

Visualizing the translation landscape in human cells at high resolution

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This manuscript has been previously reviewed at another journal. This document only contains information relating to versions considered at Nature Communications.

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The revised manuscript is now suitable for publication in Nature Communications. The results are interesting and will make an excellent contribution to the field.

Reviewer #2

(Remarks to the Author)

The authors have significantly improved their manuscript by tailoring their claims to the presented data and replacing their work in the context of previous publications in the field. However, I am still concerned about the level of evidence supporting the assignation of SERBP1 in short stretches of density on the ribosomes.

Main point

The authors have now described in the method section the procedure they used to assign short stretches of density around the 80S ribosome to pieces of SERBP1.

CryoID is designed as an unbiased tool for protein identification in longer stretches of high resolution cryoEM maps, but the way it is used here and the entire approach for SERBP1 identification is not unbiased. With this level of data I think it is only possible to conclude that SERBP1 is a likely hit for the protein explaining these densities. Of course one might be very tempted to believe this result but I think that the confidence level is a bit low for publication and some kind of orthogonal piece of data would typically be needed to support the claim. Given that SERBP1 is not an essential gene I think that at least a SERBP1 KO followed by in-cell ribosome SPA reconstruction would be needed. It is not an unrealistic experiment as the new reconstruction does not need to yield extremely high resolution to compare the presence or absence of these densities on 80S ribosomes (in maps reconstructed from the same number of particles at the same - very low, threshold). I estimate that this is doable in a typical 3 months review timing and would give sufficient support to the SERBP1 assignation for publication.

Other points

The authors answered that they have no claim on the amount of hibernating ribosomes.

However, if the cells are in a situation with this high amount of hibernating 80S it is also likely that other observations especially the observation of SERBP1 on translating ribosomes (and potentially any other observation including the presence of some Z tRNA states etc.) are also a consequence of the unhealthy cellular state rather than a normal feature of translation in human cells.

L51-54: "Recent applications of cryo-ET to thin cellular samples, such as small bacteria or cellular lamellae milled by a

focused ion beam (FIB)19,20,21, have revealed remarkable aspects of ribosome dynamics inside cells, including drug interactions and their impact on translation22,23,12” add citation PMID: 38340715

L54: “albeit with limited detail” actually this is not very true, PMID: 39668257 needs to be cited here and the sentence adjusted

L57: “However, the full potential of in situ single-particle cryo-EM for new biological discovery had not yet been demonstrated.” not sure what this exactly means. PMID: 39626661 needs to be cited here.

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L433: I would also recommend the deposition of the raw micrographs of this study in EMPIAR to enable further computational method development

Reviewer #3

(Remarks to the Author)

The revised version of the manuscript by Zheng et al. has improved with regards to more analysis and comparisons with previous work but it could still try to better explain the mechanistic implications of the findings. It now toned down some previously rather general statements or overstatements, which is more reasonable. A problematic aspect remains, as also pointed out by the other referees, that the sample preparation procedure is likely to generate cellular stress, i.e. the observations and statistics of ribosome populations for given functional (or blocked) states may not be functional or representative.

Detailed remarks:

- figures and orientation of the ribosome: the fact that others (possibly newer to the field) have rotated the view compared to standard representations in the field is not a reason to do the same. This can also come from software using certain default settings / orientations.

In fact, the 2 standard orientations of ribosome figures have a good reason: the front view shows the factor binding site in the middle between the small ribosomal subunit (upright, left) and the large ribosomal subunit (upright, right). To show tRNAs as the authors mention, the top view is ideal (as properly used in Fig. 1g and others), with the small ribosomal subunit at the bottom and the large ribosomal subunit at the top, so that the orientation makes sense with regards to the translation direction, i.e. 5' left, 3' right on the mRNA. Hence, only Fig. 1b, 2a and 7a would need to be rotated 90° right (clockwise).

