THE EMBO JOURNAL

Manuscript EMBO-2010-75520

The SUMO system controls nucleolar partitioning of a novel mammalian ribosome biogenesis complex

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Review timeline:

Submission date:	30 July 2010
Editorial Decision:	15 September 2010
Revision received:	17 December 2010
Editorial Decision:	17 January 2011
Revision received:	20 January 2011
Accepted:	21 January 2011

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 14 September 2010

Thank you for submitting your manuscript for consideration by The EMBO Journal, and again I am very sorry for the unusual delay with its evaluation. We still have not received any comments from referee 3, despite multiple reminders sent by our office and an initial acknowledgement that the comments would arrive shortly. Upon close study of the two reports at hand, I have therefore decided to forward them to you at this stage together with a preliminary decision, in order to avoid further loss of time. Based on the two reports we received so far, this decision is however not a fully straightforward one. As the referees acknowledge, your identification of mammalian orthologs of the Rix complex and Rea1 does in principle provide an important advance from the ribosome biogenesis side, pending more complete characterization of these factors and their roles; at the same time however the referees point out that from the SUMO side, the study does currently not offer major new insights regarding the functional significance of PELP1 sumolylation or on how SENP3 and SUMO regulate ribosome biogenesis.

My preliminary conclusion (still subject to change should the missing third report come in and bring up serious additional concerns) is therefore that we should be able to consider a revised version further for publication, but only if you will be able to substantially improve these criticized aspects. Importantly, it will be essential to further address the relevance of PELP1 sumoylation for ribosome biogenesis by at least investigating the role of PELP1 SIMs vs. direct sumoylation (SIM/sumoylation site mutagenesis) for PELP1 partitioning, but also by testing whether such alterations would also directly impact on ribosome biogenesis similar to PELP1 knockdown (e.g. through RNAi-rescue experiments with non-sumoylatable or SIM-mutated PELP1). In addition, adding the more detailed characterization of the Rix complex orthologs as requested by referee 1, and adequately addressing the various more specific points of both reviewers will also be important to make this study a more suitable candidate for an EMBO J paper. I realize that this may likely

require quite some additional time and effort and would also understand if you decided to publish the study with minor changes elsewhere, but should you be able to improve and extend it along the lines discussed above, then please resubmit a revised manuscript using the link below. Before doing so, please however briefly consult with us on whether there may be any news from the outstanding third reviewer; in turn I will communicate any comments we may still get (and their possible consequences for the final decision) as soon as possible.

I should add that it is EMBO Journal policy to allow a single round of revision only, and that it is thus essential that you completely answer the points raised if you wish the manuscript ultimately to be accepted. When preparing your letter of response, please also bear in mind that this will form part of the Peer Review Process File, and will therefore be available online to the community in the case of publication (for more details on our Transparent Editorial Process initiative, please visit our website: http://www.nature.com/emboj/about/process.html). Finally, please also briefly specify the individual author contributions, either in the acknowledgements section or in an adjacent separate section, as we are attempting to adopt this as a common policy now. In any case, please do not hesitate to get back to us should you need feedback on any issue regarding your revision.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1 (Remarks to the Author):

The de-sumoylating enzyme SENP3 has been previously described the Müller and Dasso laboratories to be involved in the biogenesis of ribosomal subunits. However, whether this is a direct effect of de-sumolyation or due to an indirect effect of depleted cellular SUMO pool has remained unclear. Exploiting a biochemical approach, here Müller and co-workers have uncovered a new interaction of the de-sumoylating enzyme SENP3 with a sub-complex of factors (PELP1, TEX10 and WDR18) that are directly involved in the maturation of the large subunit of the ribosome (60S) at multiple steps. Further, they show that shuttling of PELP1 between the nucleolus and nucleoplasm requires SUMO 2 modification and SENP3 de-sumoylation. These findings suggest that the effect of SENP3 on 60S maturation is direct. The manuscript clearly describes a new interaction that is of interest to both the SUMO and ribosome biogenesis field. However, their findings need to be strengthened by performing additional controls and biochemical/cell-biology experiments.

Figure 1 & 2: The association of SENP3 with PELP1-WDR18-TEX10, the mammalian forms of Rix1-Ipi1-Ipi3, is clearly important and novel. Since this is most important finding in the manuscript, the authors need to clarify better characterize the interaction.
1. Is SENP3 associated with 60S pre-ribosomes? Or is it part of only the free PELP1-WDR18-TEX10 sub-complex? Sucrose or glycerol gradient analysis of the isolated complex followed by Western analysis can be performed to address this issue.
2. Is the interaction with the complex or stability of the complex RNAseA dependent?
3. How does the complex behave upon siRNA treatment of other known pre-60S associated factors?
Finally, it would be important to place the SENP3 IP and the above experiments as Figure 1 (and not the in Supplementary information), since this is really the most novel aspect of the manuscript.

Figure 4: The blots in 4B and S3 don't exactly support the statement "loss of PELP1 results in significantly lower amounts of WDR18". It could be that loss of WDR18 leads to dramatic decrease in PELP1 levels, but deletion of PELP1 does not result in similar changes in WDR18 levels. This issue needs to be discussed.
Figure 4E: Specificity of the L27 nucleolar accumulation: Is L27-GFP also nucleolar enriched in siSENP3, siSUMO 1 and siSUMO 2 treated cells? Would a small subunit reporter construct (for eg. either S2-GFP or S3-GFP) mislocalised in siPELP1, siTEX10, siWDR18 and siSENP3 treated cells.

Figure 6: PELP1 localization is controlled through SUMO2/3. But whether it's controlled by the

modification or because of possible PELP1 SIM - SUMO2 interactions is unclear to me. Figure 6 could be also explained by localization of PELP1 into the compartment where it forms the most SIM-SUMO2 interactions. In order to clarify this, either the sumoylation deficient PELP1 (possibly form in which all lysines are mutated if the mutation of single lysines has turned out unsuccessful) or SIM-lacking version of PELP1 should be checked in localization experiments.

Do siSUMO 1 treated cells also mislocalize PELP1?

Is PELP1 still bound to pre60S ribosomes in siSUMO2 and siSENP3 treated cells? Sucrose gradient analysis followed by Western analysis could be performed to assess this.

