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Dom34-Hbs1 mediated dissociation of inactive 80S ribosomes promotes restart of translation after stress

Antonia M.G. van den Elzen, Anthony Schuller, Rachel Green, and Bertrand Séraphin

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|---------------------|-----------------|
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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.)

Editor: Anne Nielsen

1st Editorial Decision

07 August 2013

Thank you for submitting your manuscript for consideration by the EMBO Journal and my apologies for the unusually long duration of the review period in this case. Your study has now been seen by three referees whose comments are shown below.

As you will see, while refs #2 and #3 are mainly positive about the findings presented and raise only minor concerns, ref#1 is considerably more negative and raises a number of experimental issues that need to be resolved before this person can support publication of your manuscript here.

Concerning the specific points raised by ref #1, we understand that a full clarification of the mechanism of ribosome recycling (as requested by the referee) would rather be the topic of a separate manuscript. However, we do find that the current manuscript should be strengthened by including evidence for the physiological significance of ribosome recycling in the form of growth assays as well as by describing the interaction and distribution of the recycling factors in more detail. In addition, we would ask you to provide experimental data in response to the 7 specific points raised by ref#1 and to the minor comments made by refs #2 and #3.

Given the overall positive recommendations from the referees we would thus invite you to submit a revised version of the manuscript, addressing the comments of all three reviewers as outlined above. I should add that it is EMBO Journal policy to allow only a single round of revision, and acceptance of your manuscript will therefore depend on the completeness of your responses in this revised

version. Should you experience difficulties in addressing specific points, I would be happy to engage in further discussions.

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, 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. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

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

REFEREE REPORTS:

Referee #1:

In this manuscript, the authors investigate the role of the Dom34-Hbs1 complex, along with Rli1, in ribosome recycling via the removal of Stm1 following the alleviation of glucose starvation. They additionally observe a function for the Dom34-Hbs1 complex in general translation. Moreover, they assess the role of the individual components of the complex and the different regions of the Hbs1 in ribosome recycling. The observation that this complex is involved in the separation of inactive ribosomes upon stress relief is clearly shown using polysome assays. However these experiments do not show that the ribosomes are recycled and the data presented only hints at this without proving it. I feel the authors need to show mechanistically how the complex is acting to recycle ribosomes. It would be of benefit to see the distribution of these proteins across polysome gradients at the various time points and additional confirmation that these proteins associate with Stm1 through immunoprecipitation studies. Another global criticism would be that the authors do not show that this complex is important for cellular physiology. Therefore, it would also be beneficial to show the impact of disrupting the complex on the growth of *S. cerevisiae* following stress alleviation. As well as these global comments more specific comments are detailed below.

More specific comments:

1. Throughout the manuscript the authors fail to quantify any of the polysome profiles. Some of the differences noted by the authors are minimal at best e.g. Figure 5a, and quantitation/ statistics on the monosome to polysome ratios would verify the observations made.

2. In Figure 2a, the overlay of the different traces is confusing and hard to differentiate. The plots need to be shown as separate, smaller traces.

Also the authors show complexes minus the constituents Rli1 or Dom34, but do not show a complex where Hbs1 is absent. For completeness it would be useful to see this trace.

3. In Figure 2b, the authors show the effect of removing Rli1 or Hbs1 on ribosome dissociation but not Dom34, this needs including.

The authors also fail to adequately explain why deletion of Hbs1 has no impact the rate of ribosome splitting yet blocking Hbs1 GTPase activity by the inclusion of GDPNP diminishes the rate of splitting by 3 fold. The suggestion that excess Dom34 in the reaction mix minimizes the contribution required for Hbs1 needs supporting experimentally.

4. In Figure 4, the authors need to confirm that the mutations to Hbs1 (R517E) and Dom34 (E361R) disrupt their association given the contradictory nature of their data. Also, the continued inhibition of translation following stress relief for the Dom34 E361R strain is not complete and therefore over interpreted by the authors.

5. In Figures 5a and 5b the overlay of the traces is difficult to see. Color needs including to differentiate between the traces.

6. The authors comment on a role for Dom34-Hbs1 in general translation, commenting on traces in Figures 1, 3 and 4. A separate figure is required to adequately show this observation.

