nature portfolio

Peer Review File

Ribosomes hibernate on mitochondria during cellular stress

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

Cellular ribosomes are known to adhere to outer mitochondrial membrane (OMM) in cellular stress conditions, including glucose deprivation and infections. However, nature of interactions between the cellular ribosomes and OMM under such stress conditions were generally speculative and poorly understood. The authors of the present study combined single particle cryo-EM and electron tomography (ET) to examine how 80S ribosomes tether to fragmented mitochondrial outer membranes during prolonged glucose depletion. They used a fission yeast, Schizosaccharomyces pombe, as the model system.

To better understand the interactions, first the cryo-EM structures of the S. pombe ribosomes isolated under glucose depleted conditions and actively growing cells were determined in 1.9 Å - 2.96 Å resolution range and a complete atomic model was developed, using the available structure of homologous S. cerevisiae as a template and de novo modeling. High resolution of their structure allowed authors to build a previously unknown segment of the S. pombe 28S rRNA. One of the major findings from the structure of the stressed conditioned ribosome included the absence of tRNAs and a large conformational change in the 28S rRNA helix 69 (H69), which rotates by about 60 degrees towards the large ribosomal subunit (LSU)'s peptidyl-transferase center (PTC). Since H69 lies at the center of ribosome structure and is known to be involved in inter-subunit bridge formation, tRNA interactions, and translocation, its dramatically twisted conformation would make the ribosome inactive and adopt to a hibernation state.

In the second part of story, they studied interactions between the OMM and tethered ribosomes, using cryo-ET of focused-ion beam-milled wild-type and mutated cells, and sub-tomogram averaging. They obtained multiple tomograms to derive their conclusions. These include, (1) unlike previous presumptions, the 80S ribosomes tether to OMM through small ribosomal subunit (SSU), (2) clusters of 2-5 80S ribosomes are formed through specific intermolecular interactions involving eS7 and uS13 proteins from SSU and eL27 and uL18 proteins from LSU, (3) the ribosome tethering to mitochondrial membrane is independent of stress-related mitochondrial fragmentation, shown by ET of a knockout strain of Δdnm1, which shows intact OMM with tethered ribosomes, and (4) by fitting the high-resolution ribosome structure into corresponding ET densities they predicted that the ribosome interacts with OMM through SSU protein Cpc2, a homologue of Rack1. ET of Δcpc2 cells confirmed the role of Cpc2 in tethering. Animations of the ribosome-tethered OMM tomograms are nicely done.

In my view authors have made several discoveries by elegantly complementing their high-quality structural results with genetic and biochemical experiments. The study will be of immense interest to researcher working in the fields of translation, eukaryotic stress response, and mitochondrial homeostasis. Therefore, I strongly recommend publication in Nature Communications.

Minor Point: H69 has nothing to do with PTC. So, its multiple mentions in the PTC context should be removed. The role of H69 without linking it to PTC is significant enough.

Reviewer #2 (Remarks to the Author):

In this work, Gemin, Gluc et al. study from a structural perspective how glucose starvation impacts on the structure and supramolecular organization of ribosomes in Saccharomyces pombe. The authors purify ribosomes from starved and unstarved S. pombe cells and subject them to cryoEM analysis to

identify structural differences of ribosomes. After starvation, the authors observe an altered conformation of rRNA h69, which is sterically incompatible with tRNA binding to the ribosome. By imaging starved S. pombe cells using cryoEM tomography, the authors characterize inactive ribosomes tethered to the surface of mitochondria by Cpc2/RACK1. Ribosomes form higher-order assemblies on the mitochondrial surface, which the authors interpret as ribosome storage structures.

From a methodological perspective, cryoEM and cryoEM tomography analysis are sound and follow state-of-the-art procedures. In contrast, conclusions drawn from the data are largely speculative and require further validation, as well as more careful analysis of the data. The function of ribosome tethering to the mitochondrial membrane remains completely unclear, limiting biological insight.

There are several points that should be addressed in a revised version of the manuscript before publication:

1) The authors observe an altered conformation of rRNA h69 in ribosomes isolated from starved S. pombe cells. According to their processing scheme, this conformation is present in less than 10% of analyzed ribosomes, which seems not enough to qualify h69 conformation as a general regulator for ribosome activity. The authors may want to tone down the functional link between h69 conformation and ribosome inactivity.

2) It is completely unclear, whether h69 actively blocks tRNA binding to the ribosome or – in the absence of tRNAs – simply samples an alternative conformation that is not related at all to cellular stress.
- The authors should analyze whether the altered h69 conformation can also be observed in tRNA-lacking particles in their cryoEM dataset of ribosomes isolated from non-starved S. pombe cells and include this analysis into the manuscript.
- No information is given at all about how h69 is stabilized in the observed conformation. The authors should analyze the interactions in detal. Can any functional mechanism for h69 rearrangement be proposed based on the observed interactions?

3) The authors determine the high-resolution cryoEM structure of an inactive ribosome from starved S. pombe cells, but do not comment about classical ribosome hibernation factors. The authors should analyze their structure for the presence of such known hibernation factors and include the analysis into the manuscript.