Additionally, monitoring the localization of the other components of the complex and importantly few other components of the 60S biogenesis pathway will improve the quality of the manuscript (by tagging if antibodies against endogenous proteins don't work). Especially studying the localization of the other components under different PELP1 (PELP1 deletion, PELP1 SIM deletion and non-sumoylatable PELP1) and sumoylation (siRNA-SENP3, siRNA-SUMO) conditions will provide additional information about the complex formation and dynamics. Is the distribution of other nucleolar/nucleoplasmic pre60S biogenesis factors affected in siSUMO2 and siSENP3 cells?

Discussion:
The discussion can definitely be shortened with emphasis on SENP3 interactions. In particular, the "cancer "aspect which appears rather speculative can be excluded (also from the abstract).

In summary, the manuscript is interesting and the findings made by the authors are novel and important to both the SUMO and ribosome field. However, it would be important to provide controls and additional experimental data to add specificity and strength to their findings.

Referee #2 (Remarks to the Author):

In their manuscript, Finkbeiner et al. extend previous studies of SUMO specific protease SENP3 in rRNA processing by identifying SENP3 associated proteins and investigating their functions in ribosome biogenesis. They identify PELP1, TEX10 and WDR18 as SENP3 associated proteins and identify these proteins as orthologs of the yeast Rix1-Ipi1-Ipi3 complex. Using RNAi knockdown, the authors show that, similar to the yeast complex, PELP1, TEX10 and WDR18 are involved in 28S rRNA maturation and localization of Rpl27, part of the large ribosomal subunit. Knockdown of MDN1, a homolog of yeast Rea1, is also shown to reduce rRNA processing and Rpl27 export from the nucleolus. Identification of human proteins that function similarly to the yeast Rix1-Ipi1-Ipi3 complex in ribosome biogenesis represents an important finding. In addition to these studies, the authors also present data that PELP1 can be SUMOylated and show that the distribution of PELP1 in the nucleoplasm vs. the nucleolus is regulated by the SUMOylation pathway. While the localization data are convincing, the functional significance of PELP1 modification by SUMO or non-covalent binding to SUMO are not addressed.

Suggestions to improve the manuscript:

•The possibility that SUMO modification of PELP1 regulates its nucleolar localization should be presented as one of several models consistent with the data and not overstated. Additional data to address this issue would strengthen the manuscript. Similarly, it is not clear that PELP1 is a "major" SUMO substrate in the nucleolus (p.11).

•Figure 4 presents the key data showing function of PELP1, TEX10 and WDR18. 4C presents "Fold change of 28S/32S ratio"; what are the range of ratios observed? P values should be presented to indicate statistical significance.

•Does SENP3 RNAi regulate Rpl27 localization, as predicted?

•Data in Figure 5 support SUMOylation of PELP1, however, this largely relies on the appearance of a higher molecular weight species rather than IP/westerns. The His-SUMO-2 pull-down without SENP3 RNAi should be shown.

Additional points to consider:

•Previous studies from this lab suggested a major role for NPM1 in regulation of rRNA processing by SENP3. Is PELP1 in the SENP3-NPM1 pathway or does it act in parallel?

•Additional data to support association of MDN1 with the endogenous PELP1, TEX10, WDR18 complex would strengthen the paper.

Additional Correspondence 15 September 2010

Thank you for your response to my preliminary decision. Just today, we did now receive the outstanding third report on your manuscript, which I am forwarding to you copied below. Overall, this reviewer raises similar concerns as the first two while also acknowledging the potential importance of your findings, thus reenforcing our initial editorial conclusion that major revision work would be required for the study to become a good candidate for publication in The EMBO Journal. I can therefore confirm my preliminary decision of yesterday, asking you to also incorporate referee 3's concerns and comments during your revision work and when preparing the revised manuscript. As mentioned before, should there be any questions relating to this revision then please don't hesitate to get back to me.

Yours sincerely,

REFEREE REPORT

Referee 3 (comments to the authors):

In the manuscript, "Nucleolar partitioning of PELP1- a regulator of ribosome biogenesis-is controlled by the SUMO system", the role of specific proteins in ribosome biogenesis in a metazoan system. The authors identify a protein complex using affinity chromatography and pulldowns that contains SENP3, PELP1, TEX10 and WDR18. This complex shares some similarities with a yeast complex that has been implicated in ribosome biogenesis. PELP1 was (at least partially) localized to the nucleolus and the authors suggest that rRNA processing and L27 localization are altered upon depletion of the SEN3P-associated proteins. Radioactive experiments suggest that PELP1 is modified by SUMO and the authors suggest that SENP3 is involved in removal of SUMO. The authors propose that modification of PELP3 is involved in compartmentalization of the protein.

The authors have some convincing evidence for the presence of the proposed complex, but their discussion and additional work lead to some unresolved and interesting questions. Throughout the manuscript, many other factors that interact with PELP3 and SENP3 are mentioned. Why are these not seen in the proteomic experiments? How would the authors predict that the complexes would change if the catalytically inactive form of SENP3 was used? (This allele is mentioned but is not referenced and there is no data to show that it blocks SUMO removal from a given substrate). Some follow up experiments along these lines would strengthen the manuscript.

The authors also convincing show nuclear localization of some of these proteins and changes in the localization under different conditions. What is not so convincing demonstrated is the change in ribosome biogenesis. The changes that the authors claim do not seem to be supported by the gel in Fig. 4B. On page 8, it is stated that the amount of mature 28S rRNA to precursor ratio is decreased by 70% (ie it is at 30% as compared to controls) and this can not be comfirmed from the experiment as presented. The gel is not of high enough quality and the changes appear to be marginal and the distinction between the top and middle panel are not clearly discussed. What was used as a loading control or for normalization? This is a major weakness of the manuscript.

While this work could offer some insight into a area where there is very little data, regulation of ribosome biogenesis in higher eukaryotes, the conclusions are not supported strongly enough by the

presented data to be convincing.

