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TORC1 phosphorylates and inhibits the ribosome preservation factor Stm1 to activate dormant ribosomes

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(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. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

Thank you again for submitting your manuscript to The EMBO Journal. Please excuse the delay in getting back to you with a decision, which was due to a delayed referee report as mentioned. We have now however received four reports on your study, which are copied below. In light of the overall positive referee comments, we invite you to prepare and submit a revised manuscript.

As you will see, all referees appreciate the findings and their interest to the field. Nonetheless, they do raise several points that should be addressed in the revised version of the manuscript. In particular the phosphorylation of Stm1 by TORC1 and its functional role should be further strengthened (ref #1- point 2; ref #2- points 1, 2, 3, 4; ref #3- point 11). Furthermore, referee #1's major concern 6 regarding the formation of non-translating 80S ribosomes vs. stabilization of dormant 80S ribosomes should be addressed, either experimentally or by critical discussion. In addition, please also revise the polysome profiling figures, as three referees bring this issue up on different instances and include all requested additional information on methodologies and controls. In our view, it will not be essential for publication to experimentally test conservation of all aspects of the model in mammalian cells (i.e. ref #3- point 1, 3, 10, 11). However, please carefully consider these points and include experimental data where ever possible and revise text and discussion to accurately reflect available data vs. discussion. Finally, please also carefully consider all other referee comments and revise the manuscript and figures as needed, as well as providing a detailed response to each comment. Please also remember that the revised manuscript must fulfill all EMBO Journal formatting requirements when it is next submitted (please see below and: https://www.embopress.org/page/journal/14602075/authorguide#submissionofrevisions). In the current submission, the referenced Table S2 is for example missing and no Table S1 is present, please update this and also remember to provide a list of yeast strains with genotypes. As noted again below, a data availability section must furthermore be included and all datasets have to be accessible to the referees during the revision (either using a token/link or set to public).

Referee #1:

In this manuscript Shetty et al., provide evidence for the evolutionary conserved mechanism of regulation/preservation of dormant ribosomes. Specifically, the authors report that that in yeast, inhibition of TORC1 is paralleled by Stm1-dependent formation of dormant 80S ribosomes, whereby Stm1 protects their integrity. The authors' model is further substantiated by the findings demonstrating that upon activation, TORC1 phosphorylates and suppresses Stm1, thereby stimulating protein synthesis. Finally, Shetty et al., provide data suggesting that mammalian ortholog of Stm1, SERBP1 also participates in dormant 80S ribosome assembly in mTORC1-dependent manner. Overall, it was found that this manuscript is of potentially broad interest inasmuch as it provides previously unappreciated insights into the mechanisms underpinning ribosome dormancy and more generally translational regulation. Altogether, it was thought that the results adequately support major conclusion of the article whereby the quality of the data was found to be optimal. Notwithstanding these clear strengths of the manuscript, several relatively minor issues were observed. My specific comments and concerns are outlined below.
Major comments

- In addition to disrupting the non-specific interactions of proteins with ribosomes, high KCL concentrations are known to disrupt 80S ribosomes that are not associated with mRNA (e.g., J Biol Chem 1970 Mar 25;245(6):1504-6.). To this end, it may be pertinent to show UV tracings of gradients (polysome profiles) prepared in the presence of different KCL concentrations (i.e., polysome profiles corresponding to the Western blots shown in Supp Fig 1C) and use higher KCL concentrations (0.8mM to 1M) in experiments presented in figure 1 to distinguish between mRNA associated and mRNA-free 80S ribosomal subunits that are assumed to be dormant. For instance, if the authors predictions are correct, 0.8-1M KCL should diminish 80S peak in experiments in figures 1D, E and F.
-In figure 3A, it is not clear why mTORC1 was used in the kinase assay with Stm1 and not TORC1. The authors should clarify this. In addition, the phosphorylation of the AAA Stm1 mutant is barely reduced, and under these conditions it appears that the 50KDa band, which is referred to as "auto-phosphorylation of a mTORC1 component" is reduced to the same extent thus suggesting that the activity of mTORC1 may be lower in the AAA Stm1 mutant vs. WT Stm1 in vitro kinase assay. INK128 data are convincing, but nonetheless, I would suggest toning down the claims based on the in vitro kinase experiment shown if figure 3A. In addition, the plausibility that AAA or EEE mutation may affect the folding of Stm1 and thus its function should also be considered and/or commented on.

-In Fig. 3D the polysome profiles obtained in Stm1 mutants are compared to those from WT but it appears that this was only done under rapamycin treatment. It was thought that making latter comparison under control conditions is warranted as this would allow estimating the effects of Stm1 mutation on 80S and polysome assembly at baseline.
-In figure S4A there appears to be 3 peaks (one seems to be cut off), whereas there are only 2 peaks in figure S4B. The authors should label the peaks and show the full absorbance profile for figure S4A. In addition, Stm1 is potentially implicated in ribosome biogenesis (DOI:https://doi.org/10.1016/j.celrep.2022.110353). Although the time scales of the treatments are likely to be too short to exert significant impact on polysome profiles via modulation of ribosome biogenesis, it may be pertinent to discuss this point especially considering the established role of TORC1 in the latter process.

- It was previously reported that rapamycin reduces Stm1 levels (Van Dyke, 2006). Consistent with this, 24h rapamycin treatment decreased Stm1 levels substantially (e.g., Fig.4J). This should be clearly pointed out and discussed. Does 24h rapamycin treatment decrease the amount of dormant monosomes compared to the shorter treatment, and does shorter rapamycin treatment decrease Stm1 levels?
-Based on provided data it remains unclear whether Stm1 stimulates formation of non-translating 80S ribosomes, or whether it may stabilize and/or prevent disassembly of dormant 80S ribosomes, after their formation. Taking this into account, the authors should perhaps consider rephrasing some of their conclusions.

Minor comments
-The authors should consider adding a reference for the sentence on the page 3, paragraph 2, "In budding yeast, TORC1 comprises TOR1/TOR2, Kog1, and Lst8 while mammalian TORC1 (mTORC1) consists of mTOR, the Kog1 ortholog RAPTOR, and mLST8."
-Type of statistical tests that were used should be clearly indicated in figure legends (e.g., Figs. 5E, 6H, 6J, S6D).

I hope that the authors will find my comments constructive and of sufficient pathos

Sincerely

I/Topisirovic

Referee #2:

The translational capacity of the cell tightly linked to nutrient availability, a process directly regulated by the TOR kinase pathway. Previous work has demonstrated that under starvation yeast ribosomes put into a hibernating via interaction with a conserved protein Stm1. This trick was employed in the past by the Yusupov group to isolate empty 80S ribosomes and solve a high resolution structure, which revealed how Stm1 locked the two ribosomal subunits. Yet, how ribosomal subunits are released upon nutrient availability remained unknown.

Here, Shetty et al. suggest that Stm1 is a direct substrate of the TORC1 kinase complex. They provide evidence that phosphorylation of specific residues within Stm1 license the breakdown of the hibernated 80S state and consequently the release of the ribosomal subunits into the translating pool. In my opinion, this is the major finding of the manuscript, and is of strong interest to the translation and signalling communities. Technically the work has been carefully carried, and the experiments are well-controlled and planned. The data are clean and clear cut.