4) In their cryoEM tomography data, the authors completely exclude ribosomes not associated with the mitochondrial membrane from the analysis. The authors should structurally characterize these non-associated ribosomes as well and compare their structure to the structures obtained from cryoEM analysis under the same conditions. Are central findings from cryoEM analysis of purified ribosomes recapitulated in cryoEM tomography data, e.g. h69 conformation and factor binding?

5) Related: eEF2 is detected on inactive ribosomes during glucose starvation in situ, but not after purification. Was eEF2 lost during purification? Could other relevant hibernation factors mediating ribosome inactivity in addition or instead of h69 have been lost as well?

6) In their cryoEM tomography data, the authors observe ribosomes tethered to the mitochondrial membrane in defined higher-order assemblies. They suggest that these assemblies ‘safeguard’ and ‘shield’ active sites of ribosomes. Classical hibernation factors accomplish this by directly binding and blocking such active sites, but it is not clear how such a safeguarding function could be accomplished by higher-order oligomer formation as observed here. The authors should clearly explain the proposed mechanism, or tone down this statement.

7) Analyzing S. pombe cells lacking Cpc2/RACK1, the tethering factor, the authors seem not to observe ribosome tethering to mitochondria anymore. However, this should be properly quantified e.g. by comparing the density of ribosomes within a given shell around the mitochondrial membrane in the cryoEM tomography data of Cpc2/RACK1-containing and lacking cells.

Minor points:
• The layout of Extended Data Fig. 9 should be improved. It is difficult to follow the experimental workflow.

• Extended Data Fig. 12: it is impossible to judge presence or absence of tRNAs in the view depicted. The authors should include a cut-open view like in Extended Data Fig. 8. Why is eL8 highlighted?

• p. 13, lines 10-13: the authors propose that ribosome tethering to the mitochondrial membrane protects mitochondria from autophagy and stabilizes the mitochondrial membrane potential. The authors should clearly explain the proposed mechanism.

• In their cryoEM tomography data, the authors do not observe clear density for the mitochondrial membrane, which they attribute to a flexible interaction. Narrowing down the set of particles for averaging based on the distance and orientation towards the mitochondrial membrane may help to capture a more defined structure at lower resolution.

• p. 6, line 16: "Prolonged glucose depletion leads to sequestration of cytosolic ribosomes on fragmented mitochondria 5, possibly due to an overload of the ISR pathway". Please explain in more detail.

• The authors suggest that the arrangement of ribosomes in higher order assemblies may promote initiation of translation as polysome. Is the arrangement compatible with translation initiation? Please discuss.

Reviewer #3 (Remarks to the Author):

The manuscript by Gemin and colleagues reports the accumulation of 80S ribosomes on mitochondrial membranes in response to starvation of S. pombe cells. Glucose starvation leads to halted protein synthesis, which on the structural level is organized by tethering of the ribosomes to fragmented mitochondria, as the authors show by cryo-ET imaging of FIB-milled lamellae. Interestingly, the ribosomes were attached to the mitochondria via the small ribosomal subunits. This enabled the authors to identify the tether protein Cpc2.

Overall, it is an excellent manuscript providing insights into an important process in yeast, including a molecular link, using high-end imaging. Some technical challenges should be worked out before the manuscript should be published.

1. Tethering of ribosomes to mitochondria in response to starvation is known for 50 years, strangely the authors do not discuss the reports by Kellems and colleagues from the 70s.
2. Page 8, line 6: the authors state that they observed clusters of ribosomes, however, I do not see clusters in fig 2d,e. To me, the distribution looks more like even distribution on a 2D surface. If the authors believe that the clusters are there and are functionally relevant, they need to perform quantitative statistical analysis. Among other tools, angular/distance distribution of neighboring

ribosomes may analyzed, similarly to Figure 3 of Brandt et al Mol Cell 2010, P 560-569
3. In the methods, page 22: it is not clear how exactly the initial positions on the surface of mitochondria were generated. The writing is not specific enough for the procedures to be reproduced, please rewrite.
4. Page 25, line 5: github/BlobPicking.py is inaccessible, this limits the reproducibility of the report.
5. Critically important, no PDF validation reports for structures were provided for review. I would like to take a look at them.

Point by point answer to reviewers:

Reviewer #1 (Remarks to the Author):

In my view authors have made several discoveries by elegantly complementing their high-quality structural results with genetic and biochemical experiments. The study will be of immense interest to researcher working in the fields of translation, eukaryotic stress response, and mitochondrial homeostasis. Therefore, I strongly recommend publication in Nature Communications.

Minor Point: H69 has nothing to do with PTC. So, its multiple mentions in the PTC context should be removed. The role of H69 without linking it to PTC is significant enough.

We very much thank the reviewer for their comments regarding the significance and quality of our work and results. In regard to the reviewer's comment on H69, we acknowledge that this is indeed correct, and we have changed the references in the text to point only to H69.

Reviewer #2 (Remarks to the Author):

From a methodological perspective, cryoEM and cryoEM tomography analysis are sound and follow state-of-the-art procedures. In contrast, conclusions drawn from the data are largely speculative and require further validation, as well as more careful analysis of the data. The function of ribosome tethering to the mitochondrial membrane remains completely unclear, limiting biological insight.

