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Structural basis of mitoribosomal tRNA translocation catalyzed by mammalian mitochondrial elongation factor G1 (mtEFG1)

Eva Kummer and Nenad Ban.

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 7th April 2020

Thank you again for submitting your manuscript reporting cryo-EM structures of the mammalian mitochondrial ribosome with EFG1 for consideration by The EMBO Journal. Please apologize the delay in communicating this decision to you. As mentioned, we had been waiting for a repeatedly delayed report, which we have unfortunately still not received. Given the expertise of this particular referee in cryo-EM analyses, which we felt is needed, and in the interest of time, I meanwhile contacted another referee with such technical expertise. The comments of these referees are now included below for your information.

As you will see, the reviewers are overall positive and acknowledge the study's contribution to the field and its quality. Nonetheless they also raise some concerns that would need to be addressed by revising the figures/text or adding to the discussion in a revised manuscript. Therefore, I would now like to invite you to prepare and submit a revised manuscript.

REFEREE REPORTS

Referee #2:

This work by Krummer and Ban is focused on the structural insights from the two cryo-EM structures of the mammalian mitochondrial ribosome in complex with the translation GTPase EF-G and captured in two consecutive stages of ribosome translocation. Due to the enormous effort of the Ban research group as well as other groups, over the last several years a number of excellent publications revealed the detailed structures of mitochondrial ribosomes from various species. And now, when we more-or-less know how mitoribosomes are structurally organized, it is really curious

to see how they function and whether or not there are any conceptual differences in functioning between mitoribosomes and prokaryotic and/or eukaryotic cytoplasmic ribosomes? This work represents a long-awaited logical continuation of the previous work by the same group and reveals many interesting aspects of ribosome translocation in mitochondria. Studying ribosome translocation in mitochondria is crucial not only from a fundamental point of view but also has far-reaching medical implications because many antibacterial antibiotics exhibit their side effects by targeting human mitochondrial ribosomes. Here, the authors provide a rational explanation of why, for example, such antibiotic as Fusidic Acid that targets bacterial EF-G protein does not act upon mitochondrial EF-G. Also, detailed analyses of the obtained structures revealed a number of important differences between bacterial and mitochondrial ribosome translocation.

Overall this work is an excellent and exemplary research study that was accomplished in the best traditions of structural biology! In my opinion, this work represents a significant conceptual advance in the field and also answers several long-standing questions. The main findings of this study merit publication in The EMBO Journal and are ideally suited for this journal in terms of scope. Moreover, the manuscript is very well written and organized. It has simple and bright illustrations that are self-explanatory. Actually, I was able to get the main message and the key findings of this work just by looking at the figures, even without reading the main text or figure legends. To conclude, this reviewer is enthusiastically in favor of publishing this work. Also, this reviewer has several minor critical points, which the authors might wish to address:

Comments, suggestions, and questions to the authors:

1. For structural studies, the authors used mitoribosomes from a pig (Sus scrofa) complexed with human mtEFG1. What was the rationale for assembling such chimeric complex instead of using swine mtEFG1 (especially, with the desired ORF being synthesized)? How similar are swine and human mtEFG1s? What are the chances that human mtEFG1 would bind any different to a swine mitoribosome? I think these are pretty obvious questions that might pop-up in the heads of many readers, especially given the extremely broad readership of EMBO Journal, and authors might wish to include the answers to these questions at the beginning of the results section.

2. For structural studies, the authors used a short synthetic mRNA which contained only two codons (CUG AUG). If I understand everything correctly, the first CUG codon appears in the E site of the mitoribosome while the second AUG codon appears in the P site and forms canonical codon-anticodon interactions with the initiator tRNA in the P site (Figure 2B). Apparently, there was no mRNA present in the A site and, therefore, the entire complex is not strictly a post-translocation state. What was the rationale for not including at least one extra codon for the A site to make it more physiologically relevant?

3. Overall the "Results" section of this manuscript is written more as results already combined with the discussion. Personally, I like this format way more than a traditional format with the two separate sections "Results" and "Discussion". Therefore, I would like to suggest to the authors to rename their "Results" section to "Results and Discussion" and their current "Discussion" section is really nothing else but "Conclusions" and summary of the important findings.