Concerns:

1. This reviewer is concerned about the use of recombinant protein alone (in the absence of ribosomal subunits) to determine TORC1 phosphorylation sites of Stm1. According to the model presented, the right substrate for TORC1 kinase complex is the 80S bound to Stm1, the hibernating ribosome. The authors need to demonstrate phosphorylation of the Stm1 residues in vitro using the 80S-Stm1 complex isolated from yeast under starvation conditions. This is a key experiment that should nail the importance of TORC1 in activating the translational capacity of the cell.
2. The authors base their major conclusion using two Stm1 mutants (phospho-deficient/mimic). Are the yeast strains expressing Stm1-AAA and Stm1-EEE rapamycin sensitive? This is also a clear prediction from the model.
3. What are the protein levels of Stm1-AAA and Stm1-EEE mutants in vivo? This control is important.
4. Does increased expression of Stm1-AAA and Stm1-EEE impact the recovery from starvation in a dominant manner?
5. It would be useful to depict a structure of the 80S ribosome bound to Stm1 and point out the residues that are modified by TORC1. This reviewer had a quick look at the 80S-Stm1 structure and the phospho-sites, and indeed the position of the modifications could point to a mechanism by which the ribosomal subunits are unlocked from the hibernating state.

Referee #3:

In the manuscript titled "TORC1 phosphorylates and inhibits the ribosome preservation factor Stm1 to activate dormant ribosomes", the authors identified a new TORC1 substrate Stm1 (SERBP1 in mammalian cells) which can be phosphorylated at multiple sites and inhibited by TORC1. Upon TORC1 suppression or nutrient starvation, Stm1 forms dormant 80S ribosomes and therefore protects them from proteasomal degradation which subsequently contributes to translation resumption upon re-feeding. Overall, the manuscript was written and described clearly and reveals a potentially important regulation of mTORC1 signaling in protein translation. This manuscript could be improved by addressing the following comments.

Comment for the authors to address:

1. The authors should consider making similar phosphorylation-site mutations in SERBP1 for functional conservation of mTORC1-regulated phosphorylation.

2. In figures 1F, S1F, 2D, the authors use cycloheximide. As this treatment can promote the accumulation of AA's, the authors should check if TORC1 is activated under these conditions. This might confound interpretation of this data. Also, there is no Fig. S1F (referenced on pg 4).

3. Does SERBP1 knockdown in HEK293T suppress cell proliferation as in yeast?

4. The authors show that Stm1 mainly binds to 80S when mTORC1 is inhibited such as with rapamycin or by nitrogen starvation (Figures 1D and E). The authors should show that adding back nitrogen would rescue Stm1 distribution in polysome profiling.

5. Overall, polysome profiling figures (Ex: Figure. 2 A-E and others) need the y axis properly labeled so that the reader can compare whether the various treatments affect 40S, 60S, 80S, and polysome amounts.

6. Can the authors provide measurements of mTORC1 activity with markers (phospho-antibodies?) in yeast treated with rapamycin or during nitrogen starvation and restimulation?

7. The Stm1 region of 9-40 binds to 60S subunits and 60-141 associates with 40S subunits. But the polysome profiling patterns in Figure. S2 are very similar between Δ2-40 and Δ81-120 mutants. The authors should discuss this. Along these lines, deletion of 60-141 should not bind to 40S subunits, however, in figure. S2, the western blotting analysis shows that Δ41-80 and Δ81-120 still bind to 40S. Please clarify.

8. In Figure. 4J, Bortezomib treatment rescued Rpl3 ribosome levels upon rapamycin treatment. In figure S4 N, Rpl3 levels were further decreased by Bortezomib after GFP-IP. The authors need to explain this.

9. In Figure 4E, the technical details of the method that the authors used to monitor ribophagy should be described with greater detail as well as the showing differences in normal vs starved or rapamycin treatment.

10. In yeast, rescue of Stm1 decreased ubiquitination in the ribosome pellet (Figure. 4H). Including a SERBP1 rescue experiment would highlight the important role and conservation of SERBP1 in regulating protein synthesis.

11. Stm1 AAA mutant cells displayed delayed recovery of translation similar with delta-Stm1 and Stm1 EEE mutant (Figure 5A,

B) despite higher levels of ribosomes. Please explain why? Since the authors already claimed that Stm1 had no role in normally growing cells, this also raises another critical question: what are the biological consequences of TORC1/Stm1 phosphorylation if AAA and EEE mutants exhibit the same level of translational resumption upon re-feeding?

12. In figure 6, can the authors please provide a schematic of Stm1 vs. SERBP1 with its various domains illustrated so that the authors can discuss, and the reader better understand, the potential evolutionary conservation and function of all the TORC1-regulated phosphorylation sites for each region. Additionally, it would be important to show whether the phosphorylation sites in SERBP1 are conserved with the yeast Stm1 sites by showing the phosphorylation site sequences. Are the SERBP1 sites in the linker region spanning the space between the 40S and 60S subunits the same as for Stm1?

Referee #4:

The study by Shetty and colleagues examines how TORC1 regulates the status of dormant 80S ribosomes facilitated by yeast protein Stm1. Stm1 binds in the intersubunit space of 80S ribosomes to preserve them 80S in the absence of mRNA and tRNA during starvation and other stresses. Functional interaction between rapamycin and Stm1 in earlier studies suggested involvement of TOR pathways in Stm1 regulation, but the mechanisms of such involvement have been unexplored.
This work reveals that TORC1 is required to phosphorylate Stm1 to reduce its association with the ribosome and thus resume translation. Several phosphorylation sites are identified on Stm1, including those accessible within the 80S*Stm1 complex (serines 41, 45, and 55), and mutational analyses are used to test them. Phosphomimetic mutant (Stm1EEE) phenotype is similar to Delta-Stm1 in that it disrupts 80S*Stm1 formation, further corroborating the importance of the phosphorylation in inhibiting dormant 80S formation. The authors then use cell assays with Stm1 mutants/deletion to show that Stm1 binding to 80S ribosomes during stress is required to protect 80S from ubiquitinylation and proteasomal degradation. Finally, the study demonstrates that the mammalian homolog of Stm1 (SERBP1) is also regulated by TORC1, underscoring the evolutionarily conserved mechanism. Overall, this is a well-executed and clearly presented work, supported by experimental data reflected in well-organized figures. I support the publication of this manuscript.

Referee #1:

In this manuscript Shetty et al., provide evidence for the evolutionary conserved mechanism of regulation/preservation of dormant ribosomes. Specifically, the authors report that that in yeast, inhibition of TORC1 is paralleled by Stm1-dependent formation of dormant 80S ribosomes, whereby Stm1 protects their integrity. The authors' model is further substantiated by the findings demonstrating that upon activation, TORC1 phosphorylates and suppresses Stm1, thereby stimulating protein synthesis. Finally, Shetty et al., provide data suggesting that mammalian ortholog of Stm1, SERBP1 also participates in dormant 80S ribosome assembly in mTORC1-dependent manner. Overall, it was found that this manuscript is of potentially broad interest inasmuch as it provides previously unappreciated insights into the mechanisms underpinning ribosome dormancy and more generally translational regulation. Altogether, it was thought that the results adequately support major conclusion of the article whereby the quality of the data was found to be optimal. Notwithstanding these clear strengths of the manuscript, several relatively minor issues were observed. My specific comments and concerns are outlined below.