- 2 digits after the comma for resolution values make no sense: the precision obtained from cryo-EM is not in the 0.01 Å range. One has to consider that the output of software (cryoSPARC or other) serves to guide or evaluate the refinement, but the absolute resolution values estimated on the final cryo-EM maps should say 2.2 Å, 3.1 Å etc. throughout the manuscript.

- regarding HHT complexes, reference G. de Loubresse et al. is for the yeast ribosome; there is also an HHT complex with the human ribosome (see doi: 10.1038/nature24482. PMID: 29143818)

- Ext. Fig. 4: if the mRNA region is at lower resolution (which can be expected due to its mixed nature), then this should be explained in the text (which otherwise gives a false impression) and in addition a local resolution map for the mRNA region should be shown.

- SERBP1: if after checking the structure only ~10 amino acids are visible (referee 3), most of the conclusions on that factor are likely to be unreliable (e.g. localisation of the N-domain, which could have been novel, but now turns out not to be); hence, also the prediction is to be taken more carefully. This should be clarified. In this context, the phrase in the abstract regarding SERBP1 also appears confusing.

- cryo electron microscopy (without hyphen; hyphen to be used only for the abbreviation: cryo-EM)

- Figure 7a: is the scale the same in x and y directions? it looks extended in the y direction (which would become x direction after rotating 90° right/clockwise).

- validation report of atomic model: what is chain CA (60S)? The quality of the atomic model would be expected to be better at the claimed resolution. For the A-P complex, there are many chains with medium fit; similar issues in some chains of the other atomic models, some show strong deviations, which should be corrected prior to PDB/EMBD deposition for all entries.

Version 1:

Reviewer comments:

Reviewer #2

(Remarks to the Author)

The revised manuscript is now appropriate for publication in Nature Communications and will provide an interesting contribution to the field.

Reviewer #3

(Remarks to the Author)

I went through the point to point response and it appears that all suggestions have been taken into account. I have no further comments.

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Reviewer #1 (Remarks to the Author):

The revised manuscript is now suitable for publication in Nature Communications. The results are interesting and will make an excellent contribution to the field.

We thank the reviewer for the effort rereviewing our manuscript and the positive assessment.

Reviewer #2 (Remarks to the Author):

The authors have significantly improved their manuscript by tailoring their claims to the presented data and replacing their work in the context of previous publications in the field. However, I am still concerned about the level of evidence supporting the assignment of SERBP1 in short stretches of density on the ribosomes.

We thank the reviewer for their positive feedback on our revisions. In response to the concerns regarding the SERBP1 assignment, we have revised the manuscript to adopt a more conservative interpretation. Specifically, we have removed the section discussing definitive identification of SERBP1 and now refer to the two densities in the updated Figure 4 as “unknown density 1” and “unknown density 2.” We report only that the built models for these densities closely resemble AlphaFold2 predictions for various segments of SERBP1, without asserting a definitive assignment or biological interpretation.

Main point

The authors have now described in the method section the procedure they used to assign short stretches of density around the 80S ribosome to pieces of SERBP1. CryoID is designed as an unbiased tool for protein identification in longer stretches of high resolution cryoEM maps, but the way it is used here and the entire approach for SERBP1 identification is not unbiased. With this level of data I think it is only possible to conclude that SERBP1 is a likely hit for the protein explaining these densities. Of course one might be very tempted to believe this result but I think that the confidence level is a bit low for publication and some kind of orthogonal piece of data would typically be needed to support the claim. Given that SERBP1 is not an essential gene I think that at least a SERBP1 KO followed by in-cell ribosome SPA reconstruction would be needed. It is not an unrealistic experiment as the new reconstruction does not need to yield extremely high resolution to compare the presence or absence of these densities on 80S ribosomes (in maps reconstructed from the same number of particles at the same - very low, threshold). I estimate that this is doable in a typical 3 months review timing and would give sufficient support to the SERBP1 assignment for publication.