4. Panel A in Figure 1 is fantastic with excellent graphics! However, what I find missing is another panel with a scheme of translocation stages. Because this is not a review article, but actually a research paper, this scheme doe not need to include all the stages of translation but rather be focused mainly on the steps of translocation and schematically depict what are the steps known, what are the steps visualized in the current work, and what are the differences between the steps? In think, the introduction section of this work would also benefit from having such a scheme (either as apanel in Figure 1 or as a separate figure). The authors are welcome to ignore this comment because the desired scheme is partially shpown in Figure 6.

5. Figures 2 and 3: Especially for panel A, I would like to suggest including small insets showing the direction of the view relative to the zoomed-out ribosome.

6. Figure 4: Panel A looks too busy. The main point of this figure is to show that the area around the FA binding site is densely packed in mtEFG1 and not so constrained in the case of bacterial EFG. For this purpose, showing the rRNA at the background might not be necessary and even distracting.

7. Lastly, the structural comparison (superposition) of mtEFG1 and bacterial EFG could be included as a panel in one of the main-text figures. One of the questions that almost every reader of this work will have is "What are the main differences between the ribosome-bound mtEFG1 and the ribosome-bound bacterial EFG?"

Referee #3:

- general summary and opinion about the principle significance of the study, its questions and findings

There are many intricacies of mammalian mitochondrial translation that await understanding and this manuscript addresses one of these, namely translocation of the mt-tRNAs and mt-mRNAs through the mitoribosome.
The abstract is clearly written and the introduction sets out what is known about this process in other systems and the role of elongation factor G before detailing the differences in mitochondria. The only aspect of the introduction that I believe should be addressed is where the authors state "This strict task sharing is in stark contrast to bacterial EFG that plays a role not only in the elongation phase but is also crucially involved in ribosome recycling.". This is a little disingenuous as there have been an increasing number of instances where a second EFG has been found in different bacterial species (>140 species: PMID: 21829651) a number of which are involved in ribosome recycling. One such report indicating this second form is given below. In some cases this observation has followed on the back of identifying the mitochondrial form.

A bacterial elongation factor G homologue exclusively functions in ribosome recycling in the spirochaete Borrelia burgdorferi.
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An important observation is that the identification of how EFG2 interacts with bL12m-CTD shows similarity to the interaction between these proteins in bacteria. Likewise seeing the impact of how different antibiotics might interact differently with bacterial and mitochondria ribosomes in of clinical value.

The mammalian mitoribosome has many features that distinguish it from other ribosomes. This includes the acquisition of many mito-specific proteins as well as the loss of regions of rRNAs. The data in this report is of high quality and substantiates the authors proposal that the loss of one such region in the 16S rRNA affects the binding of the mt-tRNA elbow regions and therefore the hinge around which the L1 stalk moves during translocation. The outcome of which is that the E-site mt-tRNA is not stabilised as fully as in the bacterial counterpart. This premise was made by Prof R Agrawal a number of years prior to the reference cited and this should be added for completeness to the referencing.

- specific major concerns essential to be addressed to support the conclusions

The use of a short RNA to simulate the mt-mRNA to prime the mitoribosome is experimentally appropriate. However, the hexanucleotide used does not seem to correspond to any 5' initiating sequence that this reviewer could find in the mtDNA sequence for S. scrofa (GenBank: AJ002189.1). There do not appear to be any monocistrons that have nucleotides preceding the start codon, so an explanation for the choice of this sequence should be inserted in to the text.

Another reason to explain the choice of hexanucleotide is that for initiation of translation, the fmet-tRNAmet is positioned in the P-site and so translocation would move the hexanucleotide out of the ribosome rather than onto a second coding triplet, which is slightly less physiological. Perhaps some text to explain why this template was used and not a slightly longer one, or one lacking nucleotides 5' to the start codon.

With this in mind Fig 2 D makes it difficult to see how the fmet-tRNAmet aligns with the AUG, but this is probably just the positioning of the A, U and G in the figure. Perhaps this could be adjusted ?

- minor concerns that should be addressed

The details depicted in Fig 3 are difficult to follow as all that is being described as the features being pointed out in the text are not marked in the figure. Fig 3c mentions the binding of mtEFG1 to GAC but the latter is not introduced for context (not the hexanucleotide as it is the wrong sequence or in the wrong orientation) nor clearly marked. Similarly, the cluster that includes 16S helices 43, 44 and 89, only H43 is labelled making it difficult to coordinate the text and the images 3D and E.