We are glad the reviewer found our work is of broad interest to the readership of the *EMBO Journal*. We also thank the reviewer for the constructive comments.

Major comments
- In addition to disrupting the non-specific interactions of proteins with ribosomes, high KCL concentrations are known to disrupt 80S ribosomes that are not associated with mRNA (e.g., J Biol Chem 1970 Mar 25;245(6):1504-6.). To this end, it may be pertinent to show UV tracings of gradients (polysome profiles) prepared in the presence of different KCL concentrations (i.e., polysome profiles corresponding to the Western blots shown in Supp Fig 1C) and use higher KCL concentrations (0.8mM to 1M) in experiments presented in figure 1 to distinguish between mRNA associated and mRNA-free 80S ribosomal subunits that are assumed to be dormant. For instance, if the authors predictions are correct, 0.8-1M KCL should diminish 80S peak in experiments in figures 1D, E and F.

We agree with the reviewer that high KCl (in our case 300 mM) disrupts empty 80S ribosomes. As requested, we now show polysome profiles of control and nitrogen starved cells, using different KCl concentrations (50, 150, 300 and 800 mM KCl). We also show the corresponding western blots of Stm1 and Rpl3, in Fig EV1C. As the reviewer predicted, high salt concentrations (300 and 800 mM KCl) disrupted empty 80S ribosomes formed upon nitrogen starvation while 50 and 150 mM KCl had no effect on such empty ribosomes. We have discussed these observations in the Results section (Page 4).

-In figure 3A, it is not clear why mTORC1 was used in the kinase assay with Stm1 and not TORC1. The authors should clarify this. In addition, the phosphorylation of the AAA Stm1 mutant is barely reduced, and under these conditions it appears that the 50KDa band, which is referred to as "auto-phosphorylation of a mTORC1 component" is reduced to the same extent thus suggesting that the activity of mTORC1 may be lower in the AAA Stm1 mutant vs. WT Stm1 in vitro kinase assay. INK128 data are convincing, but nonetheless, I would suggest toning down the claims based on the in vitro kinase experiment shown if figure 3A. In addition, the plausibility that AAA or EEE mutation may affect the folding of Stm1 and thus its function should also be considered and/or commented on.

The catalytic centre of TORC1 is conserved from yeast to mammals. Hence, we used available recombinant mTORC1 for the in vitro kinase assays. We do not have purified yeast TORC1 available. We note that merely immunoprecipitating yeast TORC1 would not yield sufficiently pure TORC1 for the experiment in question.

We have noticed that autophosphorylation of TORC1 is also reduced in the presence of mutant Stm1. Although we do not know the reason for this, it is possible that non-

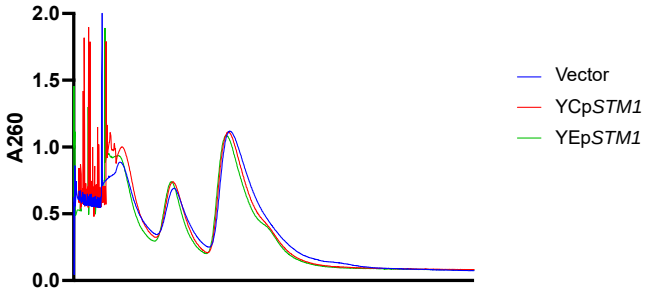
phosphorylated substrate might be interfering with TORC1 activity. Furthermore, we agree with the reviewer that the weak decrease in the phosphorylation of Stm1^{AAA} is likely due to the presence of other phosphorylation sites on Stm1. We have mentioned this point in the text (Page 6, para 2). In addition, as the reviewer recommends, we have toned down the claims based on the in vitro kinase experiment (Page 6, para 2). We also mentioned the potential effect of phosphorylation on the structure of Stm1 (Discussion para 2, page 11).

-In Fig. 3D the polysome profiles obtained in Stm1 mutants are compared to those from WT but it appears that this was only done under rapamycin treatment. It was thought that making latter comparison under control conditions is warranted as this would allow estimating the effects of Stm1 mutation on 80S and polysome assembly at baseline.

Since Stm1 is important mainly, if not exclusively, under TORC1 inhibited conditions, we previously showed ribosome profiles only from rapamycin-treated cells (Fig 3D). In the revised manuscript, we now include the polysome profiles of untreated control and Stm1 mutant cells (Fig EV3D). The profiles of rapamycin-untreated control and Stm1 mutant cells are indistinguishable, again suggesting that Stm1 is important only upon TORC1 inhibition.

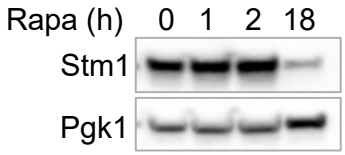
-In figure S4A there appears to be 3 peaks (one seems to be cut off), whereas there are only 2 peaks in figure S4B. The authors should label the peaks and show the full absorbance profile for figure S4A. In addition, Stm1 is potentially implicated in ribosome biogenesis (DOI:https://doi.org/10.1016/j.celrep.2022.110353). Although the time scales of the treatments are likely to be too short to exert significant impact on polysome profiles via modulation of ribosome biogenesis, it may be pertinent to discuss this point especially considering the established role of TORC1 in the latter process.

The first peak in Fig. S4A (Fig EV4A in the revised manuscript) is a characteristic ribosome free peak. For the reviewer, we show the full polysome profile below (not included in the manuscript). As per the reviewer's suggestion, we have now labelled the peaks in the manuscript. Also, since we did not see any ribosomal defect in Stm1 mutant cells grown in normal conditions (Fig EV3D), it is unlikely that Stm1 has a role ribosome biogenesis.

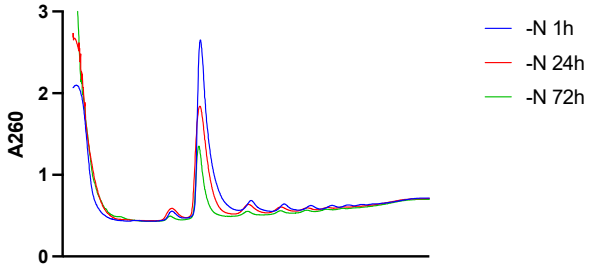


- It was previously reported that rapamycin reduces Stm1 levels (Van Dyke, 2006). Consistent with this, 24h rapamycin treatment decreased Stm1 levels substantially (e.g., Fig.4J). This should be clearly pointed out and discussed. Does 24h rapamycin treatment decrease the amount of dormant monosomes compared to the shorter treatment, and does shorter rapamycin treatment decrease Stm1 levels?

As described by Van Dyke, we also observed a gradual decrease in Stm1 upon long-term TORC1 inhibition (Fig. EV4J). As suggested by the reviewer, we have included this point in the discussion (Page 12, para 1). However, short-term TORC1 inhibition did not affect Stm1 levels (see figure below, not included in the manuscript).



The level of dormant 80S ribosomes gradually decreased during starvation, as turn-over of ribosomes is an important source of nutrients during starvation (see figure below). These data are shown for the reviewer's reference.