7. In Figure 5C, the authors need to show Dom34 alone. Additionally, statistical comparison of the different traces would be beneficial given the larger standard deviation for the Dom34+Hbs1.

Referee #2:

The eukaryotic proteins Pelota and Hbs1 (in *S. cerevisiae*, Dom34 and Hbs1) are involved in the process of no-go decay, functioning with the NTPase ABCE1 (in *S. cerevisiae*, Rli1) to release stalled ribosomes from mRNA. Previous *in vitro* studies (Pisareva et al., 2011) have shown that these factors can also split 'empty' 80S ribosomes that are not bound to mRNA, and promote the re-entry of the resulting ribosomal subunits into the translation process. Empty 80S ribosomes are known to accumulate during mitosis, meiosis and conditions of stress, and it was previously suggested (Pisareva et al., 2011) that Pelota/Hbs1/ABCE1 would function to promote resumption of translation following recovery from stress.

In the present clearly presented and straightforward manuscript, van der Elzen et al. provide evidence from *in vivo* experiments that is entirely consistent with this hypothesis. They report that dissociation of empty 80S ribosomes and resumption of translation following recovery from glucose starvation are both impaired in dom34 and Hbs1 deletion strains of *S. cerevisiae*, and in *in vitro* experiments, show that Dom34/Hbs1 and Rli1 cooperate in splitting of empty 80S ribosomes from glucose-starved yeast. These ribosomes are associated with the stress-response factor Stm1, which is located in the ribosomal mRNA-binding channel, preventing mRNA entry and stabilizing ribosomal subunit interactions. Consistent with an earlier report (Pisareva et al., 2011), Hbs1 was not essential for ribosomal splitting *in vitro*, but *in vivo* studies showed that it enhances resumption of translation following recovery from stress. Hbs1's GTPase activity was required for this function, whereas its extended N-terminal region and the ability to interact stably with Dom34 were not. Further experiments showed that Dom34-Hbs1 and Rli1 may also function to dissociate mRNA-free 80S ribosomes in non-starvation conditions. In these experiments, it would be informative if the authors showed directly whether empty 80S ribosomes are also bound by Stm1 in non-starvation conditions. Taken together, these data advance knowledge of the translation process in eukaryotes and merit publication in EMBO Journal.

Minor comments

1. It is more accurate to describe Rli1/ABCE1 as an NTPase, rather than as an ATPase (p2, line 7; p3, line 9).

2. It would be more generally informative to the non-specialist reader to mention in the abstract that Rli1 is known as ABCE1 in other eukaryotes.

3. The authors should explain what is meant by NRD (p4, line 27).

4. Typographical errors.

p10, line 3. "The higher 80S peak could BE due"

p11, line 3. "stall translation" instead of "cause inefficient translation"?

Referee #3:

In many translation inhibiting stress conditions, free ribosomal subunits reassociate to form a large pool of nontranslating or hibernating 80S ribosomes stabilized by Stm1 in eukaryotes. The subunits of these inactive ribosomes need to be mobilized for translation restart upon stress relief. In addition, recent studies in yeast and mammalian systems identified Dom34 (Pelota in humans) and the GTPase Hbs1, forming a complex structurally similar to eRF1 and eRF3, that together with Rli1 similarly promote subunit dissociation of mRNA-bound ribosomes but in a codon-independent manner. In light of these observations, in this study the authors set out to address a key question as to whether this process, in analogy to ribosome recycling after termination, depends on recycling factor activity. They discovered a new function for Dom34-Hbs1. The authors observe that Dom34-Hbs1 stimulates the dissociation of non-translating ribosomes that accumulate upon glucose starvation in yeast. Then, the biological relevance for this activity was demonstrated by showing the dependence on Dom34-Hbs1-Rli1 proteins for translational recovery in yeast cells after glucose deprivation. Moreover, the authors showed that Dom34-Hbs1 mediated dissociation of non-translating ribosomes can also stimulate translation in non-stressed conditions. Collectively, these observations reveal a seemingly general mechanism for the control of translation wherein ribosomes stored in an unproductive state can be readily reversed by the activities of Dom34, Hbs1 and Rli1.

