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Dissociation by Pelota, Hbs1 and ABCE1 of mammalian vacant 80S ribosomes and stalled elongation complexes

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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 January 2011

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 while referees 2 and 3 are clearly supportive of publication of the study here referee 1 raises one major concern that in his/her view precludes publication of the study at this point. In the meantime referee 3 has commented on this point and agrees that this could be a major concern. He/she therefore feels that both concerns raised by referee 1 (partial activity of bacterially expressed Pelota and the steric clash issue) should be addressed by further experimentation, i.e. by using Pelota that has been isolated from mammalian cells. We should thus be able to consider a revised version of the manuscript if the concerns raised by referee 1 can be sorted out beyond doubt and the other points can be addressed in an adequate manner. The revised manuscript will need to be seen by the referees again and a final decision can only be made at that stage.

I should remind you that it is EMBO Journal policy to allow a single round of revision only and that, therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript as well as on the final assessment by the referees.

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

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

Yours sincerely,

Editor
The EMBO Journal

Referee #1 (Remarks to the Author):

The manuscript by Pisareva et al describes a biochemical study investigating the mehcanism of action of Pelota and Hbs1, the mammalian homologues of the yeast no-go decay factors Dom34 and Hbs1.

The authors report that in a recosntituted in-vitro system, bacterially expressed Pelota can dissociate 80S ribosomal couples, or mRNA-bound ribosomes if these are either at the very end of an mRNA or if the ribosomal mRNA has been damaged through the action of the bacterial RelE toxin. All of these activities are stimuated by Hbs1 and require ABCE1. Crucially, they do not observe dissociation of elongating ribosomes with signficant 3'-mRNA length, and in this respect their findings differ from reports for the yeast factors.

There is a central problem with this study in that the only really novel finding, namely that mammalian pelota does not act as a bona fide no-go-decay factor on elongating ribosomes, may very easily be an artefact of the experimental procedures used by the authors. For example, if the bacterially expressed Pelota has only partial activity (due to missing post-translational modifications or other reasons), it might only act on loosely bound ribosomes such as mRNA-free couples, RelE-damaged ribosomes, or ribosomes with unusally short mRNA portions, but not on potentially more tightly bound ribosomes on full mRNAs. This would not necessarily mean that mammalian Pelota does not have NGD activity in vivo.

The argument of the authors that RelE removes a steric hindrance with the A-site mRNA is particularly confusing, as the same steric clash should prevent Pelota action on ribosomes containing short mRNAs?

Overall, I think the extensive and detailed mechanistic discussion that the authors develop based on a negative result from a single experimental approach is a premature, and for this reason I think this study in its current form is not publishable.

Referee #2 (Remarks to the Author):

During the elongation stage of translation, various circumstances can cause a ribosome to stall. A prolonged elongation stall leads to recruitment of the Dom34/Hbs1 complex to the empty A site of the stalled ribosome. This triggers release of the peptidyl-tRNA and dissociation of the stalled ribosome. The No-Go decay (NGD) pathway is triggered when elongation complexes stall within the ORF, for example because of the presence of stable secondary structures. In this case, the mRNA is cleaved in the vicinity of the stalled ribosome by an unknown endonuclease. A related pathway is the non-stop decay (NSD) pathway in which ribosomes become stalled at the end of the transcript due to the absence of a stop codon. Although these pathways have been investigated in yeast, and functional and structural information is available for the yeast Dom34/Hbs1 complex, the homologous complex in multicellular eukaryotes, Pelota/Hbs1, is much less well characterized.

