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40S subunit dissociation and proteasome-dependent RNA degradation in nonfunctional 25S rRNA decay

Kotaro Fujii, Makoto Kitabatake, Tomoko Sakata and Mutsuhito Ohno

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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 18 December 2011

Thank you for submitting your manuscript for consideration by The EMBO Journal. Let me first of all apologise for the delay in getting back to you with a decision. Unfortunately, one of the referees was not able to return his/her report as quickly as initially expected.

In the meantime, three referees have now evaluated the manuscript, and their comments are shown below. You will see that all three referees are generally very positive about the paper and would support publication after appropriate revision. Given the referees' positive recommendations, I would like to invite you to submit a revised version of the manuscript that addresses the points raised by the referees in an adequate manner. It would strengthen the paper significantly if point 1 of referee 1 could be addressed by additional experimentation. I should add that it is EMBO Journal policy to allow only a single round of revision and that acceptance of your manuscript will therefore depend on the completeness of your responses in this revised version. Please do not hesitate to get back to me at any time in case you would like to consult on any aspect of the revision in more depth.

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

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

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

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1

In this report, M. Ohno and colleagues characterize the degradation process of non-functional ribosomal 60S subunits (25S NRD). In particular, the authors nicely show that non-functional 60S subunits are selectively conjugated to ubiquitin, dissociated from the 40S subunits and degraded in a Cdc48/Ufd1/Npl4 complex and proteasome-dependent manner. In general, I found this manuscript extremely interesting and well done. In particular, the quality of data is remarkable. However, I have two major criticisms that should be raised before I can recommend this paper for publication in the EMBO Journal.

1. The authors clearly show that ribosomes with the A2451U mutation (non functional ribosomes) are selectively ubiquitylated (Figure 2B and C). In a previous paper, they show that the Mms1-Rtt101 (cullin 4) ligase is specifically responsible for this process. At this stage of knowledge, it is now essential to determine which ribosomal protein(s) are target(s) for this ligase when 25S rRNAs carry the A2451U mutation. I realize that finding the substrate for a ligase is never trivial but in this specific case where the choice is restricted to 60S ribosomal proteins, it should be feasible in a reasonable time. In addition, it would important to analyze whether non-functional ribosomes are mono or polyubiquitylated, and in this case, with which type of chains. Considering the involvement of Cdc48 and the proteasome, one would expect of course a K48-mediated chain. Preventing 25S NRD by preventing ubiquitylation of non-functional ribosomes (with an approach different that deletion of MMS1) would be the final proof of the degradation process they propose.

2. The authors should make an effort to better describe their experiments in the result section. In Figure 1B, I guess the complementation has been done in galactose. In Figure 1C-E, which strain has been used, wt or mutant? Lanes 4, 8 and 12 and more generally purification using Rpl28-Flag are not commented. The panel B of Figure 2 is not commented, reading the text and looking at the figure is not sufficient to understand that again, it corresponds to purification using Rpl28-Flag. What has been probed in in situ hybridization experiments shown in Figure 4E? Comments on Figure 5B (p15, Section on Proteasomal degradation of key..., 1st paragraph) is really unclear.

Minor question:
The author found that the 25S NRD does not strictly depend on Ufd3 but is it controled by Ubp3/Bre5 deubiquitylation complex?

Referee #2

This manuscript addresses the mechanism by which large ribosomal subunits harboring defective 25S rRNA are targeted for degradation in a process referred to as Non-functional Ribosomal Decay (NRD). This work provides evidence that defective 60S subunits are dissociated from small ribosomal subunits in a process involving Ub modification and the action of the Cdc48 complex. Subsequently, these defective subunits appear to be degraded by the proteasome. Finally, it appears that this pathway functions at low levels even in normal cells. In general, the experiments are sound and well controlled, the work interesting and important. Thus, I am enthusiastic about publication of this work in EMBO. Specific comments to improve the work are detailed below.

This review is from Roy Parker and I would be willing to clarify my comments for the authors if need be.

Specific Comments.

1) P4: lines 8-9. No-go decay is not limited to mRNA with strong structures and also acts on mRNAs with runs of rare codons, sites of depurination etc (reviewed in Chen et al., 2010, NSMB). As such, the authors might want to adjust this sentence to be broader.