The model in Fig 6 is rather busy and makes it hard to see which section of the figure the bullet point 'conserved elements in tEFG1 . . . . .' refers to. Why is one arrow dashed the other solid ? If the fig had designated A, B and C then some of the bullet points cod be placed in the legend and referred to the panels making it clearer and easier to follow.

Referee #4
As far as I can tell, this is excellent work and they have obtained important new insights into the operation of mitochondrial ribosomes. I have some technical issues with the presentation of the structural analysis, mostly minor:

The panels in fig 1A are simply described as "structures". Are these the experimentally determined cryo EM maps, or has the density been generated from the fitted atomic structures? The primary experimental results should be shown, even if they are segmented and coloured according to the fitted components.

The FSC resolution curve for POST in Fig EV2 is not correct. It is cut off at 0 FSC but clearly goes negative. The whole curve should be shown. The lack of a smooth transition to 0 value may indicate some errors in the analysis and this should be checked.

Fig 3A and D are connected by boxes suggesting that the view in D is an enlargement of the boxed area in A, but they don't correspond either in view or colour code and the inset is described as an overview. This is very confusing.

Data collection & image processing p12 line 9: words missing "unsupervised 3D classification using a the 55S mitoribosome"

1st Revision - authors' response 15th April 2020

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view but also has far-reaching medical implications because many antibacterial antibiotics exhibit their side effects by targeting human mitochondrial ribosomes. Here, the authors provide a rational explanation of why, for example, such antibiotic as Fusidic Acid that targets bacterial EF-G protein does not act upon mitochondrial EF-G. Also, detailed analyses of the obtained structures revealed a number of important differences between bacterial and mitochondrial ribosome translocation.

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Comments, suggestions, and questions to the authors:

1. For structural studies, the authors used mitoribosomes from a pig (Sus scrofa) complexed with human mtEFG1. What was the rationale for assembling such chimeric complex instead of using swine mtEFG1 (especially, with the desired ORF being synthesized)? How similar are swine and human mtEFG1s? What are the chances that human mtEFG1 would bind any different to a swine mitoribosome? I think these are pretty obvious questions that might pop-up in the heads of many readers, especially given the extremely broad readership of EMBO Journal, and authors might wish to include the answers to these questions at the beginning of the results section.

ANSWER: Mitochondrial ribosomes are present in low abundance. For this reason, we isolate mammalian mitoribosomes from pig liver tissue as it supplies us with rather large amounts of mitochondria and thus good yields for mitochondrial ribosomes that would be more difficult to obtain from cultured human cells. Human and swine mitochondrial ribosomes are highly similar especially with respect to functionally important sites of the ribosome. Therefore, porcine mitoribosomes are an excellent model for mammalian mitochondrial translation (Greber et al. Science 2015, Amunts et al. Science 2015). Swine and human mitochondrial EFG1 are as well highly similar with 91.7 % identity and 96.7 % similarity in their primary sequences. Residues that differ between swine and human are not located within functional sites important for nucleotide binding, FA resistance as well as interaction with the ribosomal stalk base, the tRNA-mRNA module or the bL12m CTD. Therefore, there is no indication that swine and human mtEFG1 would function differently. We decided to use the human protein to help in mapping disease-causing mutations in human proteins and as a logical continuation of our earlier published work on the mitochondrial initiation complex that employed a chimeric approach as well. Moreover, the ultimate goal will be to use the structural information to develop better antibiotics with less side effects in human mitochondria. Here minor changes in amino acid side chains of translation factors may make a difference when rationalizing good compound candidates and for this purpose having a structure of the human mtEFG1 on the mammalian mitoribosomes might be advantageous.
We have added a comment to the Materials and Methods section and a reference to it in the main text: "Of note: Swine and human mitochondrial EFG1 are highly similar with 91.7 % identity and 96.7 % similarity in their primary sequences indicating their strong