-Based on provided data it remains unclear whether Stm1 stimulates formation of non-translating 80S ribosomes, or whether it may stabilize and/or prevent disassembly of dormant 80S ribosomes, after their formation. Taking this into account, the authors should perhaps consider rephrasing some of their conclusions.

We agree that our data do not clearly distinguish whether Stm1 stimulates the formation of dormant 80S ribosomes or just stabilizes 80S ribosomes. To address this concern, we analyzed the level of dormant 80S ribosomes upon formaldehyde cross-linking. In vivo trapping of dormant ribosomes by formaldehyde cross-linking would tell us whether dormant ribosomes are formed in vivo in the absence of Stm1. In agreement with our hypothesis, we found decreased levels of dormant ribosomes in the absence of Stm1 (Figure 2E). In Figure 2F, the same formaldehyde cross-linked cell-extracts were analyzed in 300 mM KCl containing sucrose density gradient to confirm that the dormant ribosomes are stabilized by formaldehyde unlike formaldehyde untreated dormant 80S ribosomes (Figure EV1C). These data suggest that Stm1 is required for the formation of dormant 80S ribosomes in vivo. If Stm1 was only involved in stabilization of dormant 80S ribosomes, formaldehyde cross-linking should have stabilized these dormant ribosomes in the absence of Stm1. We have discussed this in the results section (Page 5).

Minor comments
-The authors should consider adding a reference for the sentence on the page 3, paragraph 2, "In budding yeast, TORC1 comprises TOR1/TOR2, Kog1, and Lst8 while mammalian TORC1 (mTORC1) consists of mTOR, the Kog1 ortholog RAPTOR, and mLST8."
Correction included.

-Type of statistical tests that were used should be clearly indicated in figure legends (e.g., Figs. 5E, 6H, 6J, S6D).
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I hope that the authors will find my comments constructive and of sufficient pathos
Sincerely

I/Topisirovic

Referee #2:

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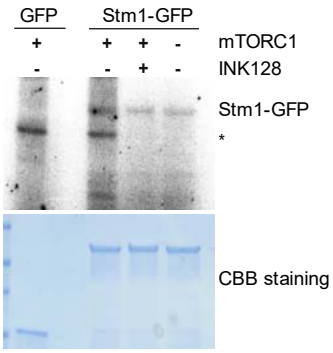
Here, Shetty et al. suggest that Stm1 is a direct substrate of the TORC1 kinase complex. They provide evidence that phosphorylation of specific residues within Stm1 license the breakdown of the hibernated 80S state and consequently the release of the ribosomal subunits into the translating pool. In my opinion, this is the major finding of the manuscript, and is of strong interest to the translation and signalling communities. Technically the work has been carefully carried, and the experiments are well-controlled and planned. The data are clean and clear cut.

We are glad that the reviewer found our work is of strong interest to the translation and signaling communities. Also, we acknowledge the work of Yusupov's group that has been a key resource for investigation of Stm1.

Concerns:
1. This reviewer is concerned about the use of recombinant protein alone (in the absence of ribosomal subunits) to determine TORC1 phosphorylation sites of Stm1. According to the model presented, the right substrate for TORC1 kinase complex is the 80S bound to Stm1, the hibernating ribosome. The authors need to demonstrate phosphorylation of the Stm1 residues in vitro using the 80S-Stm1 complex isolated from yeast under starvation conditions. This is a key experiment that should nail the importance of TORC1 in activating the translational capacity of the cell.

We have observed that Stm1, including newly made Stm1, is phosphorylated under nutrient-sufficient conditions where there is active translation (Fig S3A). This suggests that free Stm1, which is not bound to dormant ribosomes in growing cells, is phosphorylated by TORC1 and thus inhibited from forming the dormant ribosomes. Hence, we used recombinant Stm1. We agree with the reviewer that ribosome bound Stm1 could also be a substrate of TORC1. However, we do not know whether Stm1 is phosphorylated in 80S ribosomes or upon splitting of the subunits (perhaps mediated by Dom34-Hbs1 complex). Initially, we performed in vitro kinase assays with Stm1-GFP immunoprecipitated from yeast (Figure, see below, not included in the manuscript). Unfortunately, we observed significant phosphorylation of Stm1 even in the absence of added recombinant mTORC1, suggesting co-purification of an unknown kinase in addition to ribosomes. Hence, we used recombinant free Stm1 to show that it is phosphorylated by mTORC1.



2. The authors base their major conclusion using two Stm1 mutants (phospho-deficient/mimic). Are the yeast strains expressing Stm1-AAA and Stm1-EEE rapamycin sensitive? This is also a clear prediction from the model.

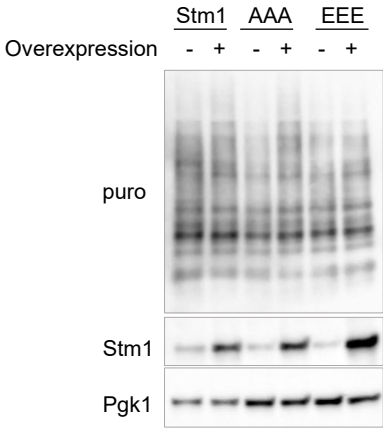
As requested, we have included data on the rapamycin responsiveness of Stm1-AAA and Stm1-EEE strains in the revised manuscript (Fig EV3E). Stm1-AAA cells show no change in rapamycin sensitivity while Stm1-EEE cells show rapamycin hypersensitivity, similar to *stm1*Δ cells. This agrees with our model that Stm1-EEE cells are defective in preserving ribosomes upon TORC1 inhibition.

3. What are the protein levels of Stm1-AAA and Stm1-EEE mutants in vivo? This control is important.

As shown in the Fig 5A and EV5B, expression of Stm1-AAA and Stm1-EEE was similar to that of wild-type Stm1 under TORC1-inhibited or control conditions.

4. Does increased expression of Stm1-AAA and Stm1-EEE impact the recovery from starvation in a dominant manner?

We analyzed the effect of overexpression of Stm1-AAA and Stm1-EEE on translation during recovery (see figure below). We did not see a significant effect of AAA or EEE overexpression on translation during recovery from starvation.



5. It would be useful to depict a structure of the 80S ribosome bound to Stm1 and point out the residues that are modified by TORC1. This reviewer had a quick look at the 80S-Stm1 structure and the phospho-sites, and indeed the position of the modifications could point to a mechanism by which the ribosomal subunits are unlocked from the hibernating state.

We thank the reviewer for analyze the structure of Stm1-bound ribosomes. As suggested, we analyzed the structure of Stm1 bound to ribosomes (Fig. EV3C). The structure indeed suggests that phosphorylation at serine 41 and 55 would sterically interfere with Stm1 binding to ribosomes.

Referee #3:

In the manuscript titled "TORC1 phosphorylates and inhibits the ribosome preservation factor Stm1 to activate dormant ribosomes", the authors identified a new TORC1 substrate Stm1 (SERBP1 in mammalian cells) which can be phosphorylated at multiple sites and inhibited by TORC1. Upon TORC1 suppression or nutrient starvation, Stm1 forms dormant

80S ribosomes and therefore protects them from proteasomal degradation which subsequently contributes to translation resumption upon re-feeding. Overall, the manuscript was written and described clearly and reveals a potentially important regulation of mTORC1 signaling in protein translation. This manuscript could be improved by addressing the following comments.