There are several points that should be addressed in a revised version of the manuscript before publication:

1) The authors observe an altered conformation of rRNA h69 in ribosomes isolated from starved S. pombe cells. According to their processing scheme, this conformation is present in less than 10% of analyzed ribosomes, which seems not enough to qualify h69 conformation as a general regulator for ribosome activity. The authors may want to tone down the functional link between h69 conformation and ribosome inactivity.

We very much thank the reviewer for their comments and suggestions to improve our manuscript. As suggested by the reviewer, we toned down throughout the text the discussion in regard to H69 as a regulator of ribosome activity as follows:

In the abstract we changed the sentence,

" *causing ribosomes to enter an inactive state characterized by a conformational change* "

into

" Cryo-electron microscopy structure of the ribosomes show that they are devoid of both tRNA and mRNA, and a subset of the particles depicted a conformational change in rRNA H69 that could prevent tRNA binding. "

In the discussion, on page 11,

"*Such alterations in ribosome conformation likely mediate a downregulation of protein synthesis, enabling cells to sustain unfavorable conditions such as nutrient depletion*"

to

"The conformational change observed in a subset of ribosomes isolated on day 7 of glucose depletion may lead to obstruction of tRNA binding and contribute to repression of protein synthesis."

2) It is completely unclear, whether h69 actively blocks tRNA binding to the ribosome or – in the absence of tRNAs – simply samples an alternative conformation that is not related at all to cellular stress.
- The authors should analyze whether the altered h69 conformation can also be observed in tRNA-lacking particles in their cryoEM dataset of ribosomes isolated from non-starved S. pombe cells and include this analysis into the manuscript.

No information is given at all about how h69 is stabilized in the observed conformation. The authors should analyze the interactions in detail. Can any functional mechanism for h69 rearrangement be proposed based on the observed interactions?

Thank you for the comment. Based on the reviewer's suggestion, and to assess whether the H69 conformational state is unique to ribosomes isolated under cellular stress, we performed further 3D variability analysis on a subset of ribosomes lacking density for tRNA. These ribosomes were isolated from cells growing under rich-glucose conditions, where we do not expect to observe this conformational change.

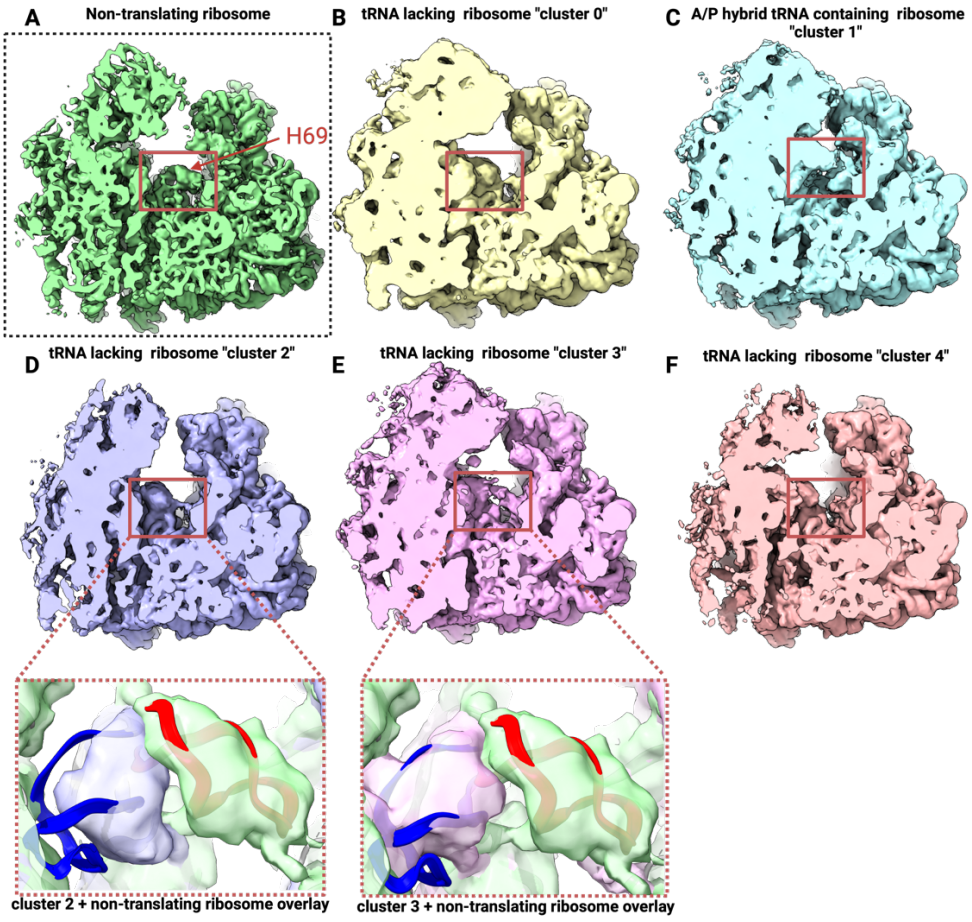
In the 3D classification, we used the same mask around H69 and the PTC as we did for the dataset of ribosomes isolated after 7 days of glucose depletion. Since most ribosomes isolated from rich media contained A- and P-site tRNA, we focused our classification on a subset of 68,060 particles out of the 220,243 initially picked that displayed no tRNA density in this dataset. The classification results are summarized in the figure below. While some dynamics were observed for H69 in ribosomes isolated from high-glucose media, we did not observe H69 tilting (Panels B, D-F) to the same degree as observed on day 7 with cells growing under stress from glucose depletion (Panel A). Notably, within this subset of particles, we also obtained a new class with A/P hybrid tRNA density that was not observed in our initial 3D classification (Panel C-cluster1).