1st Revision - authors' response 17 December 2010

In the following I will give a point-by-point answer on the issues raised by the referees:

Referee #1

The referee stated that the "manuscript clearly describes an new interaction that is of interest to both the SUMO and ribosome biogenesis field". At the same time he/she also mentions that our findings need to be strengthened by performing additional controls and biochemical/ cell biological experiments. We thank the referee for his/her constructive suggestions and accordingly have performed a series of experiments, as outlined in detail below.

Specific points:

Figure 1 & 2: The association of SENP3 with PELP1-WDR18-TEX10, the mammalian forms of Rix1-Ipi1-Ipi3, is clearly important and novel. Since this is most important finding in the manuscript, the authors need to clarify better characterize the interaction.

1. Is SENP3 associated with 60S pre-ribosomes? Or is it part of only the free PELP1-WDR18-TEX10 sub-complex? Sucrose or glycerol gradient analysis of the isolated complex followed by Western analysis can be performed to address this issue.

To address this question we have performed density gradient centrifugations as suggested by the referee. Pre-ribosomal particles were isolated from cell nuclei by sucrose gradient centrifugation (new Figure 4E and Supplementary Figure 9). Fractions of sucrose gradients were collected and the presence of BOP1, a component of the PES1-BOP1-WDR12 (Pe-BoW) rRNA processing complex (Holzel et al., 2005; Lapik et al., 2004), served as a marker for pre-60S complexes (new Figure 4E, fractions 10-13). This analysis revealed that endogenous PELP1 and WDR18 perfectly co-fractionate with BOP1 demonstrating that both proteins are associated with pre-60S particles. A significant fraction of SENP3 also cosediments with PELP1, WDR18 and BOP1. These findings, which were included on page 10 of the MS, thus strongly suggest that SENP3 interacts with the PELP1-TEX10-WDR18 complex at pre-60S particles.

2. Is the interaction with the complex or stability of the complex RNAseA dependent?

To address this point we performed co-immunoprecitation experiments from control or RNase treated cell extracts. As shown in new Supplementary Figure S3A removal of RNA does not affect the interaction of PELP1 and WDR18.

3. How does the complex behave upon siRNA treatment of other known pre-60S associated factors?

To see whether knock-down of other pre-60 associated factors affects the complex we depleted cells from PES1, a component of the 60S-associated PeBoW complex (see above).
As shown in new Supplementary Figure 12, when compared to control we did not observe an altered binding of PELP1 to WDR18 upon loss of PES1.

Finally, it would be important to place the SENP3 IP and the above experiments as Figure 1 (and not the in Supplementary information), since this is really the most novel aspect of the manuscript.

We appreciate the positive evaluation of this data, but due to the addition of several other figures and space limitations we would like to keep this figure in the Supplementary part.

Figure 4: The blots in 4B and S3 don't exactly support the statement "loss of PELP1 results

in significantly lower amounts of WDR18". It could be that loss of WDR18 leads to dramatic decrease in PELP1 levels, but deletion of PELP1 does not result in similar changes in WDR18 levels. This issue needs to be discussed.

We agree with the referee that loss of WDR18 does affect the levels of PELP1 more strongly than *vice versa* (Figure 4B bottom panels and Supplementary Figure 3B). However, comparing the amount of WDR18 in PELP1 depleted cells and control cells shows a reproducible reduction of WDR18 levels.

Figure 4E: Specificity of the L27 nucleolar accumulation: Is L27-GFP also nucleolar enriched in siSENP3, siSUMO 1 and siSUMO 2 treated cells?

We have now included an experiment where we monitored YFP-L27 (we erroneously stated that this was a GFP tagged construct) upon depletion of SENP3. As can be seen in Figure 4F depletion of SENP3 also leads to the nucleolar accumulation of the YFP-L27 reporter and a complete loss of cytoplasmic or nucleoplasmic staining. Interestingly, the nucleolar shape is changed in a subset of SENP3 depleted cells. Note that compared to experiments, where delocalization of PELP1 or other components was monitored upon depletion of SENP3, the YFP-L27 assays were performed at a later time point after SENP3 depletion. The deformation of the nucleoli is thus as late event occurring after SENP3 depletion.

Would a small subunit reporter construct (for eg. either S2-GFP or S3-GFP) be mislocalised in siPELP1, siTEX10, siWDR18 and siSENP3 treated cells.

The results from the sucrose gradient fractionation (new Figure 4E) make it unlikely that PELP1-TEX10-WDR18 are involved in the 40S ribosome biogenesis pathway. By contrast, the presence of SENP3 on 40S particles points to a role of SENP3 in the 40S maturation pathway. Accordingly, we have previously described an interaction of SENP3 with proteins of the small ribosomal subunit (Haindl et al., 2008). However, we feel that in the context of PELP1-TEX10-WDR18 it is beyond the scope of this manuscript to further elaborate on this point.

Figure 6: PELP1 localization is controlled through SUMO2/3. But whether it's controlled by the modification or because of possible PELP1 SIM - SUMO2 interactions is unclear to me. Figure 6 could be also explained by localization of PELP1 into the compartment where it forms the most SIM-SUMO2 interactions. In order to clarify this, either the sumoylation deficient PELP1 (possibly form in which all lysines are mutated if the mutation of single lysines has turned out unsuccessful) or SIM-lacking version of PELP1 should be checked in localization experiments.

We agree that at the initial stage our model was largely based on the simple correlation between the enhanced SUMO modification of PELP1 and its nucleolar delocalization upon depletion of SENP3. To strengthen this point and to determine whether SUMO-modification of PELP1 directly mediates its subnuclear partitioning we mimicked constitutive modification by linearly fusing SUMO2 to the C-terminus of PELP1 (new Figure 6E). This approach of fusing SUMO linearly to the target protein has been used in several cases to dissect the functional outcome of SUMO modification. Importantly, in contrast to PELP1WT, which accumulated in the nucleolus, the PELP1-SUMO2 fusion protein exhibited a nucleoplasmic distribution and was largely excluded from the nucleolus (new Figure 6E). Moreover, PELP1-associated factors, such as LAS1L and SENP3, were also released from the nucleolus in the presence of PELP1-SUMO2 (new Figure 6F). The SIM-deficient PELP1-SUMO2 fusion exhibits a similar localization suggesting that the covalent attachment of SUMO to PELP1 is sufficient to determine its sub-nuclear distribution (Supplementary Figure 13A). These data thus indicate that SUMO modification of PELP1 directly affects its nucleolar partitioning and the compartmentalization of PELP1-associated proteins.
To further examine whether PELP1 is the only critical target in this process we first mapped lysine 826 as the major SUMO attachment site in PELP1 (new Figure 5D and Supplemenntary Figure 10C). We next expressed PELP1K826R, where this site has been replaced by an arginine, in control cells and SENP3 depleted cells. Notably, like wild-type PELP1, PELP1K826R (as well as the SUMO-binding deficient mutant (PELP1IV790/1AA,VI880/1AA) is