This manuscript investigates the mechanism and role of mammalian Pelota/Hbs1 in the dissociation of elongation complexes. The authors show that, in contrast to yeast, in mammals this dissociation requires an additional protein, ABCE1. In fact both ABCE1 and Pelota are required for this process, whereas Hbs1 is not necessary but plays a stimulatory role. Remarkably, and again in contrast to the

yeast proteins, the ABCE1/Pelota/Hbs1 complex only dissociates elongation complexes when these are in close proximity to the mRNA 3' end, suggesting that ABCE1/Pelota/Hbs1 plays a role in NSD. Since it is possible that during NGD, mRNA cleavage converts an NGD target into an NSD target, it could be that in mammals ABCE1/Pelota/Hbs1 drives removal of stalled ribosomes in a similar manner in both pathways.

The authors also show that ABCE1/Pelota/Hbs1 dissociates vacant 80S ribosomes. This activity may play an important role during some physiological conditions, such as meiosis or mitosis, during which translation is inhibited and vacant 80S ribosomes accumulate. These accumulated ribosomes must be dissociated into ribosomal subunits in order to allow translation to resume, for example after meiosis is completed. This may provide an explanation for the observed meiotic and mitotic defects of Pelota mutants.

In summary, the manuscript provides important insights into the mechanism of ribosome dissociation when the A site codon is not a natural stop. In addition the manuscript is clearly written and the experiments are of high technical quality.

Referee #3 (Remarks to the Author):

This submission focuses on mammalian Pelota/Hbs1 function in vitro and its need for ABCE1 to dissociate stalled elongation complexes in an A-site independent manner. The work also demonstrates the consequential release of peptidyl-tRNA (rather than peptide release). All of this is provided the template RNA contains no more than 9-nts downstream of the P site. The submission nicely interfaces with the recent (2010) report of Shoemaker et al. from Rachel Green's lab, which showed many of the same things for yeast Dom34/Hbs1. However, yeast Dom34/Hbs1 does not involve ABCE1 (in contrast to yeast eRF1/eRF3), and treatment of yeast elongation complexes with Dom34/Hbs1 results in mRNA (which had a 24-nt 3'UTR) largely remaining bound to 40S subunits. In fact, the finding in mammals that incubation of elongation complexes with Pelota/Hbs1 and ABCE1 resulted in only a tiny fraction of mRNA with 40S subunits compared to in yeast, could reflect the presence of a longer 3'UTR in yeast, which the authors note may stabilize the association of mRNA with 40S subunits.

The paper is well-written with an informative Introduction. Its novelty lies in part in its study of mammalian Pelota/Hbs1 and their ABCE1-dependent role in promoting the nearly complete dissociation of vacant 80S ribosomes formed in vitro. Formation was achieved by assembling 40S subunits, [32P]-labeled 60S subunits phosphorylated using casein kinase II and, after formation, eIF6 to prevent the reassociation of dissociated complexes. This dissociation reaction cannot be accomplished by eRF1/eRF3 and ABCE1 and, for unclear reasons, it does not require Hbs1 when higher concentrations of Pelota are provided. In related studies, Hbs1 was found to inhibit 80S ribosome dissociation in the presence of GMPPNP, which was shown not to inhibit ABCE1 activity. However, the reaction including GMPPNP without Hbs1 is missing, and the mechanistic significance of this finding is unclear. The authors go on to show that Pelota/Hbs1 and ABCE1 do not dissociate 80 complexes formed on mRNA containing a 3'UTR of ~400 nt. The finding that Pelota and Hbs1 did not induce shifts in the toe-prints of pre-TCs or ECs could indicate that they did not bind or bind productively. This would be consistent with the Kobayashi et al (2010) finding that archeal Pelota docked with archeal EF1a onto the bacterial 70 ribosome clashes with the A-site of mRNA. Cleavage of the mRNA A site with the bacterial toxin RelE resulted in approximately 50% dissociation of pre-TCs. The authors proposed that incomplete dissociation was due to competition between Pelota and RelE for A-site binding. Pelota/Hbs1 result in release of peptidyl-tRNA and dissociation of mRNA from 40S subunits. The authors also show that the dissociation of 80S complexes by Pelota/Hbs1 and ABCE1 falls off after 9 nts (i.e, with 13 or 17 nts), indicating that Pelota/Hbs1 and ABCE1 do not function to recycle ribosomes on most NGD targets unless there is mRNA cleavage immediately downstream of the stalled ribosome.