Overall, this study is both significant and of general interest in that it expands the biochemical and physiological roles of Dom34-Hbs1 in co-translational quality control on RNAs. All findings go into making an important story. This reviewer has no major concerns.

The following minor points are for authors' attention:

1. A reference(s) should be cited after the following sentence in page 3: "Ribosomal subunits that are released through recycling may not engage in new rounds of protein synthesis, but instead associate to form a large pool of non-translating, inactive ribosomes (refs).
2. Please define "NRD" when it first appears in page 4 (bottom).
3. In page 10, line 3: The higher 80S peak could (be?) due to some amount of Stm1-bound ribosomes ...
4. The experiment in Fig. 5C would be more thorough if addition of Dom34 alone in the in vitro experiment is also tested.
5. Page 12, bottom, unclear sentence: "both Dom34 and Hbs1 are important for normal growth of yeast strains with (double mutant strains showing?) reduced amounts of 40S subunits likely because these double mutant strains have too few ribosomes available to function.

1st Revision - authors' response

14 October 2013

Referee #1:

In this manuscript, the authors investigate the role of the Dom34-Hbs1 complex, along with Rli1, in ribosome recycling via the removal of Stm1 following the alleviation of glucose starvation. They additionally observe a function for the Dom34-Hbs1 complex in general translation. Moreover, they assess the role of the individual components of the complex and the different regions of the Hbs1 in ribosome recycling. The observation that this complex is involved in the separation of inactive ribosomes upon stress relief is clearly shown using polysome assays. However these experiments do not show that the ribosomes are recycled and the data presented only hints at this without proving it.

I feel the authors need to show mechanistically how the complex is acting to recycle ribosomes.

It would be of benefit to see the distribution of these proteins across polysome gradients at the various time points and additional confirmation that these proteins associate with Stm1 through immunoprecipitation studies.

For several reasons, we were somewhat surprised by this request from the referee. First, the binding of Dom34-Hbs1 to inactive ribosomes, inducing their dissociation, is likely to be highly transient

and has not been seen previously *in vivo*. Second, we never stated that Dom34 and/or Hbs1 would interact with Stm1 and our conclusions never imposed such an interaction.

We nevertheless monitored HA-tagged Dom34 and protein A-tagged Hbs1 distribution across polysome gradients, at different time points before, during and after glucose depletion. We observed (see figures attached) that in all conditions tested, the large majority of these proteins was consistently found in non-ribosomal fractions. Very small amounts of Dom34 co-sediment with 80S ribosomes, making it impossible to assess any differences in 80S bound levels between different conditions or even to exclude that the signals resulted from diffusion of Dom34 from the top of the sucrose gradients. Consistent with these results, we could not detect any (ribosome-bound) Stm1 or ribosomal protein Rpl1A co-precipitating with protein A-tagged Hbs1. We cannot exclude that ribosome bound Dom34-Hbs1 may dissociate during the lengthy cell lysis, sedimentation or immunoprecipitation.

Having said this, we emphasize that these findings are by no means contradictory to our interpretation of our data: Dom34-Hbs1 are likely to bind transiently to inactive ribosomes, inducing quickly their dissociation and thus no direct interaction with Stm1 (or localization in the same fraction) is required to explain their action.

*Another global criticism would be that the authors do not show that this complex is important for cellular physiology. Therefore, it would also be beneficial to show the impact of disrupting the complex on the growth of *S. cerevisiae* following stress alleviation.*

We have now added data to show that yeast lacking Dom34 is delayed in growth when recovering from glucose starvation (Supplementary Figure 1).

As well as these global comments more specific comments are detailed below.

More specific comments:

1. Throughout the manuscript the authors fail to quantify any of the polysome profiles. Some of the differences noted by the authors are minimal at best e.g. Figure 5a, and quantitation/ statistics on the monosome to polysome ratios would verify the observations made.

We have now included quantifications of monosome to polysome ratios in Figures 1, 3, and 5. Given the large size of Figure 4, quantifications are included in the Supplementary Information.

2. In Figure 2a, the overlay of the different traces is confusing and hard to differentiate. The plots need to be shown as separate, smaller traces.

We divided the plot into four smaller plots.