2) P12: line 5. Rpt2 data reference to S2C should be S2B

3) Figure 4C and 4D -
a) It would make it clearer to the reader to explain why the erg6Δ strain is used, as not all readers will know this.
b) In Figure 4C, the 50uM of MG132, with pUbi expression, looks very similar to the DMSO control, suggesting the drug may in fact be partially acting via Ub starvation, contrary to the text in p12. I agree there is a difference at the higher concentration. However, given this data I suggest the authors either, i) soften the text somewhat, or ii) present quantitation of the northerns (decay curves with error bars), which might clarify these apparent differences.

4) Figure 4E: It would help the field to be explicit that the distributions of the defective 25S rRNAs reported in this work is different from previous reports (Cole et al., 2009) and the authors should mention why this might be.

5) Figure 4F: The 25S NRD substrate accumulation in 80S versus 60S species is not quite as clear cut as the text on p13-14 argues. This makes the interpretation that 60S subunits originate from split apart 80S ribosomes less convincing. For example, Cdc48 tet-off shows about equal enrichment in both 80S and 60S fractions, contrary to the text stating 80S enrichment (Ufd1 is certainly more convincingly in the 80S fraction). The mms1 null looks to me more enriched in the 60S, again contrary to the text stating 80S enrichment. Given this, I think a better double mutant for the epistasis experiment would involve Rpt2 and Ufd1, which is worth generating and testing if technically feasible to produce. At a minimum, the authors could make this more convincing by a) quantifying the difference distributions with error bars, and b) adjusting the text accordingly.

6) Figure 5A: It is striking that normal 60S ribosomes purify many more non-ribosomal proteins than NRD ribosomes. The work would be helped by a brief comment/speculation on why this is and what the proteins are (if known).

7) There is a discrepancy from the early experiments (where ubiquination is specific to defective ribosomes) and the later experiments (where it occurs even on wild-type ribosomes). To address this, I suggest: a) the authors be frank in the initial experiments (Figure 2), that there is a low level of ubiquination on wild-type ribosomes (referencing later part of paper), and b) clarify the differences in exposure times or procedural differences between Figure 2 and Figure 6 that cause this discrepancy.

8) It seems interesting that the mms1 null strain still shows ubiquitinated ribosomes, given this gene is described as being essential to 25S NRD. Does this imply redundancy, or an alternative Ub-ligase for normal ribosomes versus NRD ribosomes? This might be worth a short comment.

Referee #3

General Remarks:

This manuscript outlines the involvement of the proteasome and the ubiquitin binding complex Cdc48-Npl4-Ufd1 in 25S rRNA degradation. Importantly, the authors show that ribosome particles containing non-functional rRNA are selectively ubiquitinated and degraded indicating a role for proteasome-mediated quality control and turnover of 60S ribosomes. This article is well written, of

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general interest, and appropriate for publication in EMBO.

Minor Points:
Figure 4E:
This figure would be improved by choosing representative cells that are in the same phase of the cell-cycle. Furthermore, by northern blot the non-functional RNAs are already degraded at 60 minutes in wild-type cells, it would be of interest to show examples at earlier time-points. As well an MG132 treated cell should be included as a control.

Figure 4F:
The authors state that the non-functional 25S rRNA accumulate in various fractions in different mutant strains. Is the change in fractionation pattern of the 25S rRNA observed in the various mutants really due to accumulation of the 25S rRNA in different particles or is it simply due to a change in the ratio of 60S/80S particles in the mutant strains? To clarify this point the polysome profiles of each mutant should be provided in the supplemental figures.

Figure 5:
While the silver stain provides insight into the purification of the 60S using A2451U-MS2, the presence of various components of the 60S should be verified by western blot analysis.

Title:
The title at first glance indicated to me that the 40S is degraded by the NRD pathway. As such, I would suggest replacing "removal" with "dissociation" in the title.

1st Revision - authors' response 05 March 2012

Referee #1

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We agree with this comment in that it is now important to identify the ribosomal protein(s) that is ubiquitinated in 25S NRD, in order to understand the molecular mechanism by which nonfunctional ribosomes are specifically recognized by Mms1-containing E3 ligase. However, we believe that this particular point is beyond the scope of the current study, because in this manuscript, we preferred to focus on the downstream reaction after the ubiquitination step and to analyze how ubiquitinated ribosomes are degraded. Therefore, we would rather not include an analysis of the ubiquitinated ribosomal protein(s), but to leave that issue to be discussed in another paper.