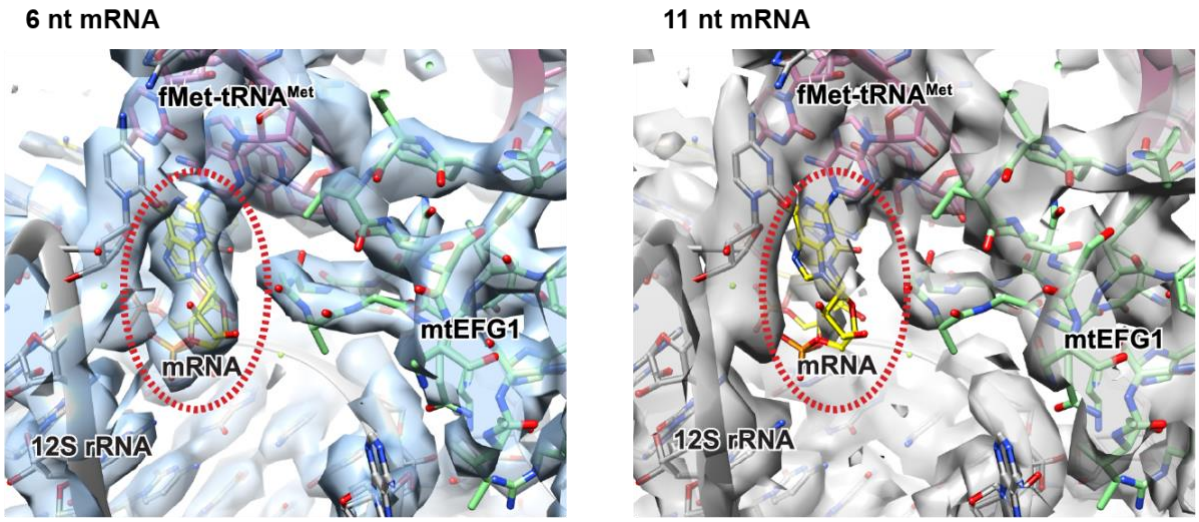
functional conservation. Therefore, we decided to reconstitute the mammalian mitochondrial translation elongation complex as a chimeric system using porcine mitochondrial ribosomes and human mtEFG1 and fMet-tRNA^{Met} so that the results would be more applicable to the scientist interested in the human system."
We have in addition added a comment to the Conclusion: "Our data may furthermore aid to rationalize reported mutations in human mtEFG1 that cause Combined oxidative phosphorylation deficiency 1 (COXPD1) – a fatal mitochondrial disease leading to early and rapidly progressive hepatoencephalopathy[84-87]."

2. For structural studies, the authors used a short synthetic mRNA which contained only two codons (CUG AUG). If I understand everything correctly, the first CUG codon appears in the E site of the mitoribosome while the second AUG codon appears in the P site and forms canonical codon-anticodon interactions with the initiator tRNA in the P site (Figure 2B). Apparently, there was no mRNA present in the A site and, therefore, the entire complex is not strictly a post-translocation state. What was the rationale for not including at least one extra codon for the A site to make it more physiologically relevant?

The answer to this question also addresses a related question posed by referee 3:
ANSWER: Reconstitution of mitochondrial translation complexes is in the field still in its infancy in comparison with bacterial and even eukaryotic systems. Our lab has managed to produce relevant complexes in recent years for the first time. While establishing the protocols, we have tested a number of constructs and conditions and knew that the short mRNA oligo can be incorporated into the translational complexes efficiently. This was one of the reasons why we opted in first place to use this oligo. However, there are more reasons to believe that this oligo is a reasonable choice for the present study. Ribosomes can encounter nucleolytically truncated mRNAs inside the cell. In such a scenario, mtEFG1 will still have to translocate the tRNA-mRNA module even when the ribosome reaches the 3' end of the message and only subsequently the stalled ribosomes will be rescued. Such a situation is actually very similar to what we see in our study and we deem our approach therefore relevant. Moreover, to our knowledge there is no indication in the literature that a codon preceding the mRNA-tRNA module is required for EFG-catalyzed translocation in the related bacterial system. In addition, in mammalian mitochondria only one type of tRNA, mtRNA^{Met}, is used both for initiation as well as elongation. We use this mtRNA^{Met} because site-specific delivery of other mtRNA species to the ribosomal P site is currently still difficult and we wanted to reconstitute a complex that carries a P site tRNA. The CUGAUG oligo enabled us to see the TI^{POST} state, in which we find two mRNAs in transit since the mtRNA^{Met} is able to establish an interaction with the CUG codon despite a base mismatch. We had referred in the text to this unusual pairing as: "Both tRNAs maintain base pairing interactions with their mRNA codons in Ti^{POST}, although the pe/E site tRNA interacts due to a mismatch of the CUG mRNA codon more weakly (Fig. 2B). " We did not anticipate binding of mtRNA^{Met} to the CUG codon in the beginning, but we believe that it may have happened in a subset of the complexes due to a tighter embedding of the mtRNA^{Met} in the ribosomal P site that stabilizes this non-canonical interaction. This is likely caused by the interaction of the unique 3 successive GC base pairs in the mtRNA^{Met} anticodon stem loop with the conserved P site element of the SSU head, the G782/A783 ridge. Therefore, this circumstance somewhat coincidentally offers us the unique opportunity to observe a true translocation scenario with two neighbouring tRNAs and provides a number of functionally important insights. As these structures are similar to translocation states observed in the bacterial, ancestral system, we believe that the complexes are physiologically relevant.