Also the authors show complexes minus the constituents Rli1 or Dom34, but do not show a complex where Hbs1 is absent. For completeness it would be useful to see this trace.

This information was mentioned as “data not shown” in the original version of our manuscript because related information was already presented in Figure 2b. At the referee’s request, we have now included this trace.

3. In Figure 2b, the authors show the effect of removing Rli1 or Hbs1 on ribosome dissociation but not Dom34, this needs including.

In the original version, related information obtained using a different assay was presented in Figure 2a. We have now included the condition requested by the referee in Figure 2b as well.

The authors also fail to adequately explain why deletion of Hbs1 has no impact the rate of ribosome splitting yet blocking Hbs1 GTPase activity by the inclusion of GDPNP diminishes the rate of splitting by 3 fold. The suggestion that excess Dom34 in the reaction mix minimizes the contribution required for Hbs1 needs supporting experimentally.

We changed the text in the discussion section to further clarify how it is possible that blocking Hbs1 GTPase activity diminishes the rate of the splitting reaction in vitro, while absence of Hbs1 has no effect in vitro. In essence, it is known from biochemical experiments that Hbs1 must depart (or hydrolyze GTP) prior to efficient splitting; so, if Hbs1 is locked onto the ribosome complex (e.g., in the presence of GDPNP), splitting can be diminished. But in the in vitro experiments, Dom34 can be supplied at a high enough concentration that Hbs1 does not further stimulate the binding reaction. Our in vivo data clearly show that in physiological conditions, the presence of Hbs1 is required for efficient recovery of translation. In vivo many factors are present and may affect Dom34/Hbs1-mediated ribosome dissociation by interacting with the ribosome, Dom34 and/or Rli1. The absence of such factors from our in vitro experiments could also explain certain differences between the systems.

4. In Figure 4, the authors need to confirm that the mutations to Hbs1 (R517E) and Dom34 (E361R) disrupt their association given the contradictory nature of their data. Also, the continued inhibition of translation following stress relief for the Dom34 E361R strain is not complete and therefore over interpreted by the authors.

We have shown in a previous publication that using yeast two hybrid analysis, the Hbs1 R517E and Dom34 E361R mutations reduce Dom34-Hbs1 interaction to background level, whereas their levels of expression are similar to those of their wild type counterparts (van den Elzen et al., NSMB 2010 Dec;17(12):1446-52, Supplementary Figure 5). In the results section of the manuscript we refer to these data.

The reviewer was correct in stating that the inhibition of recovery in the Dom34 E361R strain is not complete. We changed the text in the result and discussion sections to correct this overstatement. We also extended the explanation on the asymmetrical effect of mutating the interface (that was observed previously) in the discussion section.

5. In Figures 5a and 5b the overlay of the traces is difficult to see. Color needs including to differentiate between the traces.

We have now used colors to better distinguish the two traces.

6. The authors comment on a role for Dom34-Hbs1 in general translation, commenting on traces in Figures 1, 3 and 4. A separate figure is required to adequately show this observation.

The referee comments that we should show in a new figure that yeast lacking Dom34 or Hbs1 has an increased 80S peak. However, such a figure is already present in the manuscript (Figure 5A). The reference to figures 1, 3 and 4, as well as the references to published reports are merely made to support the highly reproducible nature of this observation. We have changed the text to clarify this point.

7. In Figure 5C, the authors need to show Dom34 alone. Additionally, statistical comparison of the different traces would be beneficial given the larger standard deviation for the Dom34+Hbs1.

We have reproduced the figure, now including a condition with Dom34 alone present. We now show that presence of both Dom34 and Hbs1 leads to a statistically significant increase in translation, when compared to conditions in which only Dom34, or only Hbs1, is present.

Referee #2:

The eukaryotic proteins Pelota and Hbs1 (in S. cerevisiae, Dom34 and Hbs1) are involved in the process of no-go decay, functioning with the NTPase ABCE1 (in S. cerevisiae, Rli1) to release stalled ribosomes from mRNA. Previous in vitro studies (Pisareva et al., 2011) have shown that these factors can also split 'empty' 80S ribosomes that are not bound to mRNA, and promote the re-entry of the resulting ribosomal subunits into the translation process. Empty 80S ribosomes are known to accumulate during mitosis, meiosis and conditions of stress, and it was previously suggested (Pisareva et al., 2011) that Pelota/Hbs1/ABCE1 would function to promote resumption of translation following recovery from stress.