In summary, this is a very nice paper that would be improved when the following issues are addressed.

Specific Issues

Abstract, line 5. While "drop-off" was used in the title of Shoemaker et al., 2010, its meaning is not obvious to those outside of the field and here would be better explained as "release" or, if need be, "drop off, i.e., release".

Page 8, line 9. Ideally, referencing "Fig. 1A, lanes 1,2" would be "Fig. 1A, compare lane 1 to lane 2" for clarity. This applies to many other references to data, and there is probably a clearer way for the authors to describe their results.

Fig 1A. "Full length" would best be changed to "Full-length mRNA" or "Full-length extension" or "mRNA". The same applies to other figures so labeled. It would have been nice to see a lane with mRNA alone. Why are there bands in the sequencing lanes above "Full length"?

Figure 2A. It seems important to repeat the reaction labeled with blue diamonds in the absence of Hbs1. Also, what is the significance of the finding that Hbs1 inhibits 80S ribosome dissociation in the presence of GMPPNP?

Experimental Procedures. It seems important to include volumes when describing, e.g., the 80S dissociation experiments.

Fig. 3D. The forward 2-nt shift should be labeled, and the position of +15 nt from UAA should be made clearer.

Page 10, first fill paragraph. Ligatin would best be better defined here, if not earlier when first mentioned.

Page 11, second-to-last line. "One type of complex" must be referring to complexes cleaved after either the first or second A-site nucleotide as the two possible types of complex. However, this should be clarified.

Fig. 5C, upper panel. It is unclear how the authors are sure of the nature of the two faster-migrating [35S]-labeled bands (except for the statement that the MVHC-tRNA complex migrated exactly like the intact complex after phenol-extraction of purified ECs, but the data are not shown; and migration at the top of a SDG in the lower panel is not particularly convincing). Along similar lines, why are there multiple bands in the vicinity of the MVHC-tRNA complex? And, why is there less radioactivity in lanes 2-4 relative to the other lanes?

Page 13. Given the potential difference between yeast and mammals, it seems important for the authors to determine if 3'UTR lengths that support 80S subunit dissociation correlate with dissociation of mRNA from 40S complexes in mammals. In other words, are 9 nts sufficient to at least partially stabilize mRNA-40S complexes (as they may be in yeast)?

Page 15. First complete paragraph. The authors could clarify why purified eRF1/eRF3 cannot mediate ribosomal disassembly of vacant 80S subunits without the help of other factors given that there is no peptide to release by referring to the next paragraph as (see below). How do the authors think eRF1 stimulate eRF3's GTPase activity in the presence of vacant ribosomes?

1st Revision - authors' response 31 January 2011

We are very grateful to the reviewers for their careful reading of our manuscript and insightful comments and suggestions. We performed all the requested experiments and modified the text and the Figures according to the reviewers' suggestions.

Reviewer 1

Specific point #1: *There is a central problem with this study in that the only really novel finding, namely that mammalian pelota does not act as a bona fide no-go-decay factor on elongating*

ribosomes, may very easily be an artifact of the experimental procedures used by the authors. For example, if the bacterially expressed Pelota has only partial activity (due to missing post-translational modifications or other reasons), it might only act on loosely bound ribosomes such as mRNA-free couples, RelE-damaged ribosomes, or ribosomes with unusually short mRNA portions, but not on potentially more tightly bound ribosomes on full mRNAs. This would not necessarily mean that mammalian Pelota does not have NGD activity in vivo.