We thank the reviewer for providing constructive suggestions on our manuscript.

Comment for the authors to address:

1. The authors should consider making similar phosphorylation-site mutations in SERBP1 for functional conservation of mTORC1-regulated phosphorylation.

We believe that this is an important suggestion. However, we find very little sequence homology between Stm1 and SERBP1. Furthermore, the identified phospho-sites on SERBP1 are not in the similar region as in Stm1. Hence, we did not perform a mutational analysis of SERBP1. A detailed investigation of SERBP1 might be of interesting for future study.

2. In figures 1F, S1F, 2D, the authors use cycloheximide. As this treatment can promote the accumulation of AA's, the authors should check if TORC1 is activated under these conditions. This might confound interpretation of this data. Also, there is no Fig. S1F (referenced on pg 4).

We would like to clarify that in figures 1F and 2D we do not use cycloheximide, while in other cases we do use cycloheximide for trapping polysomes on mRNA. We understand the reviewer's concerns from previous literature that cycloheximide treatment indirectly leads to activation of TORC1. However, during polysome profile analysis, cycloheximide treatment is only for 2 minutes prior to harvesting the cells which is, as demonstrated previously, too brief to elicit TORC1 activation. We thank the reviewer for pointing out the incorrect numbering of formerly figure S1D. It is now Fig EV1F.

3. Does SERBP1 knockdown in HEK293T suppress cell proliferation as in yeast?

SERBP1 seems to be essential for cell survival. We were unable to obtain SERBP1 knockout cell lines. Long-term depletion of SERBP1 using siRNAs (after 2 passages) showed a decrease in cell proliferation (data not included). However, for our analysis of dormant ribosomes, we have mainly focused on the early time points post-transfection.

4. The authors show that Stm1 mainly binds to 80S when mTORC1 is inhibited such as with rapamycin or by nitrogen starvation (Figures 1D and E). The authors should show that adding back nitrogen would rescue Stm1 distribution in polysome profiling.

As requested, we show that upon refeeding starved cells, Stm1 is re-distributed to ribosome free and subunit fractions (Fig EV1D).

5. Overall, polysome profiling figures (Ex: Figure. 2 A-E and others) need the y axis properly labeled so that the reader can compare whether the various treatments affect 40S, 60S, 80S, and polysome amounts.

In the revised manuscript, the y-axis of the polysome profiles is labeled to aid comparison.

6. Can the authors provide measurements of mTORC1 activity with markers (phospho-antibodies?) in yeast treated with rapamycin or during nitrogen starvation and restimulation?

TORC1 activity during nitrogen starvation and restimulation is shown in Fig EV5A.

7. The Stm1 region of 9-40 binds to 60S subunits and 60-141 associates with 40S subunits. But the polysome profiling patterns in Figure. S2 are very similar between Δ2-40 and Δ81-120 mutants. The authors should discuss this. Along these lines, deletion of 60-141 should not bind to 40S subunits, however, in figure. S2, the western blotting analysis shows that Δ41-80 and Δ81-120 still bind to 40S. Please clarify.

The statement that Stm1 region 9-40 binds to 60S subunits and 60-141 associates with 40S subunits is based mainly on the 80S ribosome structure. Presence in the polysome fractions may not be the ideal assay for determining ribosome binding. Furthermore, polysome profile-based analysis of binding of Stm1 mutants with the ribosomal subunits is tricky due to overlapping peaks. Hence, we do not claim or discuss binding of Stm1 with individual ribosomal subunits.

To address the concerns raised by the reviewer, the majority of Δ2-40 is in the 40S fraction while Δ41-80 and Δ81-120 mutant Stm1 are in the free fraction, partially supporting the structural data. Although Δ60-141 Stm1 should not bind 40S, partial binding might still be possible in mutants lacking only 40 amino acid regions (Page 5, para 2). Since the results of the deletion analysis are only suggestive, rather than conclusive, we avoid discussing this in detail in the manuscript.

8. In Figure. 4J, Bortezomib treatment rescued Rpl3 ribosome levels upon rapamycin treatment. In figure S4 N, Rpl3 levels were further decreased by Bortezomib after GFP-IP. The authors need to explain this.

Bortezomib treatment rescued the ribosome levels in the immunoblot analysis of total lysates (Fig 4J). However, when we performed the pull-down of the ribosomes, we obtained less immunoprecipitation of ribosomes from bortezomib treated samples. The decrease in Rpl3 levels is consistent with less immunoprecipitation of Rpl24-GFP in bortezomib treated cells. It is possible that polyubiquitylation of ribosomes interferes with the ribosomal-immunoprecipitation. Nevertheless, the data support our main conclusion that in the absence of Stm1 there is increased ubiquitylation of ribosomes (Fig EV4N).

9. In Figure 4E, the technical details of the method that the authors used to monitor ribophagy should be described with greater detail as well as the showing differences in normal vs starved or rapamycin treatment.

Analysis of ribophagy is performed using C-terminally GFP-tagged ribosomal protein Rpl24. In yeast, GFP is not degraded by vacuolar proteases and hence free GFP accumulates upon ribophagy. We have described this in more detail in the methods section. Furthermore, Fig EV4J-K shows ribophagy under normal and rapamycin-treated conditions.

10. In yeast, rescue of Stm1 decreased ubiquitination in the ribosome pellet (Figure. 4H). Including a SERBP1 rescue experiment would highlight the important role and conservation of SERBP1 in regulating protein synthesis.

We were not able to obtain complete knock-out of the SERBP1 in HEK293T cells and a complementation assay under partial knock-down conditions may not give clean results. Due to this technical challenge, we did not pursue the complementation analysis.

11. Stm1 AAA mutant cells displayed delayed recovery of translation similar with delta-Stm1 and Stm1 EEE mutant (Figure 5A, B) despite higher levels of ribosomes. Please explain why? Since the authors already claimed that Stm1 had no role in normally growing cells, this also raises another critical question: what are the biological consequences of TORC1/Stm1

phosphorylation if AAA and EEE mutants exhibit the same level of translational resumption upon re-feeding?

We propose that the inability of $STM1^{AAA}$ cells to efficiently resume translation during refeeding is due to their inefficiency in activation of dormant ribosomes. In the wild-type scenario, Stm1 is phosphorylated by TORC1 and thus can be released from dormant ribosomes while Stm1-AAA are less phosphorylated by TORC1 and thus allow less translation recovery. We have explained this clearly in the revised manuscript. Furthermore, from the current study, the role of TORC1-mediated phosphorylation of Stm1 is mainly to prevent formation of dormant ribosomes.

12. In figure 6, can the authors please provide a schematic of Stm1 vs. SERBP1 with its various domains illustrated so that the authors can discuss, and the reader better understand, the potential evolutionary conservation and function of all the TORC1-regulated phosphorylation sites for each region. Additionally, it would be important to show whether the phosphorylation sites in SERBP1 are conserved with the yeast Stm1 sites by showing the phosphorylation site sequences. Are the SERBP1 sites in the linker region spanning the space between the 40S and 60S subunits the same as for Stm1?