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extended N-terminal region and the ability to interact stably with Dom34 were not. Further experiments showed that Dom34-Hbs1 and Rli1 may also function to dissociate mRNA-free 80S ribosomes in non-starvation conditions. In these experiments, it would be informative if the authors showed directly whether empty 80S ribosomes are also bound by Stm1 in non-starvation conditions. Taken together, these data advance knowledge of the translation process in eukaryotes and merit publication in EMBO Journal.

Minor comments

1. It is more accurate to describe Rli1/ABCE1 as an NTPase, rather than as an ATPase (p2, line 7; p3, line 9).

Following the referee's recommendation, we replaced ATPase by NTPase at the indicated site and in the abstract.

2. It would be more generally informative to the non-specialist reader to mention in the abstract that Rli1 is known as ABCE1 in other eukaryotes.

We added this information in the abstract.

3. The authors should explain what is meant by NRD (p4, line 27).

18S NRD was changed into Non-functional 18S rRNA decay.

4. Typographical errors.

p10, line 3. "The higher 80S peak could BE due"

p11, line 3. "stall translation" instead of "cause inefficient translation"?

The typographical errors have been corrected.

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References were added.

2. Please define "NRD" when it first appears in page 4 (bottom).

18S NRD was changed into Non-functional 18S rRNA decay.

3. In page 10, line 3: The higher 80S peak could (be?) due to some amount of Stm1-bound ribosomes ...

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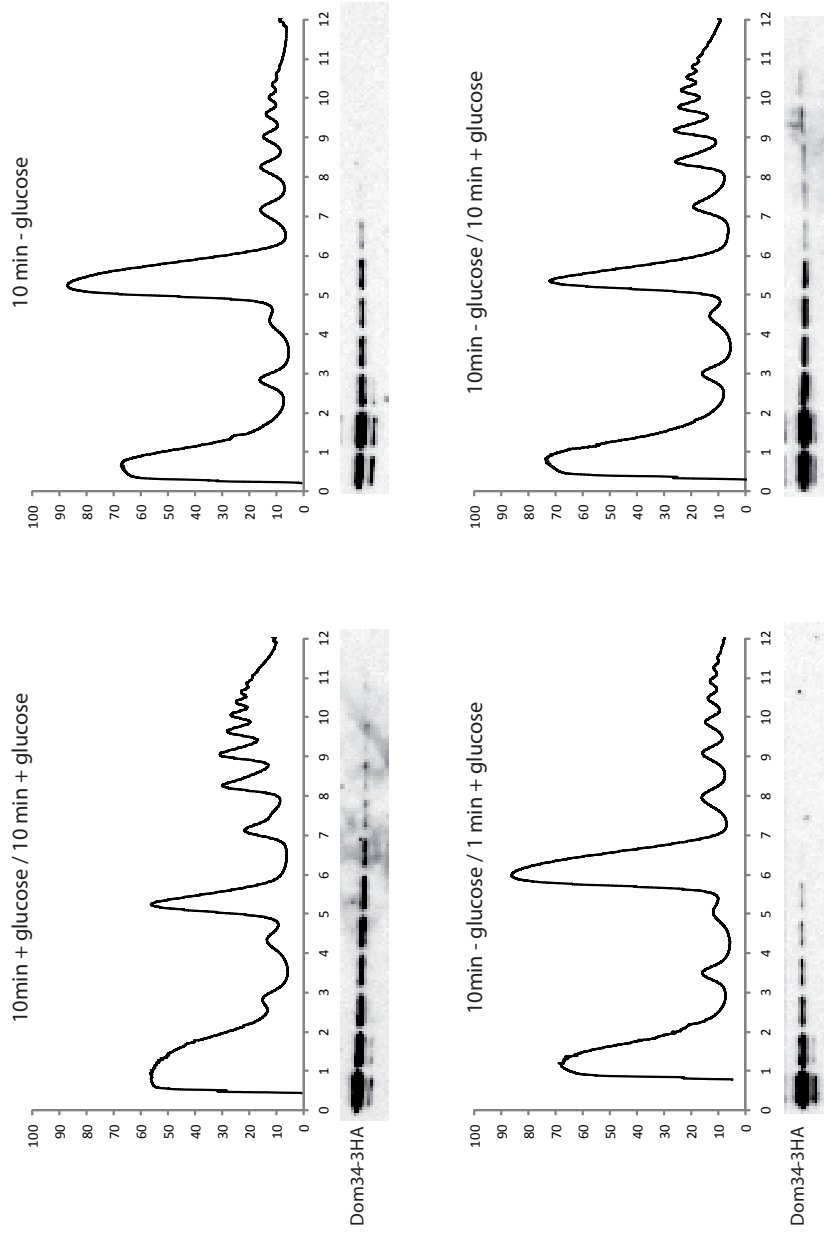
4. The experiment in Fig. 5C would be more thorough if addition of Dom34 alone in the in vitro experiment is also tested.