Answer: The reviewer raised an important question concerning the activity of recombinant Pelota. To address this question, we purified native Pelota from rabbit reticulocyte lysate (shown in the revised Figure 6D) and assayed its ability to promote dissociation of ribosomal complexes containing different numbers of nucleotides downstream of the P-site. The results of these experiments are now presented in the revised Figures 6E and 6F and discussed on page 13, last paragraph of the revised manuscript. Like the recombinant protein, native Pelota promoted dissociation of ribosomal complexes assembled on the MVHL-STOP mRNA and containing ~400nts downstream of the P-site only after their treatment with RelE (revised Fig. 6E). Native Pelota was also able to promote efficient dissociation of ribosomal complexes only if they contained ≤9 nucleotides downstream of the P-site (revised Fig. 6F). Thus, in the mammalian *in vitro* reconstituted translation system, the activities of native and recombinant Pelota were identical.

Importantly, we never claimed that Pelota does not have NGD activity. We merely stated that the fact that Pelota could not promote dissociation of ribosomal complexes containing long mRNA fragments downstream of the P-site suggests that during NGD in mammals, cleavage of mRNA might precede dissociation of stalled ribosomal complexes.

Specific point #2: *The argument of the authors that RelE removes a steric hindrance with the A-site mRNA is particularly confusing, as the same steric clash should prevent Pelota action on ribosomes containing short mRNAs?*

Answer: As suggested in the Discussion, because of the potential steric hindrance by the A-site mRNA, productive binding of Pelota might require conformational changes in ribosomal complexes that involve some repositioning of mRNA in the A-site, which would be possible in complexes containing short mRNA regions downstream of the P-site, but might be impaired by stable fixation of a longer mRNA in the mRNA-binding cleft downstream of the P-site. Treatment with RelE, on the other hand, would leave no more than 1 or 2 nts after the P-site, which could potentially completely eliminate such an impediment. We do not see any discrepancy in these arguments.

Reviewer 2

This reviewer did not raise any specific criticism.

Reviewer 3

In summary, this is a very nice paper that would be improved when the following issues are addressed.

Specific point #1: *Abstract, line 5. While "drop-off" was used in the title of Shoemaker et al., 2010, its meaning is not obvious to those outside of the field and here would be better explained as "release" or, if need be, "drop off, i.e., release".*

Answer: The suggested change has been made.

Specific point #2: *Page 8, line 9. Ideally, referencing "Fig. 1A, lanes 1,2" would be "Fig. 1A, compare lane 1 to lane 2" for clarity. This applies to many other references to data, and there is probably a clearer way for the authors to describe their results.*

Answer: The suggested changes have been introduced throughout the entire text of the manuscript.

Specific point #3: *Fig 1A. "Full length" would best be changed to "Full-length mRNA" or "Full-length extension" or "mRNA". The same applies to other figures so labeled. It would have been nice to see a lane with mRNA alone. Why are there bands in the sequencing lanes above "Full length"?*

Answer: The suggested change has been introduced into all Figures. The presence of the bands in the sequencing lanes above "Full length" is explained by the fact that corresponding plasmid DNA (not mRNA) was used to obtain reference sequences.

Specific point #4: *Figure 2A. It seems important to repeat the reaction labeled with blue diamonds in the absence of Hbs1. Also, what is the significance of the finding that Hbs1 inhibits 80S ribosome dissociation in the presence of GMPPNP?*

Answer: We performed the suggested control experiment (the result is now presented in the revised Figure 2B). The finding that in the presence of GMPPNP, Hbs1 inhibits 80S ribosome dissociation emphasizes the role of GTP hydrolysis by Hbs1 in the dissociation process and suggests the likely order of events, according to which Hbs1 probably has to dissociate from ribosomal complexes (after GTP hydrolysis) to allow ABCE1-mediated dissociation to proceed (discussed on page 15, lines 17-21 of the revised manuscript).

Specific point #5: *Experimental Procedures. It seems important to include volumes when describing, e.g., the 80S dissociation experiments.*

Answer: We made sure that all volumes of the reaction mixtures are described in Experimental procedures.