We appreciate the reviewer’s critical evaluation of our SERBP1 assignment. We agree that the identification is based on relatively short segments of density and does not reach the level of confidence typically afforded by longer, well-resolved regions. Accordingly, we have carefully revised the relevant sections and removed definitive interpretations, as noted above.

We also concur that a SERBP1 knockout followed by in-cell SPA reconstruction would provide strong validation. While we recognize the value of such an experiment, it was not feasible within the current revision cycle due to time and resource constraints. We agree the knockout experiment would be an important direction for future investigation.

Other points

The authors answered that they have no claim on the amount of hibernating ribosomes. However, if the cells are in a situation with this high amount of hibernating 80S it is also likely that other observations especially the observation of SERBP1 on translating ribosomes (and potentially any other observation including the presence of some Z tRNA states etc.) are also a consequence of the unhealthy cellular state rather than a normal feature of translation in human cells.

We agree this is an important consideration. We have added a dedicated section to the Discussion:

“In addition, the 5% glycerol used as a cryoprotectant in our protocol, although applied for only a short duration (a few minutes), may induce cellular hibernation as a result of osmotic shock⁶⁶. The observed enrichment of hibernating 80S ribosomes (Fig. 3) could reflect such stress. Consequently, the distribution of functional ribosome states (e.g., canonical elongating vs. hibernating), the prevalence of factors like SERBP1, and specific tRNA states (e.g., Z-tRNA) reported here may be influenced by the preparation conditions and may not solely represent the basal, unstressed cellular state. Future methodological improvements with faster freezing or gentler handling may help minimize these effects and more accurately preserve the native translational landscape.”

L51-54: “Recent applications of cryo-ET to thin cellular samples, such as small bacteria or cellular lamellae milled by a focused ion beam (FIB)^{19,20,21}, have revealed remarkable aspects of ribosome dynamics inside cells, including drug interactions and their impact on translation^{22,23,12}” add citation PMID: 38340715

We have cited the reference.

L54: “albeit with limited detail” actually this is not very true, PMID: 39668257 needs to be cited here and the sentence adjusted

We have removed this sentence and cited the reference.

L57: “However, the full potential of in situ single-particle cryo-EM for new biological discovery had not yet been demonstrated. “not sure what this exactly means. PMID: 39626661 needs to be cited here.

We clarified the sentence by adding “such as identifying transient or weakly bound factors, low-abundance conformational states, or native distributions of ions and post-translational modifications within cells”. We have cited the reference.

L225: “NAC β is involved in nascent peptide processing and trafficking, but has only been observed in in vitro reconstituted samples” - Nac beta is seen already in cells PMID: 39626661

We thank the reviewer for bringing this recent publication (PMID: 39626661) to our attention. This study was published after our manuscript was submitted and thus was not included in the original version. We fully agree that the in-cell observation of NAC β strengthens the understanding of its role in nascent peptide processing. We have now cited this work in the revised manuscript and modified the relevant sentence accordingly to reflect the updated findings (lines 227-228).

L233: “Furthermore, while a previous cryo-ET study which did not observe eIF6 associated with 60S subunits¹²,” PMID: 38340715 already reported eIF6 on free 60S subunits in mammalian cells.

We thank the reviewer for highlighting this important point. While the high-resolution cryo-ET study of human untreated ribosomes (PMID:37410833) did not detect eIF6 bound to free 60S subunits, our results and the independent cryo-ET work in PMID:38340715 both provide complementary evidence for eIF6-60S association in mammalian cells. We have revised the manuscript accordingly (lines 237-238).

L249: “The N-terminal region of SERBP1 was bound to all observed 80S ribosomes (Fig. 5b) and free 60S subunits (Fig. 5b, c)”: This sentence is misleading. The 60S map especially shows a density that only comes at very low threshold indicating that the majority of free 60S particles is likely not bound by SERBP1. I think the authors meant more that the density can be seen in on all maps regardless of the elongation state.

We thank the reviewer for pointing out this ambiguity in our description. To improve clarity, we have removed the sentence in question.