In addition, it would important to analyze whether non-functional ribosomes are mono or polyubiquitylated, and in this case, with which type of chains. Considering the involvement of Cdc48 and the proteasome, one would expect of course a K48-mediated chain.

We greatly appreciate this comment. We have now added an experiment to address this issue in Fig. S3F. In this experiment, Myc–ubiquitin with a K48R or K63R mutation was expressed from a plasmid, and the ribosomal ubiquitination induced by the nonfunctional 25S rRNA expressed was examined. As shown in Fig. S3F, the ladder-like signals that are characteristic of ubiquitinated proteins were completely absent when the K48R mutant was used. However, those signals were clearly observed when the K63R mutant was used. These results indicate that the observed ubiquitination on the nonfunctional ribosomes is indeed K48-linked polyubiquitin. This is consistent with our conclusion in the main text that 25S NRD requires the Cdc48 complex and the proteasome, because these complexes generally accept this type of polyubiquitin.

Preventing 25S NRD by preventing ubiquitylation of non-functional ribosomes (with an approach different that deletion of MMS1) would be the final proof of the degradation process they propose.

An experiment similar to that suggested was already included in the original manuscript (Fig. S2B, *ufd3Δ*). In this strain, which has a ubiquitin-depleted phenotype, 25S NRD was modestly inhibited and this inhibition could be rescued by the overexpression of ubiquitin. Therefore, 25S NRD can be prevented by either the deletion of E3 ligase (Mms1) or ubiquitin depletion. We have added a statement to the main text to emphasize the significance of this result.

2. The authors should make an effort to better describe their experiments in the result section. In Figure 1B, I guess the complementation has been done in galactose. In Figure 1C-E, which strain has been used, wt or mutant? Lanes 4, 8 and 12 and more generally purification using Rpl28-Flag are not commented. The panel B of Figure 2 is not commented, reading the text and looking at the figure is not sufficient to understand that again, it corresponds to purification using Rpl28-Flag. What has been probed in in situ hybridization experiments shown in Figure 4E? Comments on Figure 5B (p15, Section on Proteasomal degradation of key..., 1st paragraph) is really unclear.

As suggested, we have modified the description of the experiments in the Results section. We have also rewritten the comments on Fig. 5 to clarify this issue. Figure S5B has been inserted to show a more accurate examination of the 3′ end of the nonfunctional 25S rRNA mutant under the Rpt2-depleted conditions.

Minor question:
The author found that the 25S NRD does not strictly depend on Ufd3 but is it controled by Ubp3/Bre5 deubiquitylation complex?

We were also interested in this point and have performed the experiments necessary to clarify it. The detailed results have already been reported in our previous paper (Fujii et al, 2009, Fig. S2). In summary, the Ubp3/Bre5 deubiquitilation complex is not relevant to 25S NRD.

Referee #2

This manuscript addresses the mechanism by which large ribosomal subunits harboring defective 25S rRNA are targeted for degradation in a process referred to as Non-functional Ribosomal Decay (NRD). This work provides evidence that defective 60S subunits are dissociated from small ribosomal subunits in a process involving Ub modification and the action of the Cdc48 complex. Subsequently, these defective subunits appear to be degraded by the proteasome. Finally, it appears that this pathway functions at low levels even in normal cells. In general, the experiments are sound and well controlled, the work interesting and important. Thus, I am enthusiastic about publication of this work in EMBO. Specific comments to improve the work are detailed below.

This review is from Roy Parker and I would be willing to clarify my comments for the authors if need be.

Specific Comments.

1) P4: lines 8-9. No-go decay is not limited to mRNA with strong structures and also acts on mRNAs with runs of rare codons, sites of depurination etc (reviewed in Chen et al., 2010, NSMB). As such, the authors might want to adjust this sentence to be broader.

The description has been improved and the suggested review is now cited.

2) P12: line 5. Rpt2 data reference to S2C should be S2B

This point has been corrected as suggested.

3) Figure 4C and 4D -
a) It would make it clearer to the reader to explain why the erg6∆ strain is used, as not all readers will know this.