Nonetheless, we have also already reconstituted the elongation complex using a longer mRNA construct with 5 nucleotides 3' of the P site AUG codon, i.e. a length of 11 nucleotides in total. We find that delivery of this mRNA in our reconstitution approaches is less efficient resulting in partial occupancy and poorer resolution of the tRNA-mRNA module and loop 1 of mtEFG1. An image showing a comparison with the map of the POST complex derived from the sample with the 6nt-long mRNA and the 11nt-long mRNA is attached (see below). As the structures employing the 11nt-long oligo do not generate different or additional insights into the translocation process and due to space limitations we decided to not include this data into the manuscript.

As our answer to this question is quite complex we added a comment into the Methods section instead of the main text: "Of note: The short CUGAUG oligo and mtRNA^{Met} were used for reconstitution due to their efficient binding to the SSU and the elevated propensity of mtRNA^{Met} to bind to the mitoribosomal P site due to a 3x CG base pair in its anticodon stem loop that interacts with the conserved P site G782/A783 ridge. Slightly longer mRNA oligonucleotides were less efficiently bound to the mitoribosomal SSU during in vitro reconstitution. Originally, we did not anticipate the non-canonical interaction of the mtRNA^{Met} with the CUG codon in the TI^{POST} state but this presumably turned out to be possible due to the tighter binding of the mtRNA^{Met} to the conserved P site element."



Comparison of the mRNA density found when reconstituting the elongation complex with the 6nt mRNA oligonucleotide presented in this study (left) and a mRNA oligo containing 5 additional nucleotides 3' the AUG codon (right). The location of the mRNA is highlighted with a red ellipse. The density is clearly weaker in case of the longer oligonucleotide indication only a partial occupancy and less efficient binding of the longer oligonucleotide in comparison to the shorter.

3. Overall the "Results" section of this manuscript is written more as results already combined with the discussion. Personally, I like this format way more than a traditional format with the two separate sections "Results" and "Discussion". Therefore, I would like to suggest to the authors to rename their "Results" section to "Results and Discussion" and their current "Discussion" section is really nothing else but "Conclusions" and summary of the important findings.

ANSWER: We fully agree and have renamed the respective sections.

4. Panel A in Figure 1 is fantastic with excellent graphics! However, what I find missing is another panel with a scheme of translocation stages. Because this is not a review article, but actually a research paper, this scheme doe not need to include all the stages of translation but rather be focused mainly on the steps of translocation and schematically depict what are the steps known, what are the steps visualized in the current work, and what are the differences between the steps? In think, the introduction section of this work would also benefit from having such a scheme (either as apanel in Figure 1 or as a separate figure). The authors are welcome to ignore this comment because the desired scheme is partially shpown in Figure 6.

ANSWER: Even though it would be nice to include a scheme that visualizes the translocation steps during mitochondrial translation elongation, we deliberately decided not to do so. The reason is that for the mitochondrial system a detailed analysis of ribosomal and tRNA motions has not been investigated prior to our study, either structurally or biochemically. Although it is likely that the mitochondrial ribosome works using a similar translocation trajectory as the bacterial system, so far only our structural data provide the first experimental evidence for this. We believe that depicting such a scheme will provide the reader with the wrong impression that these motions have actually already been studied for the mitochondrial system. For this reason, we have also not included depictions of additional, putative translocation intermediates in Fig.6 that are known from bacteria but rather a dashed arrow to indicate the existence of possible additional intermediate states.

5. Figures 2 and 3: Especially for panel A, I would like to suggest including small insets showing the direction of the view relative to the zoomed-out ribosome.

ANSWER: Small insets have been added to Fig. 2A and 3A and the respective regions have been highlighted.

6. Figure 4: Panel A looks too busy. The main point of this figure is to show that the area around the FA binding site is densely packed in mtEFG1 and not so constrained in the case of bacterial EFG. For this purpose, showing the rRNA at the background might not be necessary and even distracting.