Specific point #6: *Fig. 3D. The forward 2-nt shift should be labeled, and the position of +15 nt from UAA should be made clearer.*

Answer: The suggested changes have been introduced into Figure 3D.

Specific point #7: *Page 10, first fill paragraph. Ligatin would best be better defined here, if not earlier when first mentioned.*

Answer: As suggested, Ligatin is now defined in the Introduction section (page 4, lines 11-13 of the revised manuscript).

Specific point #8: *Page 11, second-to-last line. "One type of complex" must be referring to complexes cleaved after either the first or second A-site nucleotide as the two possible types of complex. However, this should be clarified.*

Answer: The suggested changes have been introduced into this sentence (page 11, lines 20-22 of the revised manuscript).

Specific point #9: *Fig. 5C, upper panel. It is unclear how the authors are sure of the nature of the two faster-migrating [35S]-labeled bands (except for the statement that the MVHC-tRNA complex migrated exactly like the intact complex after phenol-extraction of purified ECs, but the data are not shown; and migration at the top of a SDG in the lower panel is not particularly convincing). Along similar lines, why are there multiple bands in the vicinity of the MVHC-tRNA complex? And, why is there less radioactivity in lanes 2-4 relative to the other lanes?*

Answer: We apologize for the fact that Fig. 5C was not labeled in a manner that made it obvious that lane 1 contained MVHC-tRNA complex that had been phenol-extracted from elongation complexes, and that lane 6 contained Cys-tRNA. We have amended labeling of this panel to clarify these details. It should now be apparent that the material released from elongation complexes migrates identically to MVHC-tRNA^{Cys} that had been phenol-extracted. We also conducted additional experiments using pre-TCs assembled on MVHC-6 mRNA in the presence of [^{35}S]Cys-tRNA and investigated peptide release by TCA precipitation: no peptide was released by incubation with Pelota/Hbs1 whereas eRF1/eRF3 mediated normal peptide release. We also compared the

mobility in native gels of labeled product after incubation of such pre-TCs with Pelota/Hbs1/ABCE1 and with eRF1/eRF3/ABCE1. eRF1/eRF3/ABCE1 mediated release of MVHC tetrapeptide which migrated much more slowly than the MVHC-tRNA released by Pelota/Hbs1/ABCE1. We have not included these results because they are merely confirmatory of well-supported experiments published by Shoemaker et al (2010).

The material included in lanes 1 and 6 served solely as standards against which the mobility of material in lanes 2-5 could be compared, and no attempt was made to standardize the amount of radioactive material present in these 'reference' lanes relative to that in the 'experimental' lanes 2-5. On the other hand, the total amount of radioactive material present in each of lanes 2-5 is comparable.

We also noted the presence of additional minor bands with different mobilities in the vicinity of the principal MVHC-tRNA band, which resemble additional minor bands in gels show by Shoemaker et al. (2010), and suggest that they may represent conformational variants that migrate slightly differently in the low-percentage native gels used in these experiments.

Specific point #10: *Page 13. Given the potential difference between yeast and mammals, it seems important for the authors to determine if 3'UTR lengths that support 80S subunit dissociation correlate with dissociation of mRNA from 40S complexes in mammals. In other words, are 9 nts sufficient to at least partially stabilize mRNA-40S complexes (as they may be in yeast)?*

Answer: The suggested experiment has been performed, and the results are now shown in Figure 6C and described on page 13, lines 13-18 of the revised manuscript. We found that even 9 nts downstream of the P-site were not sufficient to stabilize mammalian mRNA-40S complexes, and that mRNA was released into solution.