We have added this information to the main text.

b) In Figure 4C, the 50uM of MG132, with pUbi expression, looks very similar to the DMSO control, suggesting the drug may in fact be partially acting via Ub starvation, contrary to the text in p12. I agree there is a difference at the higher concentration. However, given this data I suggest the authors either, i) soften the text somewhat, or ii) present quantitation of the northerns (decay curves with error bars), which might clarify these apparent differences.

We have repeated the experiments, quantified the results, and presented the data in Fig. S3D. As shown in this figure, it is now clear that even a low concentration (50 μM) of MG132 efficiently inhibits the degradation of nonfunctional 25S rRNA, regardless of whether or not ubiquitin is overexpressed. The panel for this result in Fig. 4C has been replaced with the one showing the more typical case. A Ubi-depletion effect exerted by MG132 cannot be excluded, but it seems to be too low for quantitative analysis.

4) Figure 4E: It would help the field to be explicit that the distributions of the defective 25S rRNAs reported in this work is different from previous reports (Cole et al., 2009) and the authors should mention why this might be.

When the A2451U mutant rRNA in the wild-type cell was localized by RNA–FISH, we frequently observed a few punctate signals in the proximity of the nucleus (Figs 4E and S3E), as were reported by Cole et al. and in our previous paper (Fujii et al. 2009, Fig. S4A). The punctate signals were sharper in the report of Cole et al. than in our results, presumably because of differences in the probes (Cole et al. used an LNA-based probe, whereas we used a normal oligonucleotide probe. We could not avoid a higher background signal when we used the LNA-based probe). Such punctate signals were also seen in the mutant strains, albeit less frequently. At the moment, we cannot conclude whether or not these dots are the sites of degradation of the nonfunctional rRNAs. Further careful experiments will be required in the future to clarify this issue.

5) Figure 4F: The 25S NRD substrate accumulation in 80S versus 60S species is not quite as clear cut as the text on p13-14 argues. This makes the interpretation that 60S subunits originate from split apart 80S ribosomes less convincing. For example, Cdc48 tet-off shows about equal enrichment in both 80S and 60S fractions, contrary to the text stating 80S enrichment (Ufd1 is certainly more convincingly in the 80S fraction). The mms1 null looks to me more enriched in the 60S, again contrary to the text stating 80S enrichment.
Given this, I think a better double mutant for the epistasis experiment would involve Rpt2 and Ufd1, which is worth generating and testing if technically feasible to produce. At a minimum, the authors could make this more convincing by a) quantifying the difference distributions with error bars, and b) adjusting the text accordingly.

We admit that the original results in Figure 4F were not sufficiently clear. Therefore, we have repeated the experiment and quantified the signal for each dot. The data are presented in Fig. S4A with error bars. It is now clear that the 25S mutant rRNA signals are significantly concentrated in the 60S fraction under the Rpt2 tet-off condition, whereas the signals are concentrated in the 80S fraction in the Cdc48 or *Ufd1 tet-off* strain. We have modified the main text accordingly and have replaced some panels of Fig. 4F with more typical data.

We do not know why the *mms1Δ* strain and the *mms1Δ Rpt2 tet-off* strain accumulate 25S NRD substrate equally in the 60S and 80S particles, but we do not think that this phenomenon affects our conclusion. From the result with the *mms1Δ Rpt2 tet-off* double mutant strain, it is evident that Rpt2 depletion does not inhibit 80S formation. These 80S signals are almost absent in the Rpt2-depleted condition, suggesting that the observed 60S signals in the *Rpt2 tet-off* strain derive from the 80S split. Because we observed the accumulation of the 80S signals in the Cdc48-depleted cells and

Cdc48 complex functions in various pathways upstream from the proteasome, we conclude that the nonfunctional 80S particles split in a Cdc48-dependent manner before the nonfunctional 60S particles are degraded by the proteasome. We have inserted two sentences in this paragraph to clarify this point for the reader.