Stm1 and SERBP1 share very little sequence homology. Also, SERBP1 is much larger (408 amino acids) than Stm1 (273 amino acids). Both Stm1 and SERBP1 are disordered proteins and hence structural domains are difficult to demarcate. Also, in the 80S ribosome structure, only a few parts of SERBP1 have been modelled. Thus, it is difficult to elucidate the domains and conservation of phospho-sites.

Referee #4:

The study by Shetty and colleagues examines how TORC1 regulates the status of dormant 80S ribosomes facilitated by yeast protein Stm1. Stm1 binds in the intersubunit space of 80S ribosomes to preserve them 80S in the absence of mRNA and tRNA during starvation and other stresses. Functional interaction between rapamycin and Stm1 in earlier studies suggested involvement of TOR pathways in Stm1 regulation, but the mechanisms of such involvement have been unexplored.
This work reveals that TORC1 is required to phosphorylate Stm1 to reduce its association with the ribosome and thus resume translation. Several phosphorylation sites are identified on Stm1, including those accessible within the 80S*Stm1 complex (serines 41, 45, and 55), and mutational analyses are used to test them. Phosphomimetic mutant (Stm1EEE) phenotype is similar to Delta-Stm1 in that it disrupts 80S*Stm1 formation, further corroborating the importance of the phosphorylation in inhibiting dormant 80S formation. The authors then use cell assays with Stm1 mutants/deletion to show that Stm1 binding to 80S ribosomes during stress is required to protect 80S from ubiquitinylation and proteasomal degradation. Finally, the study demonstrates that the mammalian homolog of Stm1 (SERBP1) is also regulated by TORC1, underscoring the evolutionarily conserved mechanism. Overall, this is a well-executed and clearly presented work, supported by experimental data reflected in well-organized figures. I support the publication of this manuscript.

We thank the reviewer for finding our work suitable for publication in the EMBO journal.

Thank you again for submitting your revised manuscript to The EMBO Journal. As briefly discussed, we received comments from all of the initial referees on the revised version (copied again below), and while most referees are satisfied with your response, referee #2 still has major concerns that must be resolved before we can proceed further. Therefore, I would ask you to please address these remaining comments (in particular point 2 and 4) in an exceptional second round of revision. Please incorporate the data into the revised manuscript, or provide it as part of the point-by-point response (for example additional repeats of panels). In addition, I would also ask you to please address a number of editorial issues listed below. Please use the data editor's document for all changes, keeping the "track changes" option active (please see below for details).

Referee #1:

I thought that the authors have addressed all my comments in a satisfactory manner and I have no further concerns. To this end, I would like to congratulate the authors on what I found to be a quite elegant study and an important discovery.

Sincerely,

I/Topisirovic

Referee #2:

Response to the Rebuttal letter

1. While the authors claim that the 80S:Stm1 complex is a target of TORC1, they are not able to demonstrate this (Concern Point 1). Yet, the key conclusion according to the abstract (and summarized in Fig. 6L) is that, and I quote: "Upon re-feeding, TORC1 directly phosphorylates and inhibits Stm1, thereby reactivating translation". Given that there is no direct support for their conclusions and model presented, can the authors at least show that the Stm1 phosphorylation at these serine's are altered upon rapamycin treatment for example by targeted mass spectrometry-based assays.

2. The major conclusions of this work rely on two Stm1 mutants (Stm1-AAA and Stm1-EEE). I had previously requested to investigate whether these mutants are rapamycin sensitive or not. Given that stm1Δ cells are rapamycin sensitive, according to the proposed model it is expected that Stm1-EEE should be rapamycin sensitive. The authors now claim that the Stm1-EEE expressing mutant is rapamycin sensitive, and the Stm1-AAA expressing mutant is not. While Fig. EV2 F clearly demonstrates the rapamycin sensitivity of the stm1Δ mutant, Fig. EV3 E does not. Can the authors explain this? Based on the spot assays, I cannot conclude that Stm1-EEE is rapamycin sensitive. Therefore, these data are not supportive of the model presented.
Is this because the Stm1-EEE allele is expressed from a centromeric plasmid? Given that Stm1 overexpression renders yeast strains rapamycin sensitive, it is best to integrate the alleles in the genome rather than use centromeric plasmids to get a clear-cut result.

3. This is fine.

4. Does increased expression of Stm1-AAA and Stm1-EEE impact the recovery from starvation in a dominant manner?
It appears that the authors have misunderstood my concern. Since overexpression of Stm1 also renders yeast rapamycin sensitive I would like the authors investigate the impact of overexpressing Stm1-AAA and Stm1-EEE alleles on the rapamycin sensitivity of yeast. In this case, one might expect Stm1-AAA overexpression would render wild-type yeast rapamycin sensitive, whereas Stm1-EEE not (in a dominant manner i. e. despite the WT Stm1 is around). This could be an alternative way to support their conclusions.

5. This is fine.

Referee #3:

Minor points:

1. There is no EV1F figure. (on Page 4 it is mentioned; "Furthermore, inhibition of TORC1 by rapamycin or nitrogen starvation led to an accumulation of Stm1-bound 80S ribosomes (Fig 1D, 1E, and EV1F)."

2. Some polysome profiling figures need y-axis labels including Fig. 2G; Fig. EV2B.

3. Fig EV4. G, y-axis scale font is different from other figures.

4.Fig. EV1. C, the decimal point is different from other figures.

5. In page 9, the authors mentioned Fig. 6B and Fig. EV 6A that inhibition of mTOR with INK128 caused SERBP1 to accumulate in the 80S fraction.
But there is no Fig. EV 6A.

Referee #4:

The manuscript has improved after the authors' addressing the criticisms. I support publication of this work.

Referee #1 (Report for Author)

I thought that the authors have addressed all my comments in a satisfactory manner and I have no further concerns. To this end, I would like to congratulate the authors on what I found to be a quite elegant study and an important discovery.

Sincerely,
I/Topisirovic

Thank you for the comments.

Referee #2 (Report for Author)

Response to the Rebuttal letter

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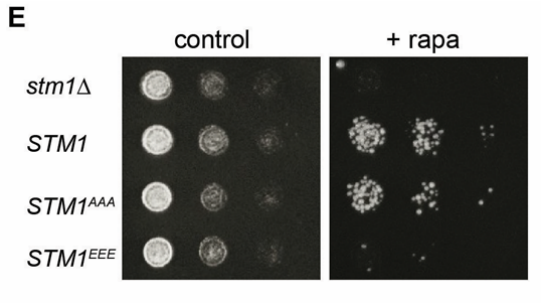
We agree with the reviewer that our study does not conclusively show that 80S:Stm1 complex is a direct target of TORC1. However, our conclusion "Upon re-feeding, TORC1 directly phosphorylates and inhibits Stm1, thereby reactivating translation" is based on the facts that Stm1 is directly phosphorylated by TORC1 in vitro (Fig 3A), Stm1 binding to 80S is reduced upon refeeding (Fig EV1D), and phosphorylation of Stm1 inhibits its binding to 80S ribosomes (Fig 3H). In the revised version, we had shown that phosphorylation of Stm1 at serine 41, 45 and 55 is reduced upon rapamycin treatment (EV3A and B).