ANSWER: Fig. 4A has been replaced with images that do not contain ribosomal RNA.

7. Lastly, the structural comparison (superposition) of mtEFG1 and bacterial EFG could be included as a panel in one of the main-text figures. One of the questions that almost every reader of this work will have is "What are the main differences between the ribosome-bound mtEFG1 and the ribosome-bound bacterial EFG?"

ANSWER: They are structurally very similar. We have added a superposition as Fig. EV4B that has been linked to the following sentence in the results section: "The overall conformation of mtEFG1 is similar to previous structural reports from the bacterial system (Fig. EV4B) [4-7, 29, 36-42]."

Referee #3:

- general summary and opinion about the principle significance of the study, its questions and findings

There are many intricacies of mammalian mitochondrial translation that await understanding and this manuscript addresses one of these, namely translocation of the mt-tRNAs and mt-mRNAs through the mitoribosome.
The abstract is clearly written and the introduction sets out what is known about this process in other systems and the role of elongation factor G before detailing the differences in mitochondria. The only aspect of the introduction that I believe should be addressed is where the authors state "This strict task sharing is in stark contrast to bacterial EFG that plays a role not only in the elongation phase but is also crucially involved in ribosome recycling.". This is a little disingenuous as there have been an increasing number of instances where a second EFG has been found in different bacterial species (>140 species: PMID: 21829651) a number of which are involved in ribosome recycling. One such report indicating this second form is given below. In some cases this observation has followed on the back of identifying the mitochondrial form.

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Suematsu T, Yokobori S, Morita H, Yoshinari S, Ueda T, Kita K, Takeuchi N, Watanabe Y
Mol Microbiol. 2010 Mar; 75(6):1445-54

ANSWER: We agree with the referee and have included now a comment and the appropriate references in the introduction as follows:
"EFG function has been extensively studied in bacteria. However, in the mitochondrial system translation elongation is poorly investigated so far and no structural information is available for mtEFG1 action during mRNA-tRNA translocation on mitochondrial ribosomes. Strikingly, mitochondria have evolved two paralogues of EFG, mtEFG1 and mtEFG2, which catalyze different steps of the translation cycle(Hammarsund, Wilson et al. 2001, Tsuboi, Morita et al. 2009). Mitochondrial EFG1 (mtEFG1) acts during translation elongation while mitochondrial EFG2 (mtEFG2) partakes in ribosome recycling(Chung and Spremulli 1990, Bhargava, Templeton et al. 2004, Tsuboi, Morita et al. 2009). This strict task sharing is in stark contrast to canonical bacterial EFG that plays a role not only in the elongation phase but is also crucially involved in ribosome recycling. The molecular basis for the separation of the dual function of canonical bacterial EFG over two separate proteins in mitochondria is not understood. However, in recent years, it has become known that also some bacterial species carry two paralogues of EFG.(Hammarsund, Wilson et al. 2001, Pandit and Srinivasan 2003, Atkinson and Baldauf 2011) While both paralogues in the spirochaete Borrelia burgdorferi show a similar task distribution as mitochondrial mtEFG1 and mtEFG2, the role of EFG2 in other bacterial species is still unclear.(Connell, Takemoto et al. 2007, Seshadri, Samhita et al. 2009, Suematsu, Yokobori et al. 2010)."

An important observation is that the identification of how EFG2 interacts with bL12m-CTD shows similarity to the interaction between these proteins in bacteria. Likewise seeing the impact of how different antibiotics might interact differently with bacterial and mitochondria ribosomes in of clinical value.

The mammalian mitoribosome has many features that distinguish it from other ribosomes. This includes the acquisition of many mito-specific proteins as well as the loss of regions of rRNAs. The data in this report is of high quality and substantiates the authors proposal that the loss of one such region in the 16S rRNA affects the binding of the mt-tRNA elbow regions and therefore the hinge around which the L1 stalk moves during translocation. The outcome of which is that the E-site mt-tRNA is not stabilised as fully as in the bacterial counterpart. This premise was made by Prof R Agrawal a number of years prior to the reference cited and this should be added for completeness to the referencing.

ANSWER: We thank the referee for careful reading of the manuscript and have added following reference to the L1 section: Sharma, M. R.; Koc, E. C.; Datta, P. P.; Booth, T. M.; Spremulli, L. L.; Agrawal, R. K., Structure of the mammalian mitochondrial ribosome reveals an expanded functional role for its component proteins. *Cell* **2003,** *115* (1), 97-108.