Therefore, we conclude that the alternative conformation of H69 is unique to ribosomes isolated under cellular stress. However, we agree that a detailed mechanism for protein synthesis inhibition

cannot be deduced from structural data alone. We also agree that it is more likely that the general regulation of protein synthesis will also depend on the presence of canonical hibernation factors that may have been lost during the purification process.

To clarify this further, an additional statement added in the discussion page 11

" However, the potential influence of additional hibernation factors during glucose depletion, which may have been lost during the purification process, cannot be excluded. Further investigation is needed to determine the mechanism of ribosome inhibition under these conditions."



A Non-translating ribosome H69 shift, 73,376 particles. Performed 3D variability on "Non-starved S. pombe ribosome" particle subset lacking tRNA, results shown in **B**-**F**. **B** "cluster 0", 18,005 particles. **C** "cluster 1", 9,198 particles, particles in this cluster contain A/P hybrid tRNA density. **D** "cluster 2", 13,184 particles. Particles in this cluster show biggest shift in H69, close-up shows overlay of cluster 2 map (blue) and non-translating ribosome map (green) with H69 atomic model of translating ribosome (blue) and non-translating ribosome (red). **E** "cluster 3", 10,837 particles. Close up colored as in D. **F** "cluster 4", 16,836 particles.

3) The authors determine the high-resolution cryoEM structure of an inactive ribosome from starved S. pombe cells, but do not comment about classical ribosome hibernation factors. The

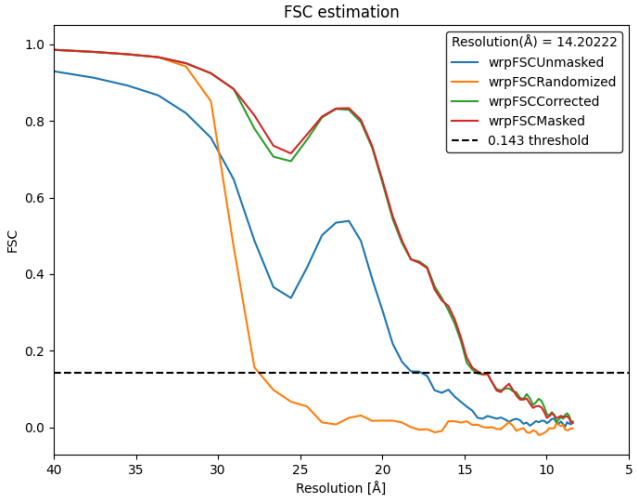
authors should analyze their structure for the presence of such known hibernation factors and include the analysis into the manuscript.

We performed a thorough classification in the cryo-EM dataset and could not find bound hibernation factors. We thus conclude that these factors were most likely lost during the purification process. A statement in the text was added to clarify this, please see answer to point 2.

4) In their cryoEM tomography data, the authors completely exclude ribosomes not associated with the mitochondrial membrane from the analysis. The authors should structurally characterize these non-associated ribosomes as well and compare their structure to the structures obtained from cryoEM analysis under the same conditions. Are central findings from cryoEM analysis of purified ribosomes recapitulated in cryoEM tomography data, e.g. h69 conformation and factor binding?

We analyzed the ribosomes present in the cytoplasm using subtomogram averaging as suggested. The results are presented in the new *Supplementary Figure S15*. The structure was obtained by picking all cytoplasmic ribosomes present in the cryo-ET dataset collected for the glucose depleted WT cells. The field of view of the acquired tomographic data was aimed at cellular regions containing mitochondria which limited the number of cytosolic regions available for analysis, thus leading to a lower total number of untethered cytoplasmic ribosomes with respect to the tethered ones. Although the resolution of the untethered ribosome structure was lower compared to the tethered ones (14 Å against 11 Å, respectively), the interpretable features of the untethered map were very similar to those of ribosomes on the mitochondrial membrane, including the presence of the factors eEF2 and Cpc2/RACK1. A thorough analysis of H69 in cryo-ET was not feasible due to the lower resolution of the cryo-ET map. Therefore, the low number of untethered particles, combined with the fact that the conformational change of H69 is present in only a subset of these particles, did not allow for an informative 3D classification of this conformational heterogeneous region.

The new obtained map of the untethered cytosolic ribosome has been deposited in the EMDB (accession code EMD-51030); the validation report is attached. For completeness, we include below the FSC curve of the untethered ribosome reconstruction.



5) Related: eEF2 is detected on inactive ribosomes during glucose starvation in situ, but not after purification. Was eEF2 lost during purification? Could other relevant hibernation factors mediating ribosome inactivity in addition or instead of h69 have been lost as well?

This is a possibility that we considered as we did not observe eEF2 and is most likely lost during one of the purification steps. It is possible that other canonical hibernation factors were also lost during purification. A statement in the discussion was added in this regard, please see point #2.