L311: Fig. 7 seems to be missing from the revised combined pdf?

In addition there seem to be no more mention in this paragraph of the “spermidine that interacts with cycloheximide bound at the E site of the ribosome” (as stated in the abstract).

We apologize for the omission in the previous combined PDF. Figure 7 (revised Fig. 6) is now included. The spermidine density near the CHX-bound E-site was described in the paragraph (lines 306-312).

L374: also cite PMID: 38340715 and adjust the discussion accordingly

We have now cited the reference.

L396: maybe more than the 5min glycerol, the live cell imaging buffer presumably also induces a change (FBS starvation, minimum nutrient). So I rather suggest to have a more general sentence on the fact that in situ structural biology is native with some limitations and possible

artefacts (especially on highly dynamics events such as translation), including the potential limitation of the freezing conditions.

We agree with the reviewer on the limitations of freezing conditions. As stated above, we have dedicated a generate discussion to this point (lines 380-388).

L433: I would also recommend the deposition of the raw micrographs of this study in EMPIAR to enable further computational method development

We thank the reviewer for the suggestion. We are currently developing new method based on this dataset and in the process of identifying additional targets. We will deposit the data when we finish the ongoing work.

Reviewer #3 (Remarks to the Author):

The revised version of the manuscript by Zheng et al. has improved with regards to more analysis and comparisons with previous work but it could still try to better explain the mechanistic implications of the findings. It now toned down some previously rather general statements or overstatements, which is more reasonable. A problematic aspect remains, as also pointed out by the other referees, that the sample preparation procedure is likely to generate cellular stress, i.e. the observations and statistics of ribosome populations for given functional (or blocked) states may not be functional or representative.

We agree with the reviewer that the observed ribosome states may reflect stressed cells. As described above, we have added clarification in the Discussion to explain that while our data reveal structural features of ribosomes *in situ*, their functional implications should be interpreted cautiously given the sample preparation conditions.

Detailed remarks:

- figures and orientation of the ribosome: the fact that others (possibly newer to the field) have rotated the view compared to standard representations in the field is not a reason to do the same. This can also come from software using certain default settings / orientations.

In fact, the 2 standard orientations of ribosome figures have a good reason: the front view shows the factor binding site in the middle between the small ribosomal subunit (upright, left) and the large ribosomal subunit (upright, right). To show tRNAs as the authors mention, the top view is ideal (as properly used in Fig. 1g and others), with the small ribosomal subunit at the bottom and the large ribosomal subunit at the top, so that the orientation makes sense with regards to the translation direction, i.e. 5' left, 3' right on the mRNA. Hence, only Fig. 1b, 2a and 7a would need to be rotated 90° right (clockwise).

We appreciate the reviewer's helpful clarification regarding standard ribosome orientations. In response, we have rotated Figures 1b by 90° clockwise to follow the conventional "front view" representation (small subunit on the left, large subunit on the right, factor binding cleft centered).

For Figure 2a, the standard orientation is not optimal due to the spatial arrangement of the highlighted features. In the canonical view, visualizing 5.8S rRNA U110 (left panel) would

require clipping away most of the 80S, while 28S rRNA G3922 (right panel) would be obscured behind the P-site tRNA. To clearly display both features, we have therefore retained a non-standard orientation.

Similarly, for Figure 7a (now updated as Figure 6a), we have intentionally retained the non-standard orientation, as it more clearly reveals the locations and densities of HHT and CHX in this specific context.

- 2 digits after the comma for resolution values make no sense: the precision obtained from cryo-EM is not in the 0.01 Å range. One has to consider that the output of software (cryoSPARC or other) serves to guide or evaluate the refinement, but the absolute resolution values estimated on the final cryo-EM maps should say 2.2 Å, 3.1 Å etc. throughout the manuscript.

All resolution values throughout the manuscript have been revised to report only one digit after the decimal point (e.g., 3.1 Å, 2.2 Å).