also excluded from the nucleolus upon depletion of SENP3 (new Supplementary Figure 13B). We therefore hypothesized that this may result from the modification of an additional SENP3-sensitive SUMO2/3 substrate that is associated with PELP1. In support of this idea in vitro and in vivo experiments demonstrated that LAS1L is modified by SUMO (new Figure 7A, B). Moreover, the modification of endogenous LAS1L by SUMO2 is drastically induced upon depletion of SENP3 (new Figure 7 B, C). We therefore suggest that enhanced sumoylation of LAS1L accounts for the nucleolar exclusion of the non-sumoylatable variant of PELP1 in SENP3-depleted cells. We thus favour a model, in which SENP3 controls nucleolar partitioning by desumoylating multiple components of this complex.
In summary, we now show that the constitutive modification of one component of the complex, i.e. PELP1, affects the sub-nuclear partitioning of other members. This scenario also explains why removal of the SUMO site on a single component is not sufficient to interfere with SENP3-controlled sub-nuclear distribution.

Do siSUMO 1 treated cells also mislocalize PELP1?

We have now added this data in Figure 6D, lower panels. As can be seen depletion of SUMO1 does not affect the localization of PELP1.

Is the distribution of other nucleolar/nucleoplasmic pre60S biogenesis factors affected in siSUMO2 and siSENP3 cells?

To address this point we monitored the localization of PES1, a component of the 60Sassociated PeBoW complex, and WDR50, the human ortholog of the SSU component Utp18, upon depletion of SENP3. In both cases we did not observe any alterations in their distribution (new Figure 6C and Supplementary Figure 11A). Accordingly, we did not observe modification of these proteins by SUMO (unpublished data).

Is PELP1 still bound to pre60S ribosomes in siSUMO2 and siSENP3 treated cells? Sucrose gradient analysis followed by Western analysis could be performed to assess this.

We agree with the referee that it is very attractive to speculate that the balanced SUMO modification assures the timely association of PELP1 and its binding partners with 60S preribosomal particles. We hypothesize that sumoylation acts as a signal to release the PELP1-TEX10-WDR18 complex from these structures. Loss of SENP3 would therefore prevent binding to the pre-60S particles or induce a premature release from these structures. We have now discussed this scenario in the discussion section.
However, it is very tedious to experimentally address this point, in particular because one requires a large number of siRNA treated cells. We therefore aim to approach this question in the future after having established cells were SUMO forms are stable down-regulated by an shRNA construct.

Additionally, monitoring the localization of the other components of the complex and importantly few other components of the 60S biogenesis pathway will improve the quality of the manuscript (by tagging if antibodies against endogenous proteins don't work).
Especially studying the localization of the other components under different ... sumoylation (siRNA-SENP3, siRNA-SUMO) conditions will provide additional information about the complex formation and dynamics.

We agree with the referee that monitoring other components of the complex in control cells as well as SENP3 depleted cells is a critical issue. While our initial experiments were hampered by the fact that suitable antibodies were not available, we meanwhile generated an antibody directed against TEX10 to localize TEX10 and used a new commercially available antibody directed against MDN1 to localize MDN1. Importantly, both MDN1 and TEX10 are found in the nucleolus in normal cells, but are largely excluded from these structures in cells depleted from SENP3 (new Figure 6C, new Supplementary Figure 7B, C, Supplementary Figure 11B). As mentioned above, this demonstrates that SENP3 not only controls the partitioning of PELP1, but also of its binding partners. To further strengthen this point we have now added new data on Las1L, which we identified as another SENP3/PELP1-associated protein (Supplementary Figures 1A and new Supplementary Figure 4D). Las1L was only very

recently described as a critical nucleolar factor of 28S rRNA maturation and ribosome biogenesis (Castle et al., 2010). We now show that Las1L is also excluded from the nucleolus upon loss of SENP3 (new Figure 6C). As outlined in detail below, we also identified Las1L as a SENP3-sensitive target of SUMO2 and propose that its modification contributes to the regulation of sub-nuclear partitioning.

Taken together, our new localization data on MDN1, TEX10 and Las1L clearly indicate that SENP3 not only controls the partitioning of PELP1.

Especially studying the localization of the other components under different PELP1 (PELP1 deletion, PELP1 SIM deletion and non-sumoylatable PELP1)....

As described above, PELP1-associated factors, such as LAS1L and SENP3, were also released from the nucleolus in the presence of PELP1-SUMO2 (new Figure 6F). The SIMdeficient PELP1-SUMO2 fusion exhibits a similar localization suggesting that the covalent attachment of SUMO to PELP1 is sufficient to determine its sub-nuclear distribution (Supplementary Figure 13A).

Discussion:

The discussion can definitely be shortened with emphasis on SENP3 interactions. In particular, the "cancer "aspect which appears rather speculative can be excluded (also from the abstract).

We have excluded the cancer aspect from the abstract and considerably shortened this part in the discussion section. However, since a large part of the literature on PELP1 concentrates on this aspect, we feel that we have to briefly discuss this aspect.

Referee #2

The referee states that the “identification of human proteins that function similarly to the yeast Rix1-Ipi1-Ipi3 complex in ribosome biogenesis represents an important finding”. He/she suggested that we should provide additional data to support our model on SUMO-regulated partitioning of the complex. As described in detail below, we have now performed additional experiments, which strengthen the idea that the covalent modification of PELP1 and/or associated factors is critically involved in the control of the PELP1-TEX10-WDR18 complex.