Specific point #11: *Page 15. First complete paragraph. The authors could clarify why purified eRF1/eRF3 cannot mediate ribosomal disassembly of vacant 80S subunits without the help of other factors given that there is no peptide to release by referring to the next paragraph as (see below). How do the authors think eRF1 stimulate eRF3's GTPase activity in the presence of vacant ribosomes?*

Answer: First, eRF1 stimulates eRF3/GTP binding by two orders of magnitude due to lowering of the dissociation rate constant (e.g. Pisareva et al., 2006). Second, structural studies of the eRF1/eRF3 complex also suggest that the middle domain of eRF1 could directly contact the switch regions of eRF3, which could contribute to stimulation of eRF3's GTPase activity (Cheng et al., 2009). Both of these outcomes of eRF1/eRF3 interaction most likely contribute to stimulation of eRF3's GTPase activity by eRF1 and vacant 80S ribosomes or pre-termination complexes. Most likely, there are also other ways in which eRF1 could contribute to stimulation of GTPase activity of eRF3 while both factors bind either to vacant 80S ribosomes or to pre-termination complexes (e.g. by proper positioning of eRF3, particularly in the case of pre-TCs). However, the ribosomal position and the structure of ribosome-bound eRF1/eRF3 complexes have not yet been determined. It is apparent from this description that the current state of knowledge concerning the mechanism of stimulation by eRF1 and by ribosomes of eRF3's GTPase activity is incomplete and still quite hypothetical. Accordingly, we have not included a discussion of this matter in the revised manuscript, because length limitations would not permit an adequate description of these various considerations, and because this topic is somewhat tangential to the principal findings presented in our report. We hope that the reviewer will understand this decision.

2nd Editorial Decision 01 March 2011

Thank you for sending us your revised manuscripts. Referees 1 and 2 have now seen it again. While referee 1 recognises the technical quality of the study and all the additional effort you undertook he/she is still not fully satisfied. Referee 2 supports publication (no further comments to the authors). All in all and given the initial positive vote by referee 3, we have come to the conclusion that the paper will now be publishable in The EMBO Journal. You will receive a formal acceptance letter

shortly.

Thank you very much again for considering our journal for publication of your work.

Yours sincerely,

Editor
The EMBO Journal

Referee #1

Response to specific point 1:

The fact that this lab managed to purify native pelota from retic lysate in a relatively short time is both testament to their experimental skills, and an important control that proves beyond reasonable doubt that the absence of activity on long mRNAs is not an artefact of bacterially expressed protein.

On re-reading the manuscript, I appear to have wrongly accused the authors of suggesting that Pelota does not have NGD activity - their arguments were clearly more sophisticated than this. My apologies.

Response to specific point 2:

The authors explanation why there would be a steric clash with pelota binding to ribosomes on long RNAs but not on short RNAs is highly hypothetical. While I agree that their explanation is not physically impossible, it involves very speculative interpretations of known data on the mRNA paths in bacterial ribosomes. I provided another explanation in my comments, namely that mRNA-free couples, RelE-damaged ribosomes, and ribosomes containing short mRNAs are held together less strongly and therefore dissociated more easily than ribosomes containing long mRNAs. This would be consistent with the authors'own statement that "Interestingly, the dissociation activity of Pelota/Hbs1 appears to be noticeably lower than that of their yeast counterparts."(p. 18) While my explanation is not more likely than the authors', it is not less likely either. I think that this point therefore remains a problem.

There was a third and unanswered point in my comments, namely that the authors draw significant mechanistic conclusions from applying a single biochemical technique to studying a biological pathway. Despite the reassurance that the observation with the native pelota gives, I still think that this is an important issue. However, I also note that the other two referees do not share my concern in this point.

Thus my overall judgement of this study is this: it is a biochemical study of very high technical standard - I would not expect anything different from this lab. It raises very interesting questions about mechanisms of the surveillance pathways, and gives POTENTIAL answers to these questions. Any further cementing of these answers, and further insights into the mechanisms, probably require complementary approaches (probably in vivo). Whether the study in its current form is sufficient for publication in the EMBO Journal may have to be the editor's decision.