- regarding HHT complexes, reference G. de Loubresse et al. is for the yeast ribosome; there is also an HHT complex with the human ribosome (see doi: 10.1038/nature24482. PMID: 29143818)

We thank the reviewer, and the citation has been added.

- Ext. Fig. 4: if the mRNA region is at lower resolution (which can be expected due to its mixed nature), then this should be explained in the text (which otherwise gives a false impression) and in addition a local resolution map for the mRNA region should be shown.

Overall, the resolution of the mRNA is generally good, with many nucleotide bases clearly resolved—particularly given its heterogeneous nature. However, certain regions, especially those not base-paired with tRNAs, exhibit reduced resolution due to increased conformational flexibility. For example, in the P–E state, the mRNA density at the A site is notably weaker. We have clarified these observations in the main text (lines 88-89) and updated the figure legend accordingly.

While we appreciate the reviewer's suggestion to include local resolution maps specifically for the mRNA, generating such maps across all states—focusing on this small and inherently dynamic region—would overly complicate the figure (with over 20 subpanels) without substantial benefit. Given the averaged and flexible nature of the mRNA density, we believe such local resolution maps would offer limited biological insight. For these reasons, we have chosen to retain the current presentation of the mRNA densities in the supplementary materials without further elaboration.

- SERBP1: if after checking the structure only ~10 amino acids are visible (referee 3), most of the conclusions on that factor are likely to be unreliable (e.g. localisation of the N-domain, which could have been novel, but now turns out not to be); hence, also the prediction is to be taken more carefully. This should be clarified. In this context, the phrase in the abstract regarding SERBP1 also appears confusing.

We thank the reviewer for raising this important point. As stated in response to Reviewer #2, to address the concerns regarding the assignment of SERBP1, we have revised the manuscript to adopt a more conservative interpretation. Specifically, we have removed the section discussing definitive identification of SERBP1 and now refer to the two densities in the updated Figure 4 as “unknown density 1” and “unknown density 2.” We report only that the built models for these densities closely resemble AlphaFold2 predictions for various segments of SERBP1, without asserting a definitive assignment or biological interpretation.

- cryo electron microscopy (without hyphen; hyphen to be used only for the abbreviation: cryo-EM)

We thank the reviewer for the comment. All instances of "cryo-electron microscopy" (full term) have been checked and corrected to remove any hyphens.

- Figure 7a: is the scale the same in x and y directions? it looks extended in the y direction (which would become x direction after rotating 90° right/clockwise).

We apologize for the confusion. The image shown in Figure 7a (now updated as Figure 6a) uses the same scale in both the x and y directions. The ribosome was intentionally displayed in a non-canonical orientation to better highlight the locations and densities of bound HHT and CHX. This specific viewing angle and clipping may give the impression of anisotropic scaling, but we have confirmed that no distortion was applied during rendering.

- validation report of atomic model: what is chain CA (60S)? The quality of the atomic model would be expected to be better at the claimed resolution. For the A-P complex, there are many chains with medium fit; similar issues in some chains of the other atomic models, some show strong deviations, which should be corrected prior to PDB/EMBD deposition for all entries.

We thank the reviewer for the valuable feedback. In response, we have taken the following comprehensive actions:

1. **Model Refinement:** We have devoted substantial effort to improve all atomic models through multiple rounds of real-space refinement and manual adjustments for each of the reported structures, with a particular focus on regions flagged for poor fit or strong deviations in the validation reports or by the reviewer. The validation reports now demonstrate high-quality model metrics.
2. **Chain CA:** In our notation, chain CA refers to EBP1. As the density for EBP1 is well resolved in the 60S structure, we have modeled EBP1 in the 60S complex.
3. **Updated Deposition:** The refined models and corresponding cryo-EM maps have been deposited in the PDB/EMDB.
4. **Validation Reports:** Official PDB validation reports for all final atomic models have been generated and are included with the resubmission.

We believe these efforts significantly improve the quality of the atomic models.