression and mutant fitness in Shewanella oneidensis MR-1. Gene Expression Omnibus GSE39462 **Referenced Data** Huang J, Brown AF, Lei M (2012). Crystal structure of the TRBD domain of TERT and the CR4/5 of TR. Protein Data Bank 4O26 AP-MS analysis of human histone deacetylase interactions in CEM-T cells (2013). PRIDE PXD000208	Not applicable
22. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	Not applicable

G- Dual use research of concern

23. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	Not applicable