2. The major conclusions of this work rely on two Stm1 mutants (Stm1-AAA and Stm1-EEE). I had previously requested to investigate whether these mutants are rapamycin sensitive or not. Given that stm1Δ cells are rapamycin sensitive, according to the proposed model it is expected that Stm1-EEE should be rapamycin sensitive. The authors now claim that the Stm1-EEE expressing mutant is rapamycin sensitive, and the Stm1-AAA expressing mutant is not. While Fig. EV2 F clearly demonstrates the rapamycin sensitivity of the stm1Δ mutant, Fig. EV3 E does not. Can the authors explain this? Based on the spot assays, I cannot conclude that Stm1-EEE is rapamycin sensitive. Therefore, these data are not supportive of the model presented.
Is this because the Stm1-EEE allele is expressed from a centromeric plasmid? Given that Stm1 overexpression renders yeast strains rapamycin sensitive, it is best to integrate the alleles in the genome rather than use centromeric plasmids to get a clear-cut result.

We realized that the reviewer's concern is based on comparing figures EV2F and EV3E, wherein EV2F shows complete loss of growth of stm1Δ cells while EV3F shows only partial reduction. This variability occurs due to the variation in the rapamycin concentration (4 ng/mL versus 6ng/mL) used in the study. To avoid any confusion, we show another replicate of the spot assay in Fig EV3E.



3. This is fine.

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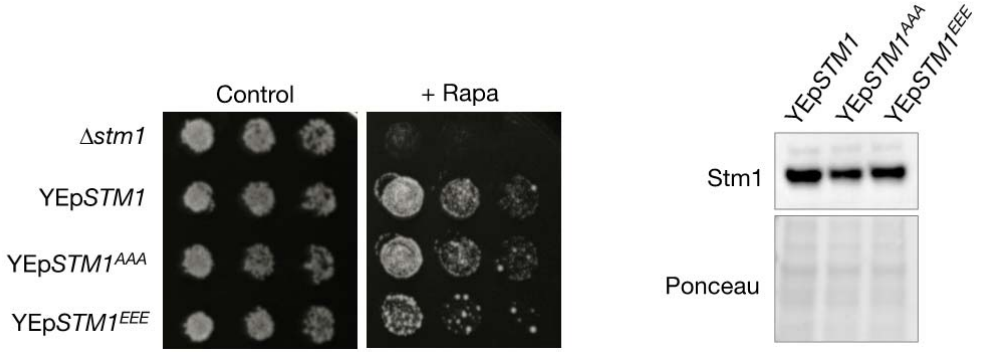
We apologize for misunderstanding the concern raised by the reviewer. We have performed the spot assay for rapamycin sensitivity upon overexpression of Stm1 mutants (See below). Overexpression of $Stm1^{EEE}$ still shows partial rapamycin hypersensitivity (rapamycin 6ng/mL 60 hours) compared to wild-type or $Stm1^{AAA}$ mutant.



5. This s fine.

Referee #3 (Report for Author)

Minor points:

1. There is no EV1F figure. (on Page 4 it is mentioned; "Furthermore, inhibition of TORC1 by rapamycin or nitrogen starvation led to an accumulation of Stm1-bound 80S ribosomes (Fig 1D, 1E, and EV1F)."
Thank you for noting the mistake. Fig EV1F refers to EV1E. Correction is included.

2. Some polysome profiling figures need y-axis labels including Fig. 2G; Fig. EV2B.
Correction is incorporated

3. Fig EV4. G, y-axis scale font is different from other figures.
Correction is incorporated

4. Fig. EV1. C, the decimal point is different from other figures.
Correction is incorporated

5. In page 9, the authors mentioned Fig. 6B and Fig. EV 6A that inhibition of mTOR with INK128 caused SERBP1 to accumulate in the 80S fraction.
But there is no Fig. EV 6A.
Thank you for noting the mistake. Fig EV6A refers to EV5I. Correction is included.

Referee #4 (Report for Author)

The manuscript has improved after the authors' addressing the criticisms. I support publication of this work.

Thank you for considering our work suitable for publication.

Thank you for submitting your revised manuscript to The EMBO Journal. As you know, we sent it back to referee #2, who now also supports publication, but noted a remaining concern. As we discussed, we would now give you the opportunity to address this issue (i.e. in an additional point-by-point response or by revising the manuscript), before we move forward with publication of the study. Please use the link below to update all files to their final version and let us know if you run into any issues doing so.

Referee #2:

The authors have satisfactorily explained and performed the necessary experiments. I support publication of the manuscript.

The colony size variability observed in the yeast growth spot assays is a worrisome for the Reviewer. I would still encourage the authors to integrate the Stm1 alleles into the yeast genome and test the sensitivity to rapamycin. This is an important experimental finding for the field.

Referee #2 (Report for Author)

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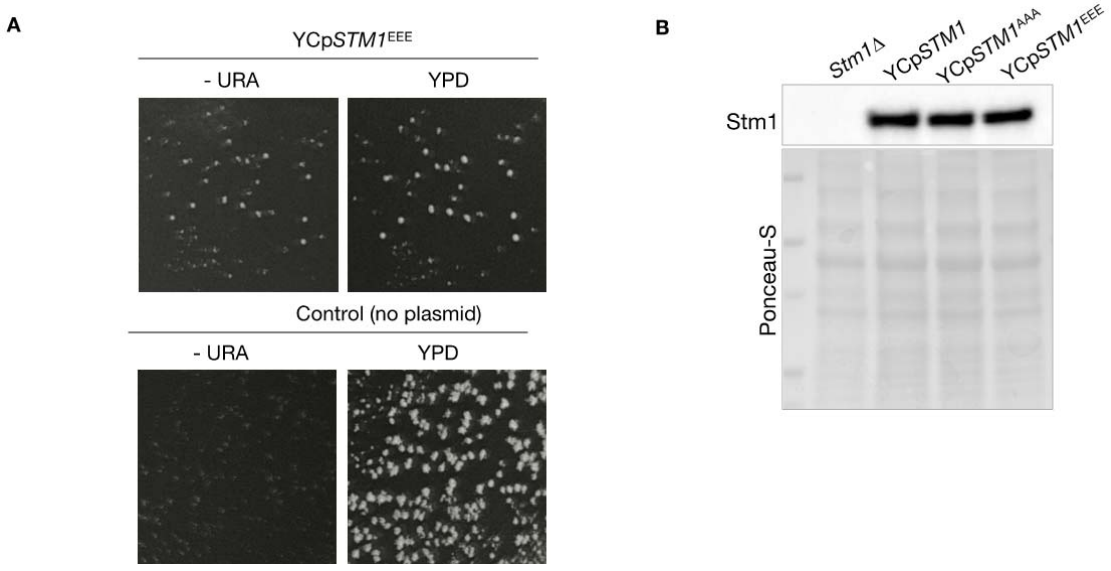
We thank the reviewer for supporting publication of our manuscript. We wish to point out that centromeric (YCp) plasmids have been used for spot assays in many previous studies and thus our spot assay experiment should not be a concern. Nonetheless, to satisfy the reviewer's concerns, we determined whether there is any loss of our YCp plasmids during the spot assays. We generally grow yeast overnight in selection media (SD-URA) and then perform the spot assays on a YPD plate with or without rapamycin and incubate for 36 h to 48 h. To confirm that there is no loss of plasmids in the absence of selection, we grew yeast cells with or without the YCp plasmid in YPD media for 36 hours and then plated on YPD agar. After 24 hours of incubation, we replica plated colonies from the YPD agar onto SD-URA and YPD agar to check for loss of the URA-containing YCp plasmid (YCp*STM1*^{EEE}). We did not observe any loss of the plasmid (Fig. A). Separately, to examine Stm1 expression in the spot assay, we also immunoblotted Stm1 from cells grown on YPD-agar for 48 h. We found no difference between the wild-type and mutant Stm1 levels (both plasmid-borne). These results confirm that use of YCp plasmids is not a concern for the spot assays.