Suggestions to improve the manuscript:

The possibility that SUMO modification of PELP1 regulates its nucleolar localization should be presented as one of several models consistent with the data and not overstated. Additional data to address this issue would strengthen the manuscript.

We agree that at the initial stage our model was largely based on the simple correlation between the enhanced SUMO modification of PELP1 and its nucleolar delocalization upon depletion of SENP3. To strengthen this point and to determine whether SUMO-modification of PELP1 directly mediates its subnuclear partitioning we mimicked constitutive modification by linearly fusing SUMO2 to the C-terminus of PELP1 (new Figure 6E). This approach of fusing SUMO linearly to the target protein has been used in several cases to dissect the functional outcome of SUMO modification. Importantly, in contrast to PELP1WT, which accumulated in the nucleolus, the PELP1-SUMO2 fusion protein exhibited a nucleoplasmic distribution and was largely excluded from the nucleolus (new Figure 6E). Moreover, PELP1-associated factors, such as LAS1L and SENP3, were also released from the nucleolus in the presence of PELP1-SUMO2 (new Figure 6F). The SIM-deficient PELP1-SUMO2 fusion exhibits a similar localization suggesting that the covalent attachment of SUMO to PELP1 is sufficient to determine its sub-nuclear distribution (Supplementary Figure 13A). These data thus indicate that SUMO modification of PELP1 directly affects its nucleolar partitioning and the compartmentalization of PELP1-associated proteins.
To further examine whether PELP1 is the only critical target in this process we first mapped lysine 826 as the major SUMO attachment site in PELP1 (new Figure 5D, Supplementary

Figure 10C). We next expressed PELP1K826R, where this site has been replaced by an arginine, in control cells and SENP3 depleted cells. Notably, like wild-type PELP1, PELP1K826R (as well as the SUMO-binding deficient mutant (PELP1IV790/1AA,VI880/1AA) is also excluded from the nucleolus upon depletion of SENP3 (new Supplementary Figure 13B). We therefore hypothesized that this may result from the modification of an additional SENP3-sensitive SUMO2/3 substrate that is associated with PELP1. In support of this idea in vitro and in vivo experiments demonstrated that LAS1L is modified by SUMO (new Figure 7A, B). Moreover, the modification of endogenous LAS1L by SUMO2 is drastically induced upon depletion of SENP3 (new Figure 7 B, C). We therefore suggest that enhanced sumoylation of LAS1L accounts for the nucleolar exclusion of the non-sumoylatable variant of PELP1 in SENP3-depleted cells. We thus favour a model, in which SENP3 controls nucleolar partitioning by desumoylating multiple components of this complex.
In summary, we now show that the constitutive modification of one component of the complex, i.e. PELP1, affects the sub-nuclear partitioning of other members. This scenario also explains why removal of the SUMO site on a single component is not sufficient to interfere with SENP3-controlled sub-nuclear distribution.

Similarly, it is not clear that PELP1 is a "major" SUMO substrate in the nucleolus (p.11).

We have now deleted the term “major” in this sentence. With regards to the additional data on LAS1L it is obvious that we cannot claim this any more.

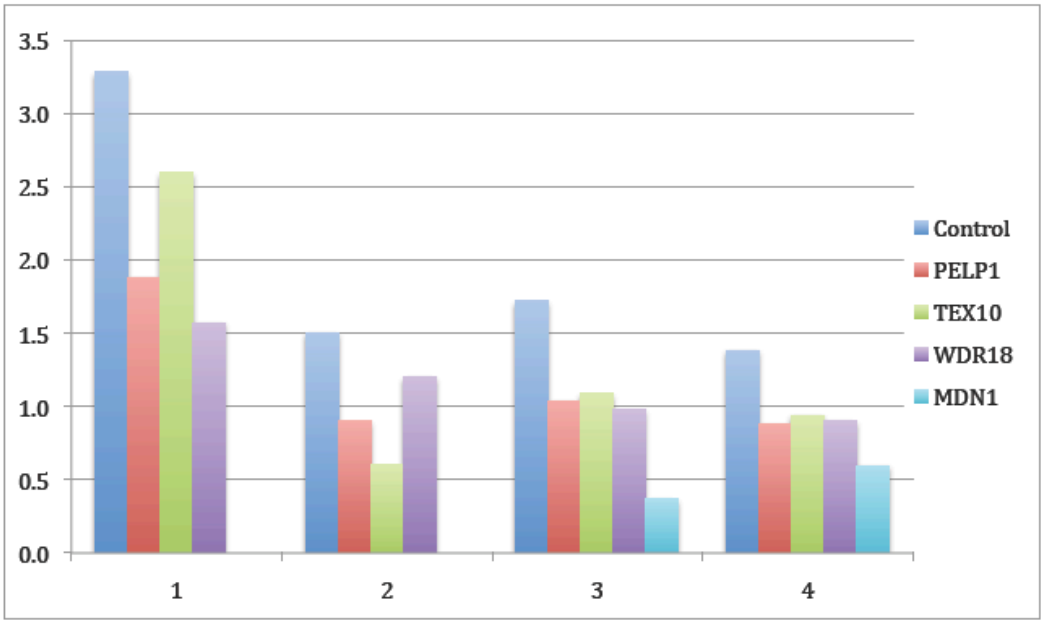
Figure 4 presents the key data showing function of PELP1, TEX10 and WDR18. 4C presents "Fold change of 28S/32S ratio"; what are the range of ratios observed? P values should be presented to indicate statistical significance.

Figure 4B shows one representative experiment from a series of four experiments. Cumulative quantitative data for all experiments is given in Figure 4C. The data are presented as relative changes in relation to the control cells, where the ratio was set to 1. To show that the data are highly reproducible and statistically significant we provide here the raw data of the four experiments. As can be seen in all individual experiments the 28S/32S rRNA ratio is higher in control cells than in cells depleted from either PELP1, TEX10, WDR18, or MDN1. Noteworthy, in the first experiment the chase time was extended to 4h thus explaining that the conversion of the 32S to the 28S rRNA was more complete leading to a higher general ratio thus not allowing p value calculations with all four experiments. If we just include experiments 2,3 and 4 in p value calculations by Student´s t-test we obtain statistically very significant data (see Extra Figure below).