6) In their cryoEM tomography data, the authors observe ribosomes tethered to the mitochondrial membrane in defined higher-order assemblies. They suggest that these assemblies 'safeguard' and 'shield' active sites of ribosomes. Classical hibernation factors accomplish this by directly binding and blocking such active sites, but it is not clear how such a safeguarding function could be accomplished by higher-order oligomer formation as observed here. The authors should clearly explain the proposed mechanism, or tone down this statement.

We thank the reviewer for this important comment. We speculate that ribosome oligomer formation could serve as further restriction of the small subunit movements, which is important during translation elongation and availability of the 40S subunit to form for initiation complexes. However, we agree that this effect could be done in parallel with the role contributed by hibernation factors, as mentioned above.

We thus toned this statement down on page 9, and it was modified to the following:

"This, combined with the presence of additional hibernation factors, may further occlude the tRNA and mRNA binding sites, restrict the movement of the 40S subunit, and potentially safeguard the translation machinery as a storage form until nutrients are restored."

7) Analyzing S. pombe cells lacking Cpc2/RACK1, the tethering factor, the authors seem not to observe ribosome tethering to mitochondria anymore. However, this should be properly quantified e.g. by comparing the density of ribosomes within a given shell around the mitochondrial membrane in the cryoEM tomography data of Cpc2/RACK1-containing and lacking cells.

We quantified the number of ribosomal particles present in the vicinity of the outer mitochondrial membrane for WT and Cpc2/RACK1 cells post glucose depletion. The results are presented in the new *Supplementary Fig. 16*. The outer mitochondrial membrane was automatically segmented using the MemBrain-seg software. Ribosomal particles were identified by template matching using PyTom and the ones within a shell of 40 nm from the outer mitochondrial membrane were selected and quantified. The ribosomal density in the vicinity of the mitochondrial membrane of WT cells was 14,806 ribosomes/μm^3 while the Cpc2/RACK1 cells showed a particle density of 1,283 ribosomes/μm^3. In addition to the stark difference of particle numbers, the few ribosomes identified in the nearby of the mitochondria in Cpc2/RACK1 cells were randomly oriented.

For a sphere with an average radius of 300 nm (typical mitochondrial size) completely packed with ribosomes (radius ~25 nm), the theoretical ribosome density is approximately 15,000 particles/μm^3. This indicates that WT mitochondria are almost entirely covered with ribosomes.

These results confirm that cytosolic ribosomal tethering to the outer mitochondrial membrane is mediated by Cpc2/RACK1 and that the Δcpc2 mutant results in minimal amounts of ribosomes found close to the outer mitochondrial membrane which are not tethered to it via their small subunit.

Minor points:
• The layout of Extended Data Fig. 9 should be improved. It is difficult to follow the experimental workflow.

As suggested by the reviewer, we have modifications done to this figure to make it clearer.

• Extended Data Fig. 12: it is impossible to judge presence or absence of tRNAs in the view depicted. The authors should include a cut-open view like in Extended Data Fig. 8. Why is eL8 highlighted?

This has been modified to include a panel with a close-up of the tRNA binding site, fitted from the cryo-EM structure of the translating ribosome. eL8 was originally highlighted to show the fit, but it has now been removed to allow for the addition of the new panel.

• p. 13, lines 10-13: the authors propose that ribosome tethering to the mitochondrial membrane protects mitochondria from autophagy and stabilizes the mitochondrial membrane potential. The authors should clearly explain the proposed mechanism.

We suggest that ribosomes completely covering the surface of the mitochondria upon stress potentially block the recruitment of protein factors responsible for marking damaged mitochondria for mitophagy. A minor clarification has been made to the respective statement/proposed mechanism in the text on page 13, and the changes are highlighted in the revised manuscript:

"It is possible that tethered ribosomes perform a moonlighting function promoting cell survival by protecting fragmented mitochondria from recruiting autophagy-related factors and preventing apoptosis by stabilizing the mitochondrial membrane potential, thus averting cell death."