The purpose of the epistasis analysis here was to analyze the effects of Rpt2 depletion on 80S formation. As discussed above, Rpt2 depletion does not inhibit the formation of the 80S particle. We have considered the possibility of making an *Rpt2 Ufd1* double tet-off mutant but have not done so for the following reason. We note that these are not temperature-sensitive strains but tet-off strains. In our experience, the growth of *Ufd1 tet-off* and *Rpt2 tet-off* strains ceases at similar times after the addition of Dox. Therefore, in a double mutant strain, the 60S degradation intermediate would accumulate to a significant extent in response to the Rpt2 tet-off effect, before the upstream factor Ufd1 is depleted, which would make interpretation difficult.

6) Figure 5A: It is striking that normal 60S ribosomes purify many more non-ribosomal proteins than NRD ribosomes. The work would be helped by a brief comment/speculation on why this is and what the proteins are (if known).

We sometimes see these nonribosomal proteins for unknown reasons. They appear not only in pW1–MS2 samples but also in pA2451U–MS2 samples. Mass spectrometric analysis has shown these nonribosomal proteins to be Ura2, Gal1, Leu2, GAPDH, etc. These proteins would be eliminated if harsh washing conditions were used. However, we preferred to use a milder buffer in this study to avoid an unintended artificial loss of protein and RNA components from the degradation intermediates in the isolation step.

7) There is a discrepancy from the early experiments (where ubiquination is specific to defective ribosomes) and the later experiments (where it occurs even on wild-type ribosomes). To address this, I suggest: a) the authors be frank in the initial experiments (Figure 2), that there is a low level of ubiquination on wild-type ribosomes (referencing later part of paper), and b) clarify the differences in exposure times or procedural differences between Figure 2 and Figure 6 that cause this discrepancy.

We have modified the descriptions for Fig. 2B and 2C and mentioned that the weak signals are also observed with wild-type ribosomes. We have mentioned this again in the comment on Fig. 6 (p. 17). In this comment, we have clarified that the signals in Fig. 2C were obtained with the MS2 pull-down, whereas the signals in Fig. 6A–D were obtained with anti-Flag immunoprecipitation.

8) It seems interesting that the mms1 null strain still shows ubiquitinated ribosomes, given this gene is described as being essential to 25S NRD. Does this imply redundancy, or an alternative Ub-ligase for normal ribosomes versus NRD ribosomes? This might be worth a short comment.

As suggested, the ubiquitination induced by H_2O_2 was clearly observed in the *mms1Δ* strain (Fig. 6A). The most straightforward interpretation of this result is that there exist multiple ubiquitin ligases that ubiquitinate damaged ribosomes. We have inserted a comment to the effect into the main text.
The ubiquitinated ribosomes observed in the *Rpt2 tet-off* cells might represent naturally generated nonfunctional ribosomes. Alternatively, they might be normal functional ribosomes stochastically selected by E3 ligase(s) for turnover. We cannot distinguish between these two possibilities, because in this case, we cannot isolate the functional and nonfunctional ribosomes. Although this is quite an interesting and important point, we must leave it to future research. In any case, it does not affect our main conclusion: ribosomes are ubiquitinated before they are degraded.

Referee #3

General Remarks:

This manuscript outlines the involvement of the proteasome and the ubiquitin binding complex Cdc48-Npl4-Ufd1 in 25S rRNA degradation. Importantly, the authors show that ribosome particles containing non-functional rRNA are selectively ubiquitinated and degraded indicating a role for proteasome-mediated quality control and turnover of 60S ribosomes. This article is well written, of

general interest, and appropriate for publication in EMBO.

Minor Points:
Figure 4E:
This figure would be improved by choosing representative cells that are in the same phase of the cell-cycle. Furthermore, by northern blot the non-functional RNAs are already degraded at 60 minutes in wild-type cells, it would be of interest to show examples at earlier time-points. As well an MG132 treated cell should be included as a control.

We have investigated the original pictures carefully and confirmed that the subcellular localization of nonfunctional 25S rRNAs is quite similar in all phases of the cell cycle. The signals are distributed in the cytoplasm in all cases examined. We have selected a clear and typical image for each case.

The reason that we used 4 h shut-off cells in Fig. 4E is because we generally isolate and analyze the nonfunctional 60S subunits from a strain grown under these conditions. It is important to confirm the cytoplasmic localization of these 60S particles to avoid analyzing immature 60S subunits retained in the nuclei of the mutants.