It is also worth noting that our conclusion of a critical involvement of PELP1-TEX10-WDR18 in ribosome biogenesis is not only based on the rRNA processing assay. We additionally used the YFP-RpL27 reporter system to more directly monitor how depletion of PELP1, TEX10, WDR18 or MDN1 affects ribosome maturation (Figure 4F). The results from this assay clearly support the idea that a loss of either protein causes a severe defect in 60S ribosome maturation. Finally, the new data from sucrose gradient fractionation (new Figure 4E) demonstrate the association of PELP1 and WDR18 with pre-60S ribosomal particles thus further underscoring their function in ribosome biogenesis.

Raw data from 4 independent rRNA in vivo labelling experiments:

Raw data Experiment	1	2	3	4	P value	Significance
Control	3.3	1.5	1.7	1.4		
PELP1	1.9	0.9	1.0	0.9	0.0031	**
TEX10	2.6	0.6	1.1	0.9	0.017	*
WDR18	1.6	1.2	1.0	0.9	0.016	*
MDN1			0.4	0.6		



Does SENP3 RNAi regulate Rpl27 localization, as predicted?

We have now included an experiment where we monitored YFP-L27 (we erroneously stated that this was a GFP tagged construct) upon depletion of SENP3. As can be seen in Figure 4F depletion of SENP3 also leads to the nucleolar accumulation of the YFP-L27 reporter and a complete loss of cytoplasmic or nucleoplasmic staining. Interestingly, the nucleolar shape is changed in a subset of SENP3 depleted cells. Note that compared to experiments, where delocalization of PELP1 or other components was monitored upon depletion of SENP3, the YFP-L27 assays were performed at a later time point after SENP3 depletion. The deformation of the nucleoli is thus as late event occurring after SENP3 depletion.

Data in Figure 5 support SUMOylation of PELP1, however, this largely relies on the appearance of a higher molecular weight species rather than IP/westerns. The His-SUMO-2 pulldown without SENP3 RNAi should be shown.

We have now added this data (Figure 5C). We had initially removed this lane from the image, but have now included it.

Additional points to consider:

Previous studies from this lab suggested a major role for NPM1 in regulation of rRNA processing by SENP3. Is PELP1 in the SENP3-NPM1 pathway or does it act in parallel?

The connection of NPM1 to the PELP1-TEX10-WDR18 complex is indeed an important question and a focus of our future work. We could now also confirm that a subfraction of endogenous PELP1 interacts with endogenous NPM1 (new Supplementary Figure 6B). Interestingly, the results from the sucrose gradient fractionation show that NPM1 is present in fractions overlapping with PELP1, WDR18 and BOP1 indicating that it is also associated with pre-60S particles. However, NPM1 was also reproducibly found in particles with lower density (Figure 4E, fractions 8, 9). This may suggest that NPM1 shares overlapping functions with PELP1-TEX10-WDR18, but likely also acts independently from this complex on distinct steps of the maturation pathway.

Additional data to support association of MDN1 with the endogenous PELP1, TEX10, WDR18 complex would strengthen the paper.

We have now included an experiment, where we demonstrate the interaction of PELP1 and MDN1 at their endogenous levels of expression (Supplementary Figure 4C).

Referee #3

The referee comments "that the authors have some convincing evidence for the presence of the proposed complex, but…some follow up experiments along these lines would strengthen the manuscript. " We appreciate the referee´s comments and according to his/her suggestions have included new data to support our conclusions. We are confident that the new data confirm and strengthen the conclusion that the PELP1-TEX10-WDR18 is critically involved in the control of ribosome biogenesis. We have also added some explanatory notes for the referee to clarify some apparent misunderstandings, for example with regard to the catalytically inactive form of SENP3.

Throughout the manuscript, many other factors that interact with PELP3 and SENP3 are mentioned. Why are these not seen in the proteomic experiments?

In our initial work we principally characterized the association of SENP3 with three binding partners, PELP1, TEX10 and WDR18, which were all identified in our "pull-down" experiment using Flag-SENP3 as a bait (Supplementary Figure 1A). By mass-spectrometric analysis we could clearly attribute the major bands migrating at 160kDa, 90kDa and 40kDa to PELP1, TEX10 and WDR18. In the revised version we now include Las1L as another SENP3-binding protein, which we also found in our affinity purification approach.
The fact that MDN1 was not identified in our SENP3-"pull-down" might be due the fact that it is not a stoichiometric component of the complex and is only transiently associated with PELP1. This is in accordance with data in lower eukaryotes, where the MDN1 homolog Rea1 is also only transiently associated with the Rix1-Ipi1-Ipi3 complex.

How would the authors predict that the complexes would change if the catalytically inactive form of SENP3 was used? (This allele is mentioned but is not referenced and there is no data to show that it blocks SUMO removal from a given substrate). Some follow up experiments along these lines would strengthen the manuscript.

The catalytically inactive form of SENP3 has been extensively characterized in several publications including work by our own group (see for example Haindl et al., 2008). The bottom line is that exchange of the catalytic cysteine residue generates an inactive protease. We used this version in the initial "pull-down"-experiment because it can act as a substrate trap and thus facilitate purification of SENP3-substrates. At high expression levels it may also exert dominant negative effects and thus enhance modification of these substrates. However, this effect is quite mild and therefore siRNA-mediated depletion of endogenous SENP3 is more suitable to convincingly show that a protein is a SENP3-sensitive SUMO target. We therefore used this approach to demonstrate that SENP3 acts on PELP1 and Las1L.

The authors also convincing show nuclear localization of some of these proteins and changes in the localization under different conditions. What is not so convincing demonstrated is the change in ribosome biogenesis. The changes that the authors claim do not seem to be supported by the gel in Fig. 4B. On page 8, it is stated that the amount of mature 28S rRNA to precursor ratio is decreased by 70% (ie it is at30% as compared to controls) and this can not be comfirmed from the experiment aspresented. The gel is not of high enough quality and the changes appear to be marginal and the distinction between the top and middle panel are not clearly discussed. What was used as a loading control or for normalization? This is a major weakness of the manuscript.