We have reproduced the figure, now including a condition with Dom34 alone present.

5. Page 12, bottom, unclear sentence: "both Dom34 and Hbs1 are important for normal growth of yeast strains with (double mutant strains showing?) reduced amounts of 40S subunits likely because these double mutant strains have too few ribosomes available to function."

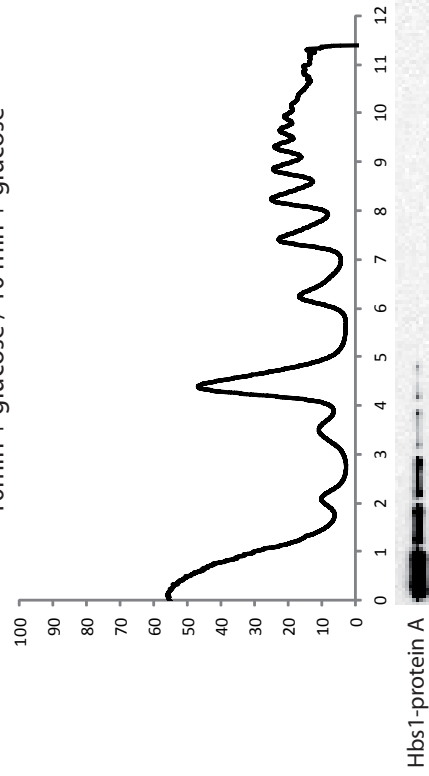
We changed the sentence.