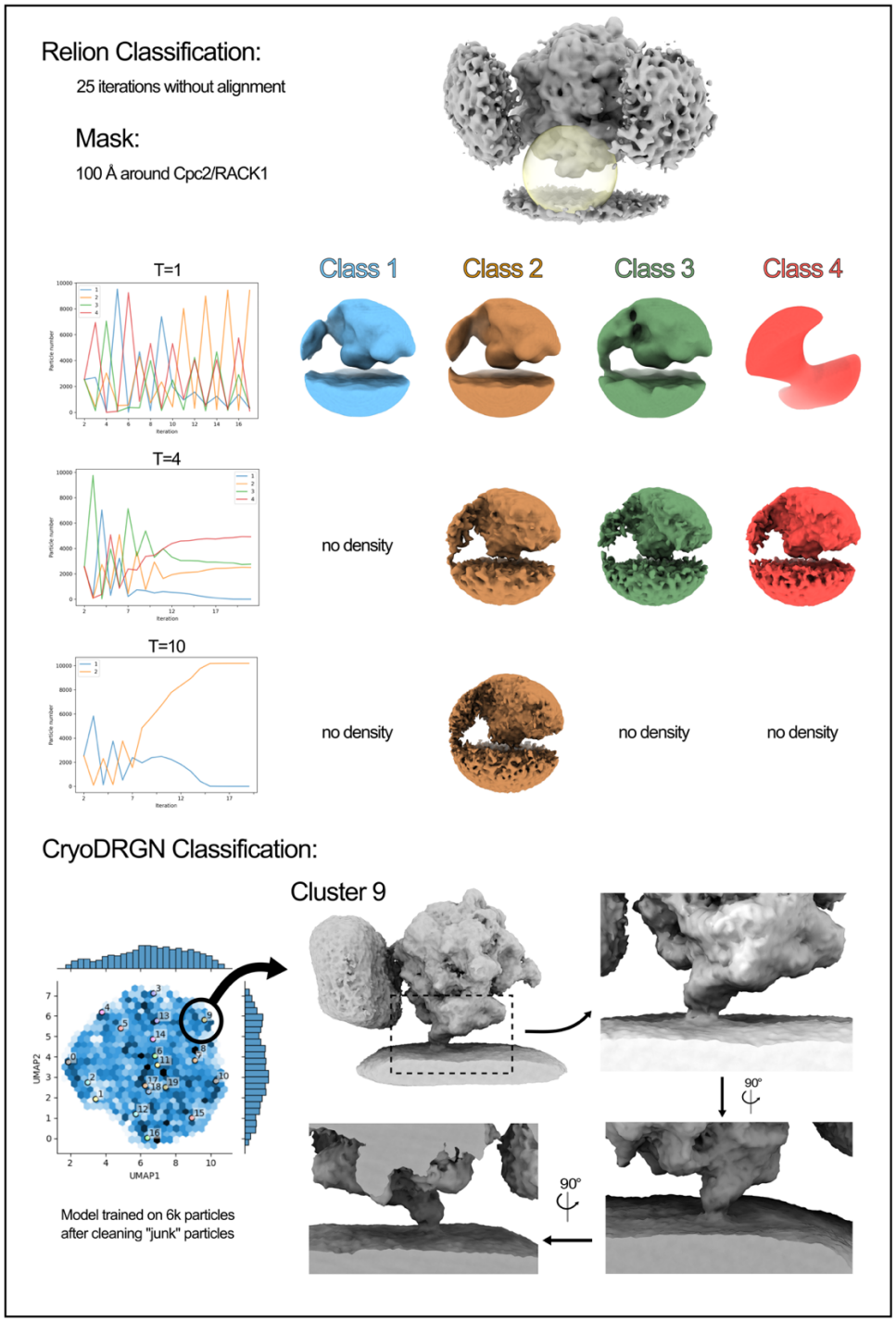
• In their cryoEM tomography data, the authors do not observe clear density for the mitochondrial membrane, which they attribute to a flexible interaction. Narrowing down the set of particles for averaging based on the distance and orientation towards the mitochondrial membrane may help to capture a more defined structure at lower resolution.

We thank the reviewer for bringing this point up. Our attempts at classification based on distance and orientation were unsuccessful and therefore not included in the manuscript.

As suggested by the reviewer, we did perform subtomogram averaging of subsets of particles based on geometrical parameters like their distance to membrane and their angular orientation with

respect to the normal to the membrane. Those analyses did not result in any density being visible that could bridge Cpc2/RACK1 to the outer mitochondrial membrane. We also tried focused 3D classification in Relion and got a similar outcome, where no additional density could be observed (see figure below).

However, this comment pushed us to perform a computational classification based on CryoDRGN for tomography (cryo-DRGN-ET). We could identify a cluster of particles that produced a map in which a weak, but clear density connects the outer mitochondrial membrane to Cpc2/RACK1.



Nevertheless, the low resolution of this map (likely due to the limited number of classified particles - 346 particles in this cluster) does not allow for the identification of the mitochondrial binding partner (see figure below) and therefore does not add any further interpretable structural information compared to what was already discussed in the manuscript.

• p. 6, line 16: "Prolonged glucose depletion leads to sequestration of cytosolic ribosomes on fragmented mitochondria 5, possibly due to an overload of the ISR pathway". Please explain in more detail.

This statement is modified, and it reads as follows: "possibly due to activation of the ISR that could lead to protein synthesis repression."

• The authors suggest that the arrangement of ribosomes in higher order assemblies may promote initiation of translation as polysome. Is the arrangement compatible with translation initiation? Please discuss.

The placement of the 40S in contact as a result of this arrangement may possibly facilitate the initiation of multiple ribosomes on the same mRNA.