First, we apologize that we may not have explained the presentation of the results in sufficient detail so that the referee could not fully capture the experimental set-up. In fact, the result of the experiment is divided in three parts. The upper panel is the autoradiography of the in vivo labelled and purified rRNA species. Because this is a pulse-chase experiment it reflects the turnover of the initial 47S rRNA in the processed versions and their intermediates.
The important thing here is to consider the ratio between the 32S and the 28S rRNA. As can be seen, in cells transfected with a control siRNA, the mature 28S rRNA species is typically more abundant than its 32S rRNA precursor. By contrast, knock-down of PELP1, WDR18,

TEX10 or MDN1 causes an accumulation of the 32S rRNA relative to the 28S form. Figure 4B shows one representative experiment from a series of four experiments. Cumulative quantitative data for all experiments is given in Figure 4C. The data are presented as relative changes in relation to the control cells, where the ratio was set to 1. To show that the data are highly reproducible and statistically significant we provide here the raw data of the four experiments. As can be seen in all individual experiments the 28S/32S rRNA ratio is higher in control cells than in cells depleted from either PELP1, TEX10, WDR18, or MDN1. Noteworthy, in the first experiment the chase time was extended to 4h thus explaining that the conversion of the 32S to the 28S rRNA was more complete leading to a higher general ratio thus not allowing p value calculations with all four experiments. If we just include experiments 2,3 and 4 in p value calculations by Student´s t-test we obtain statistically very significant data (see Extra Figure below). I would also like to stress that a reduction of the processing ratio in this range is typically observed for proteins involved in rRNA processing.

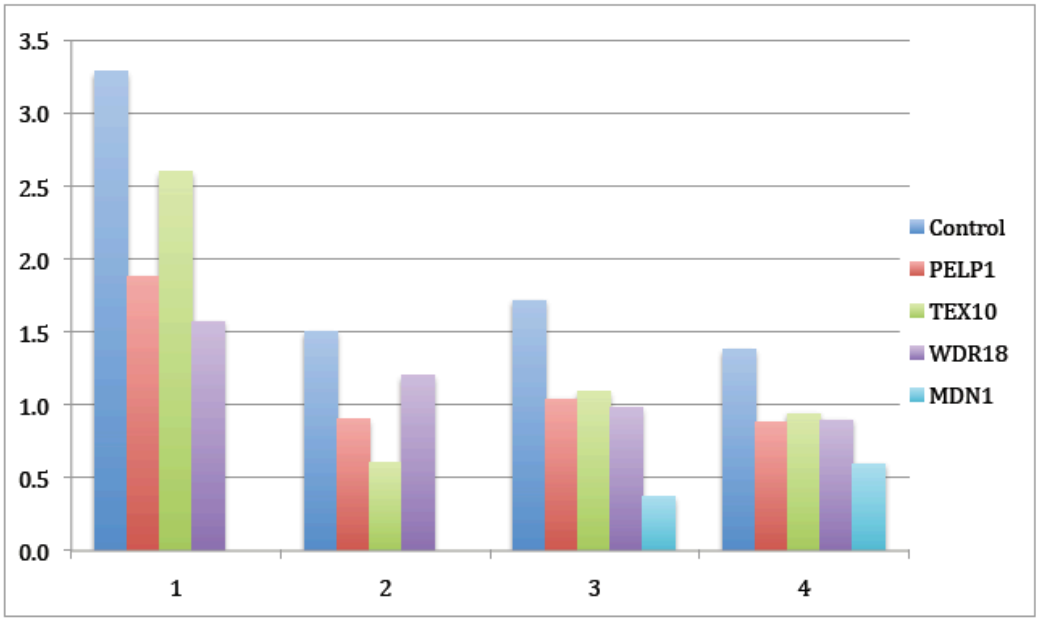
I would also like to mention that the middle panel of Figure 4B simply shows the ethidiumbromide-stained gel and is shown to demonstrate equal loading of all samples.

Finally, the three bottom panels represent Western blots in order to show efficient depletion of the respective proteins by siRNA.

It is also important to consider that our conclusion of a critical involvement of PELP1-TEX10-WDR18 in ribosome biogenesis is not only based on the rRNA processing assay. We additionally used the YFP-RpL27 reporter system to more directly monitor how depletion of PELP1, TEX10, WDR18 or MDN1 affects ribosome maturation (Figure 4F). The results from this assay clearly support the idea that a loss of either protein causes a severe defect in 60S ribosome maturation. Finally, the new data from sucrose gradient fractionation (new Figure 4E) demonstrate the association of PELP1 and WDR18 with pre-60S ribosomal particles thus further underscoring their function in ribosome biogenesis.

Raw data from 4 independent rRNA in vivo labelling experiments:

Raw data Experiment	1	2	3	4	P value	Significance
Control	3.3	1.5	1.7	1.4		
PELP1	1.9	0.9	1.0	0.9	0.0031	**
TEX10	2.6	0.6	1.1	0.9	0.017	*
WDR18	1.6	1.2	1.0	0.9	0.016	*
MDN1			0.4	0.6		



2nd Editorial Decision 17 January 2011

Thank you for submitting your revised manuscript for our consideration. It has now been assessed once more by the original reviewer 1, who considers the manuscript significantly improved in response to the initial comments and thus in principle suited for The EMBO Journal, pending some remaining minor changes to be effected through one final round of revision.

However, before we shall be able to proceed with acceptance and publication of the paper, there are some important additional issues regarding various figure panels that will need to be addressed. During our usual pre-acceptance figure check, I noted that various (mostly immunoblot) panels contain composite images assembled from splicing of lanes that are not normally adjacent to each other. This concerns not only multi-lane runs of fractionation experiments naturally requiring more than one gel (such as Figure 4E) but also various other results especially in the Supplementary Figures (e.g Fig. S1A, S4A, S5 and possibly some others where low resolution and/or potential PDF conversion artifacts make unambiguous assessment difficult, e.g. in Fig S10A). I realize that this may simply reflect removal of irrelevant in-between lanes from one and the same gel/scan/exposure - in this case it would be sufficient to provide an uncropped image of the original blot, including explanations, as a supplementary figure, and to clearly indicate the splicing through a clear black separation line between the assembled lanes, accompanied by a brief explanation in the respective figure legend. Should the assembled lanes in some cases however not stem from the same blot & exposure, I would need to kindly ask you to provide alternative figure panels in those cases - if necessary through repetition of the experiment in question - to avoid potential misrepresentation of these data.