- specific major concerns essential to be addressed to support the conclusions

The use of a short RNA to simulate the mt-mRNA to prime the mitoribosome is experimentally appropriate. However, the hexanucleotide used does not seem to correspond to any 5' initiating sequence that this reviewer could find in the mtDNA sequence for S. scrofa (GenBank: AJ002189.1). There do not appear to be any monocistrons that have nucleotides preceding the start codon, so an explanation for the choice of this sequence should be inserted in to the text.

Another reason to explain the choice of hexanucleotide is that for initiation of translation, the fmet-tRNAmet is positioned in the P-site and so translocation would move the hexanucleotide out of the ribosome rather than onto a second coding triplet, which is slightly less physiological. Perhaps some text to explain why this template was used and not a slightly longer one, or one lacking nucleotides 5' to the start codon.

ANSWER: We thank the reviewer for the opportunity to explain this point further by referring to the answer above provided in response to a highly related question by referee 2.

With this in mind Fig 2 D makes it difficult to see how the fmet-tRNAmet aligns with the AUG, but this is probably just the positioning of the A, U and G in the figure. Perhaps this could be adjusted?

ANSWER: The viewing angle in Fig. 2D cannot be adjusted without loosing the appropriate view on the interaction of the tip of mtEFG1 domain IV with the tRNA-mRNA module (loop1 and 2 here). Therefore, we have added an image of the codon-anticodon interaction of the POST state as Fig. EV4A and the interaction of both tRNAs with the mRNA in the TI^{POST} state is shown in Fig. 2B.

- minor concerns that should be addressed

The details depicted in Fig 3 are difficult to follow as all that is being described as the features being pointed out in the text are not marked in the figure. Fig 3c mentions the binding of mtEFG1 to GAC but the latter is not introduced for context (not the

hexanucleotide as it is the wrong sequence or in the wrong orientation) nor clearly marked. Similarly, the cluster that includes 16S helices 43, 44 and 89, only H43 is labelled making it difficult to coordinate the text and the images 3D and E.

ANSWER: To clarify that we refer in Fig. 3 to the GTPase-associated center (GAC) described in the text, we have included now a small inset that highlights the respective region on the large ribosomal subunit. We have also replaced Fig. 3D and made sure that the viewing directing is identical to the one shown in Fig. 3A. Moreover, we have also included elements of the GTPase-associated center (H89, H43, H44, SRL and uL11m) that have been described in the text to provide more context to the figure. Eventually, we have revised the figure legend as follows:

Fig. 3 mtEFG1 binding induces a concerted motion in the stalk base of the ribosomal GTPase-associated center.
(A) The interaction of mtEFG1 in the POST state with the GTPase-associated center (GAC) *via* domain V and with a bL12m-CTD monomer (grey) *via* the G domain are shown (view from the subunit interface onto the LSU). The respective area is highlighted on the inset as red box. mtEFG1 domains are indicated according to the color code introduced in Fig. 2D. The corresponding EM density is depicted lowpass-filtered to 5 Å and at σ=2.5.
(B) The positions of the uL11m N-terminal domain (NTD) and 16S rRNA helices H43 and H44 that form the stalk base of the GAC experience a downward motion upon binding of mtEFG1 (violet) but not upon binding of mtIF2 (orange) or in the factor-free ribosome (grey, pdb: 5AJ4[59]). Complexes have been superimposed using the 16S rRNA of the LSU. The arrows display the direction of motion.
(C) The magnitude of the downward motion of the stalk base comparing the mtIF2-bound and mtEFG1-bound mitoribosome has been calculated in Å and the stalk base components have been colored accordingly. Elements rebuilt in the current model were excluded from the calculation and are shown in grey.
(D) An enlarged view of the area in the black box of Fig. 3A is shown. mtEFG1 domain V extensively interacts with multiple elements of the GAC at 5 sites that have been color-coded. The orange, pink and blue clusters interact with 16S rRNA helices H89, H43 and H44, respectively. The red cluster stacks onto the tip of the sarcin-ricin loop (SRL) and the green cluster contacts the uL11m N-terminal domain (NTD).
(E) Close-ups of the five interaction sites of mtEFG1 domain V with the SRL (red), uL11m-NTD (green), 16S rRNA helices H43 (magenta), H44 (blue) and H89 (orange). The respective EM densities of the POST state are shown at σ=4.