The statement on Page 13 had minor modification to further discuss this:

> " *The ribosome arrays here would thus represent an advantage to initiate translation as polysomes due to proximity of the mRNA channel from neighboring 40S subunits."*

Reviewer #3 (Remarks to the Author):

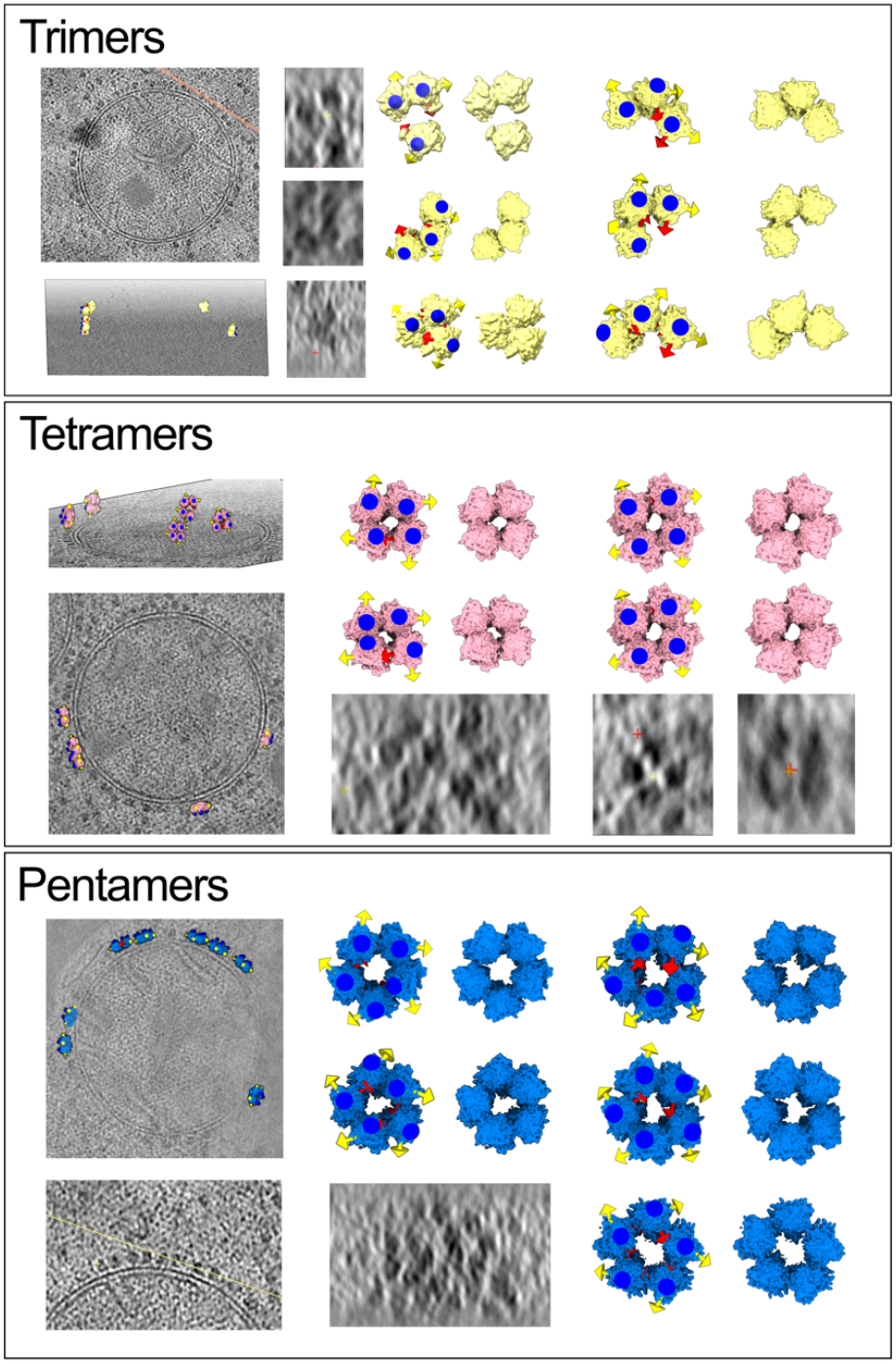
Overall, it is an excellent manuscript providing insights into an important process in yeast, including a molecular link, using high-end imaging. Some technical challenges should be worked out before the manuscript should be published.

1. Tethering of ribosomes to mitochondria in response to starvation is known for 50 years, strangely the authors do not discuss the reports by Kellems and colleagues from the 70s.

We very much thank the reviewer for their comment on our work and for bringing this important paper to our attention. It is now also cited in our manuscript in the Introduction.

2. Page 8, line 6: the authors state that they observed clusters of ribosomes, however, I do not see clusters in fig 2d,e. To me, the distribution looks more like even distribution on a 2D surface. If the authors believe that the clusters are there and are functionally relevant, they need to perform quantitative statistical analysis. Among other tools, angular/distance distribution of neighboring ribosomes may analyzed, similarly to Figure 3 of Brandt et al Mol Cell 2010, P 560-569

To adjuvate the visualization of the ribosomal cluster on the outer mitochondrial membrane, the figure below shows a gallery of these symmetrical assemblies described in the manuscript. We included representative examples of the pentameric, tetrameric and trimeric arrangements that we identified. All oligomeric species are stabilized by quasi-equivalent interaction interfaces that only differ with respect to the in-plane rotation between neighboring ribosomes.