3rd Revision - Editorial Decision **23rd Dec 2022**

Thank you for submitting the final revised version of your manuscript and responding to the remaining referee concern. Given the timing, you will hear from me again regarding the transfer of files to our publisher Wiley, but for now I am happy to inform you that we have formally accepted your study for publication in The EMBO Journal.

EMBO Press Author Checklist

Corresponding Author Name: Michael N. Hall
Journal Submitted to: The EMBO Journal
Manuscript Number: EMBOJ-2022-112344

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This checklist is adapted from Materials Design Analysis Reporting (MDAR) Checklist for Authors. MDAR establishes a minimum set of requirements in transparent reporting in the life sciences (see Statement of Task: 10.31222/osf.io/9sm4x). Please follow the journal's guidelines in preparing your
Please note that a copy of this checklist will be published alongside your article.

Abridged guidelines for figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- ideally, figure panels should include only measurements that are directly comparable to each other and obtained with the same assay.
- plots include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical
- if n<5, the individual data points from each experiment should be plotted. Any statistical test employed should be justified.
- Source Data should be included to report the data underlying figures according to the guidelines set out in the authorship guidelines on Data

2. Captions

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

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements.
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.
- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Please complete ALL of the questions below.
Select "Not Applicable" only when the requested information is not relevant for your study.

Materials

Newly Created Materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
New materials and reagents need to be available; do any restrictions apply?	Yes	Data Availability Section

Antibodies	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
For **antibodies** provide the following information: - Commercial antibodies: RRID (if possible) or supplier name, catalogue number and or/clone number - Non-commercial: RRID or citation	Yes	Material and Methods

DNA and RNA sequences	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Short novel DNA or RNA including primers, probes: provide the sequences.	Yes	Materials and Methods and Table EV3

Cell materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Cell lines: Provide species information, strain. Provide accession number in repository **OR** supplier name, catalog number, clone number, and/**OR** RRID.	Yes	Materials and Methods
Primary cultures: Provide species, strain, sex of origin, genetic modification status.	Not Applicable	
Report if the cell lines were recently **authenticated** (e.g., by STR profiling) and tested for mycoplasma contamination.	Yes	Materials and Methods

Experimental animals	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Laboratory animals or Model organisms: Provide species, strain, sex, age, genetic modification status. Provide accession number in repository **OR** supplier name, catalog number, clone number, **OR** RRID.	Not Applicable	
Animal observed in or captured from the field: Provide species, sex, and age where possible**.**	Not Applicable	
Please detail **housing and husbandry conditions**.	Not Applicable	

Plants and microbes	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Plants: provide species and strain, ecotype and cultivar where relevant, unique accession number if available, and source (including location for collected wild specimens).	Not Applicable	
Microbes: provide species and strain, unique accession number if available, and source.	Not Applicable	Material and Methods

Human research participants	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If collected and within the bounds of privacy constraints report on age, sex and gender or ethnicity for all study participants.	Not Applicable	

Core facilities	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If your work benefited from core facilities, was their service mentioned in the acknowledgments section?	Not Applicable	

Design

Study protocol	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If study protocol has been **pre-registered, provide DOI in the manuscript**. For clinical trials, provide the trial registration number **OR** cite DOI.	Not Applicable	
Report the **clinical trial registration number** (at ClinicalTrials.gov or equivalent), where applicable.	Not Applicable	

Laboratory protocol	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Provide DOI OR other citation details if **external detailed step-by-step protocols** are available.	Yes	Materials and Methods

Experimental study design and statistics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Include a statement about **sample size** estimate even if no statistical methods were used.	Not Applicable	
Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. **randomization procedure**)? If yes, have they been described?	Not Applicable	
Include a statement about **blinding** even if no blinding was done.	Not Applicable	
Describe **inclusion/exclusion criteria** if samples or animals were excluded from the analysis. Were the criteria pre-established? If sample or data points were omitted from analysis, report if this was due to attrition or intentional exclusion and provide justification.	Not Applicable	
For every figure, are **statistical tests** justified as appropriate? Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. Is there an estimate of variation within each group of data? Is the variance similar between the groups that are being statistically compared?	Yes	Figures and Material and Methods section

Sample definition and in-laboratory replication	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
In the figure legends: state number of times the experiment was **replicated** in laboratory.	Yes	Figure
In the figure legends: define whether data describe **technical or biological replicates**.	Yes	Figure

Ethics

Ethics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Studies involving **human participants**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval.	Not Applicable	
Studies involving **human participants**: Include a statement confirming that **informed consent** was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	Not Applicable	
Studies involving **human participants:** For publication of **patient photos**, include a statement confirming that consent to publish was obtained.	Not Applicable	
Studies involving experimental **animals**: State details of **authority granting ethics approva**l (IRB or equivalent committee(s), provide reference number for approval. Include a statement of compliance with ethical regulations.	Not Applicable	
Studies involving **specimen and field samples:** State if relevant **permits** obtained, provide details of authority approving study; if none were required, explain why.	Not Applicable	

Dual Use Research of Concern (DURC)	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Could your study fall under dual use research restrictions? Please check biosecurity documents and list of **select agents and toxins** (CDC): https://www.selectagents.gov/sat/list.htm	Not Applicable	
If you used a select agent, is the security level of the lab appropriate and reported in the manuscript?	Not Applicable	
If a study is subject to dual use research of concern regulations, is the name of the **authority granting approval and reference number** for the regulatory approval provided in the manuscript?	Not Applicable	

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Adherence to community standards	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
State if relevant guidelines or checklists (e.g., **ICMJE, MIBBI, ARRIVE, PRISMA**) have been followed or provided.	Not Applicable	
For **tumor marker prognostic studies,** we recommend that you follow the **REMARK** reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	Not Applicable	
For **phase II and III randomized controlled trials**, please refer to the **CONSORT** flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	Not Applicable	

Data Availability

Data availability	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Have **primary datasets** been deposited according to the journal's guidelines (see 'Data Deposition' section) and the respective accession numbers provided in the Data Availability Section?	Yes	Data availability section
Were **human clinical and genomic datasets** deposited in a public access-controlled repository in accordance to ethical obligations to the patients and to the applicable consent agreement?	Not Applicable	
Are **computational models** that are central and integral to a study available without restrictions in a machine-readable form? Were the relevant accession numbers or links provided?	Not Applicable	
If publicly available data were reused, provide the respective **data citations in the reference list**.	Not Applicable	