I am requesting these changes now in order to avoid potential problems arising after publication of your manuscript, and in accordance with the instructions for image preparation below and in our Guide for Authors. Therefore, I am returning the manuscript to you once more, hoping that you will be able to provide these clarifications and a re-revised manuscript with these figures corrected as soon as possible. When preparing this final version, in order to avoid any unnecessary further delays please also
- check other panels that I have not specifically mentioned above for correct representation of original data
- carefully check all figures for their resolution and quality and the absence of possible conversion/compression artifacts
- upload all main figures as individual files (one per figure), possibly choosing a non-compressed format such as TIF or EPS to improve quality
- add an 'Author Contribution' statement in the text, to be included adjacent to the 'acknowledgements' section
- amend the text with a 'Conflict of Interest' statement at the end
- also check the figure resolution/quality in the supplementary material
- please again include a brief letter of response to the remaining referee concerns

Pending satisfactory clarification of these issues, we should then hopefully be able to proceed swiftly with acceptance and production of the paper. In any case, please do not hesitate to contact me if you need any further clarifications.

Thank you very much for your understanding.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORT

Referee #1 (Remarks to the Author):

The revised manuscript has been clearly been improved by additional experiments, which on the other hand support the earlier findings but also bring new insights into the questions, which remained open in the original manuscript. Especially the sucrose gradient and the YFP-L27 localization experiments support and strengthen nicely the previous results about PELP1-WDR18-TEX10 and SENP3 being involved in the pre-60S maturation, and representing the mammalian homologue of yeast Rix1-Ipi1-Ipi3 complex. In addition, the new experiments on PELP1 sumoylation support the idea that SUMO2-sumoylation would be the signal for altering PELP1's subnuclear localization. On the other hand, by studying an additional 60S biogenesis pathway component, LAS1L, the authors could show, that also other 60S pathway components can be subjected to sumoylation and SENP3 mediated regulation.

Few minor concerns which the authors can easily address by easy experiments and changes in the text:

1. The authors should refrain from over-stating in the abstract : "these findings are substantial for basic understanding of ribosome biogenesis....". This authors definitely report interesting findings that clearly contribute to the ribosome biogenesis field in general, but in my opinion, by no means in any why shed light on how mammalian ribosome biogenesis and the precise role of SUMO in this process.

2. In text description of Figure 4, it would appropriate to not over-state the word „significantly" in „...loss of PELP1 results in significantly lower amounts of WDR18". There is lowering in the amount of WDR18 but not significantly.

I agree, that the localization of for example S2/S3-GFP is not directly in the scope of this paper. However, it would be a valuable negative control for the nucleolar accumulation experiment especially since, as also authors comment in the response letter, according to the sucrose gradient experiment it seems unlikely that PELP1-TEX10-WDR18 would have a role in 40S biogenesis.

Figure 6: I appreciate authors' efforts in addressing the sumoylation of PELP1 further. Especially identification of LAS1L as additional sumoylation target nicely brings the findings onto more general level, thus increasing the importance of the paper.
However, I still feel that authors should mention in the text part the possibility, that also alternative sumoylation sites might exist in PELP1. (Especially, since in figure 5D, a faint band is visible in lane 3.) This could then mean, that sumoylation on these sites functions as alternative signal for re-localization of PELP1, especially in case of PELP1K826R. Related to this, it would be important to see localization of PELP1K826R in siLAS1L cells in order to support the speculation about LAS1L's ability to affect the localization of PELP1K826R.

2nd Revision - authors' response 20 January 2011

Please find enclosed the re-revised version of our manuscript entitled "*The SUMO system controls nucleolar partitioning of a novel mammalian ribosome biogenesis complex.*
We are glad to hear that our paper can in principle be accepted for publication in EMBO Journal given that we clarify the remaining points.

First, as suggested by referee #1, we have rephrased the text to clarify three minor points:

1. In the abstract section we have changed the expression "These findings are substantial for the basic understanding of mammalian ribosome biogenesis " to "These findings contribute to the basic understanding of mammalian ribosome biogenesis" to soften this statement.

2. In the result section we have deleted the word "significantly" when we describe the reduction of WDR18 levels in the absence of PELP1.

3. As suggested by the referee, we now mention that the "we cannot totally exclude residual sumoylation of PELP1K826R in the absence of SENP3" (page 15).

In addition to these changes we have now added vertical lines in composite Figures to indicate removal of irrelevant in-between lanes. To show that in all cases the respective lanes stem from one gel we provide the original scans as supplementary Figures. In each case the details of figure assembly are given in the respective figure legends.

This applies for the following Figures:
1. The original scan and explanatory notes for Figure 7C are given in Supplementary Figure 14.
2. The original scan and explanatory notes for Supplementary Figure 1A are given in Supplementary Figure 15.
3. The original scan and explanatory notes for Supplementary Figure S4 are given in Supplementary Figure 16.
4. The original scan and explanatory notes for Supplementary Figure S5 are given in Supplementary Figure 17.
5. In addition to these changes we added vertical lines in Figure 4E, where - as you already noted – we had to run two gels due to space limitations. However, I would like to stress that the gels were transferred in parallel, membranes treated identically and exposed to a single film.
6. Similarly, for the generation of Figure S10 samples had to be loaded on separate gels. But again samples are from one experiment, gels were dried together and exposed to the same X-ray film.

We regret that we did not clearly indicate removal of lanes in the initial version. I actually was not aware what the EMBO J. policy on this issue was, but for future submission I will also follow the Rossner/Yamada guidelines.

We are now confident that we could clarify the remaining issues and hope that you are able to proceed with acceptance and publication of the paper.

Acceptance Letter 21 January 2011

Thank you for submitting your re-revised manuscript and the additionally requested clarifications. I have now had a chance to review all this material, and I am happy to inform you that there are no further objections towards publication in The EMBO Journal.

You shall receive a formal letter of acceptance shortly.