For the structural description of the interaction interfaces, we focused our analysis on neighboring ribosomes withing pentameric clusters. To identify all pentameric assemblies present in our dataset we calculated pairwise distances between a pentameric template (produced using the script https://git.embl.de/gemin/ribosonmitos/-/blob/master/MotlHandlingScripts/Fig3-AverageDimerFromPentamers/Nmer_parameter_find.m) against the final aligned positions of all ribosomes. This approach allowed us to identify sets of neighbouring ribosomes arranged around pentameric coordinated positions having pairwise distances matching the template (the used script is available at: https://git.embl.de/gemin/ribosonmitos/-/blob/master/MotlHandlingScripts/Fig3-AverageDimerFromPentamers/SM_find_pentamers_normal-editOG.m).
Within the identified pentameric clusters, we determined the relative distance and angular distribution between neighbouring ribosomes (using the script: https://git.embl.de/gemin/ribosonmitos/-/blob/master/MotlHandlingScripts/Fig3-AverageDimerFromPentamers/pentamerStats_v1.m). Therefore, our analysis is indeed based on automated, geometrical classification of pentameric-coordinated ribosomes by means of pairwise distances followed by an analysis of the angular distribution of neighbouring ribosomes.

3. In the methods, page 22: it is not clear how exactly the initial positions on the surface of mitochondria were generated. The writing is not specific enough for the procedures to be reproduced, please rewrite.

We have now included a more detailed description of how the initial extraction positions of the subtomograms were generated. This method section is modified, and it reads as follows:

"The surface of each mitochondrion was defined by manually generating a spherical model using IMOD [65]. Initial subtomogram extraction positions were uniformly distributed over the defined spherical surfaces with a spacing of 3 pixels at binning 8 (≈49.8 Å) resulting in 2,091,052 starting positions. For each extraction position three Euler angles were defined to orient the extracted volumes normal to the rendered surface (i.e. with the z axis perpendicular to the OMM pointing towards the cytosol) and the in-plane angle was randomised".

4. Page 25, line 5: github/BlobPicking.py is inaccessible, this limits the reproducibility of the report.

We thank the reviewer for pointing this out. We now fixed the URL to include the complete link: https://git.embl.de/gemin/ribosonmitos/-/blob/master/ImageAnalysis/BlobPicking.py

5. Critically important, no PDF validation reports for structures were provided for review. I would like to take a look at them.

The validation reports are now available and are included as PDF documents in attachment.

REVIEWERS' COMMENTS

Reviewer #2 (Remarks to the Author):

The authors have adequately addressed most of my comments and the manuscript is ready for publication pending some minor revisions listed below:

1) In the revised version of the manuscript, the authors have convincingly demonstrated that the conformational change of h69 observed in starved cells is not present in non-stressed cells. Considering this, could the authors identify the interactions between h69 and the rest of the ribosome structure that stabilize h69 in the new conformation and include this into the manuscript?

2) In the revised version of the manuscript, the authors suggest that a "potential influence of additional hibernation factors during glucose depletion, which may have been lost during the purification process, cannot be excluded." Considering that e.g. eEF2 completely dissociated from ribosomes during their purification protocol this statement is too weak and should be adjusted.

The authors could consider using their own phrasing from the rebuttal letter: „ it is more likely that the general regulation of protein synthesis will also depend on the presence of canonical hibernation factors."

3) Thanks to the authors for clarifying how mitochondria-tethered ribosomes may protect mitochondria from autophagy. To further support the idea, the authors could indicate in the text more explicitly that ribosomes almost completely cover the mitochondrial membrane (according to the new analysis in response to point 7), which would sterically shield the membrane surface.

Reviewer #3 (Remarks to the Author):

The authors addressed my comments in the revised version and the manuscript looks good for publication from my side.

Point by point answer to reviewers:

1) In the revised version of the manuscript, the authors have convincingly demonstrated that the conformational change of h69 observed in starved cells is not present in non-stressed cells. Considering this, could the authors identify the interactions between h69 and the rest of the ribosome structure that stabilize h69 in the new conformation and include this into the manuscript?

We very much thank the reviewer for their comments. We have included a statement regarding the interactions between h69 and the rest of the ribosome and it now reads: "In this confirmation H69 is stabilized via interactions with the ribosomal protein uL16 and the ribosomal RNA." (page 6. Lines 12-13).

2) In the revised version of the manuscript, the authors suggest that a "potential influence of additional hibernation factors during glucose depletion, which may have been lost during the purification process, cannot be excluded." Considering that e.g. eEF2 completely dissociated from ribosomes during their purification protocol this statement is too weak and should be adjusted.

The authors could consider using their own phrasing from the rebuttal letter: „ it is more likely that the general regulation of protein synthesis will also depend on the presence of canonical hibernation factors."

We very much thank the reviewer for this comment. We have revised the statement based on reviewers' suggestions.

In the manuscript we changed the sentence,

"However, the potential influence of additional hibernation factors during glucose depletion, which may have been lost during the purification process, cannot be excluded."

into

"However, the loss of canonical hibernation factors during the purification process cannot be excluded and it is likely that the general regulation of protein synthesis will also depend on the presence of these factors."

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Thank you to the reviewer for this suggestion. In the discussion we have revised the following sentence:

“It is possible that tethered ribosomes perform a moonlighting function promoting cell survival by protecting fragmented mitochondria from recruiting autophagy-related factors and preventing apoptosis by stabilizing the mitochondrial membrane potential, thus averting cell death. “

into

“It is possible that tethered ribosomes perform a moonlighting function by almost completely covering the mitochondrial membrane, which would sterically shield the membrane surface from recruiting autophagy-related factors and by stabilizing the mitochondrial membrane potential.”