dom34Δ + Dom34-3HA

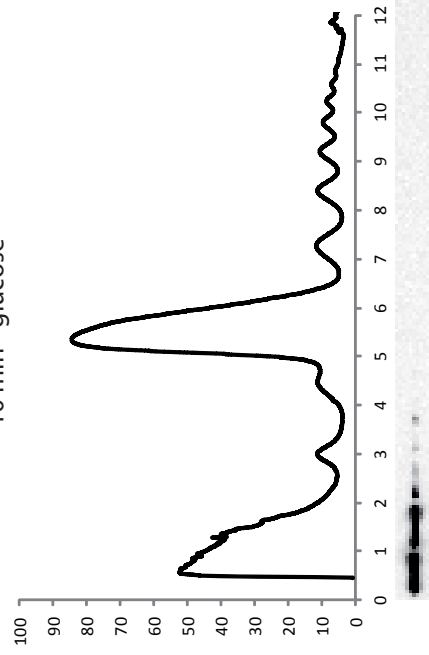


hbs1 Δ + Hbs1-protein A

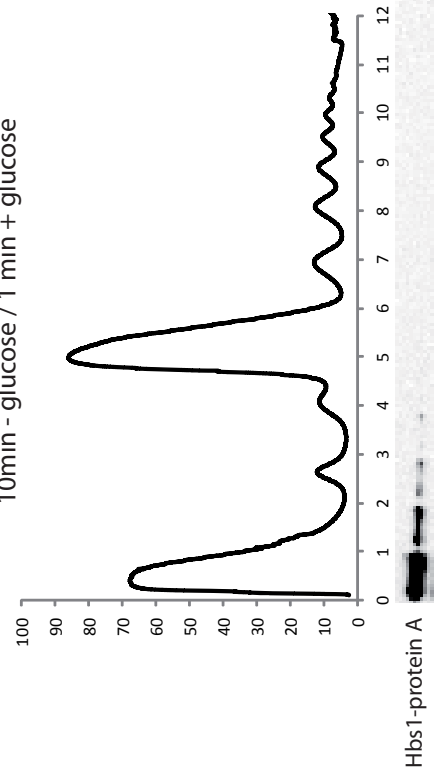
10min + glucose / 10 min + glucose



10 min - glucose



10min - glucose / 1 min + glucose



10min - glucose / 10 min + glucose

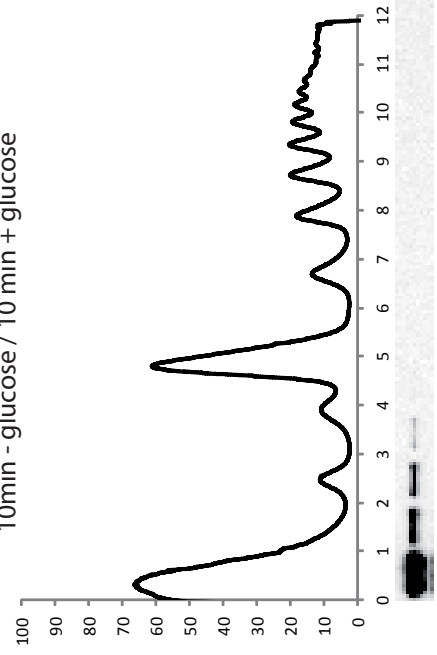
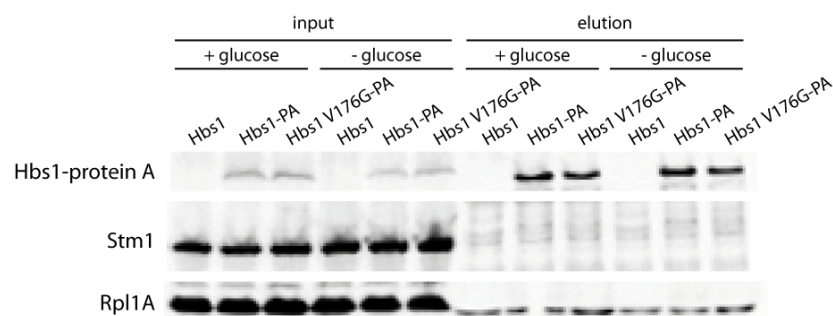


Figure A: Distribution of Dom34 and Hbs1 across polysome gradients

Polysome profiles obtained from the indicated yeast strains expressing Dom34-3HA or Hbs1-Protein A from a plasmid (controlled by their endogenous promoters). Equal size fractions were collected using an ISCO Teledyne Foxy Jr. gradient collector. The presence of Dom34-3HA or Hbs1-Protein A in each fraction was analyzed by western blotting, using anti-HA antibody (Covance MMS-101P) and PAP (Sigma P1291) respectively.

**Figure B: Interaction of Hbs1 with Stm1 containing ribosomes could not be detected.**

hbs1Δ yeast, expressing the indicated Hbs1 (wild type or mutant, tagged or not tagged) from its endogenous promoter on plasmid, was either glucose starved for 10 minutes or not glucose starved. Hbs1-protein A was precipitated using IgG Sepharose 6 Fast Flow (GE Healthcare). Input and elution of each precipitation was analyzed by western blot, detecting Stm1 and Rpl1A by polyclonal antibodies and Hbs1-protein using PAP (Sigma P1291).

Thank you for submitting your revised manuscript to The EMBO Journal, it has now been seen by two of the original referees whose comments are shown below.

As you will see the referees both find that all concerns raised have been sufficiently addressed and

they support publication of the revised manuscript. I am therefore happy to inform you that your study has been accepted for publication in *The EMBO Journal*.

REFEREE REPORTS:

Referee #2:

The authors have responded to my comments in a satisfactory manner.

Referee #3:

In this revised manuscript, the authors have adequately addressed the concerns raised by reviewers. This reviewer has no further comments and believes that the manuscript is suitable for publication in *EMBO J*.