Note that we show the distribution of nonfunctional 25S rRNAs after 2 h of transcriptional shut-off in Fig. S3E. MG132 treatment was used in this assay instead of Rpt2 tet-off. Again, most of the signals for the nonfunctional 25S rRNAs are observed in the cytoplasm.

Figure 4F:
The authors state that the non-functional 25S rRNA accumulate in various fractions in different mutant strains. Is the change in fractionation pattern of the 25S rRNA observed in the various mutants really due to accumulation of the 25S rRNA in different particles or is it simply due to a change in the ratio of 60S/80S particles in the mutant strains? To clarify this point the polysome profiles of each mutant should be provided in the supplemental figures.

We greatly appreciate this comment. We have inserted Fig, S4B showing the A_{260} patterns for the sucrose density gradient sedimentation of all the mutants. Essentially the same pattern was obtained for all the strains tested. This indicates that the size change effect of each mutant strain was restricted to the nonfunctional 25S rRNA and strengthens our conclusion. We have also added this comment to the main text.

Figure 5:
While the silver stain provides insight into the purification of the 60S using A2451U-MS2, the presence of various components of the 60S should be verified by western blot analysis.

We have constructed tagged versions of RPL3, L5, L24, and S4 and investigated whether they are also found in the nonfunctional 60S particles purified from the *Rpt2 tet-off* strain. As shown in Fig. S5A, RPL3, L5, and L24 were all recovered with the MS2 pull-down assay, whereas RPS4 was not. These results further support our conclusion that proteasome depletion causes the accumulation of the nonfunctional mutant 25S rRNA in the nearly intact 60S subunit.

Title:
The title at first glance indicated to me that the 40S is degraded by the NRD pathway. As such, I would suggest replacing "removal" with "dissociation" in the title.

We agree with this and have changed the title as suggested.

2nd Editorial Decision 13 March 2012

Thank you for sending us your revised manuscript. Referee 1 has now seen the manuscript again and is now positive about publication of your paper here (see below).

Still, prior to formal acceptance, there are a number of editorial issues that need further attention:

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* Please add an author contributions section and a conflict of interest statement into the main body of the manuscript text after the acknowledgements section.

* Please add a scale bar together with an explanation to Supplementary Figure S3E.

* Please add the statistical details including the number of independent repeats to figures S1A/B; S2A/D

* Prior to acceptance of every paper we perform a final check for figures containing lanes of gels (and equivalent data) that are assembled from cropped lanes. While cropping and pasting may be considered acceptable practices in some cases (please see Rossner and Yamada, JCB 166, 11-15, 2004) there needs to be a proper indication and explanation in the figure legend in all cases where such processing has been performed according to our editorial policies. Please note that it is our standard procedure when images appear like they have been pasted together without proper indication (like a white space or a black line between) or explanation in the figure legend to ask for the original scans. In the case of the present submission there is one panel that does not fully meet these requirements: Figure 4F. Please confirm/clarify that all dots of every lane come from the same membrane in the figure legend and amend figure 4F according to the guideline mentioned above. According to our editorial policies we also need to see the original scans for the panels in question.

* We now more generally encourage the publication of source data, particularly for electrophoretic gels and blots, with the aim of making primary data more accessible and transparent to the reader. Would you be willing to provide files comprising the original, uncropped and unprocessed scans of all gels used in the article? We would need 1 file per figure (which can be a composite of source data from several panels) in jpg, gif or PDF format, uploaded as "Source data files". The gels should be labelled with the appropriate figure/panel number, and should have molecular weight markers; further annotation would clearly be useful but is not essential. These files will be published online with the article as a supplementary "Source Data". Please let me know if you have any questions about this policy.

Thank you for your kind cooperation.

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1

In the revised version of their manuscript, Ohno and collaborators have satisfactorily answered the criticisms that I raised. I am a bit disappointed by the lack of answer to my first request (identification of the ribosomal substrate for Mms1-Rtt101) but I should admit that it is indeed beyond the scope of the present paper. I have to be patient and wait for their next study! I also think that they correctly addressed the points made by the other referees. Overall this is a remarkable study that clearly deserves publication in the EMBO Journal.

2nd Revision - authors' response 14 March 2012

Thank you for your kind assistance. Here we modified several points you requested.
Please find and confirm all the changes we made.