The model in Fig 6 is rather busy and makes it hard to see which section of the figure the bullet point 'conserved elements in tEFG1 . . . . .' refers to. Why is one arrow dashed the other solid ? If the fig had designated A, B and C then some of the bullet points cod be placed in the legend and referred to the panels making it clearer and easier to follow.

ANSWER: We have added an arrow to indicate where the bullet point belongs. The difference between the dashed and the solid arrow is that the dashed arrow indicates that there are very likely multiple additional translocation intermediates that the ribosome passes through between peptide bond formation and the TI^{POST} and POST states. We have added following sentence in the figure legend: "The dashed arrow indicates that in analogy to the bacterial system likely multiple additional translocation intermediates exist preceding the ones visualized in this study."

Referee #4

As far as I can tell, this is excellent work and they have obtained important new insights into the operation of mitochondrial ribosomes. I have some technical issues with the presentation of the structural analysis, mostly minor:

The panels in fig 1A are simply described as "structures". Are these the experimentally determined cryo EM maps, or has the density been generated from the fitted atomic structures? The primary experimental results should be shown, even if they are segmented and coloured according to the fitted components.

ANSWER: These are surface representations of the structural models. As the EM maps have varying local resolutions it is difficult to find the appropriate balance between filtering the maps to display also low resolved, flexible features and at the same time not to blur the structural details that we see at high resolution. We have therefore decided to show the structural models instead for an overview as we show the experimental densities critical for our interpretations in the respective close-up views of the other figures. In addition, we have now included images of the EM maps color-coded according to the underlying structural model as additional Fig. EV3.

The FSC resolution curve for POST in Fig EV2 is not correct. It is cut off at 0 FSC but clearly goes negative. The whole curve should be shown. The lack of a smooth transition to 0 value may indicate some errors in the analysis and this should be checked.

ANSWER: Possibly there is a misunderstanding here: the FSC curves were not plotted until FSC equals zero but rather until Nyquist, i.e. until the last significant resolution shell (in our case at 2.78 Å or 0.36 $Å^{-1}$). We have now modified the graph such that the X axis stops exactly at Nyquist and have expanded the Y axis to cover a range of -0.1 to 1.1 to clarify that the FSC curves approaches zero but does not go below until Nyquist. We believe that our data contain useful signal even at resolutions approaching Nyquist due to the very good DQE of the Falcon III detector at high spatial frequencies. This may likely cause the FSC curve to only drop towards zero at Nyquist. Possibly, if we would have collected the images using a smaller pixel size the transition towards zero would have been smoother.

Fig 3A and D are connected by boxes suggesting that the view in D is an enlargement of the boxed area in A, but they don't correspond either in view or colour code and the inset is described as an overview. This is very confusing.

ANSWER: Fig. 3D was indeed intended to show an enlarged view of the boxed area in Fig. 3A but we agree with the reviewer that this might not be entirely obvious with the chosen image. We have revised Fig. 3D now more carefully and made sure that the viewing direction is identical to the one shown in Fig. 3A. Moreover, we have also included surrounding elements of the GTPase-associated center (H89, H43, H44, SRL and uL11m) to provide more context to the figure. In addition, we show a small inset in Fig. 3A to clarify the location of the depicted region on the large ribosomal subunit. Eventually, we have revised the figure legend as follows:

Fig. 3 mtEFG1 binding induces a concerted motion in the stalk base of the ribosomal GTPase-associated center.
(A) The interaction of mtEFG1 in the POST state with the GTPase-associated center (GAC) *via* domain V and with a bL12m-CTD monomer (grey) *via* the G domain are shown (view from the subunit interface onto the LSU). The respective area is highlighted on the inset as red box. mtEFG1 domains are indicated according to the color code introduced in Fig. 2D. The corresponding EM density is depicted lowpass-filtered to 5 Å and at σ=2.5.
(B) The positions of the uL11m N-terminal domain (NTD) and 16S rRNA helices H43 and H44 that form the stalk base of the GAC experience a downward motion upon binding of mtEFG1 (violet) but not upon binding of mtIF2 (orange) or in the factor-free ribosome (grey, pdb: 5AJ4[59]). Complexes have been superimposed using the 16S rRNA of the LSU. The arrows display the direction of motion.
(C) The magnitude of the downward motion of the stalk base comparing the mtIF2-bound and mtEFG1-bound mitoribosome has been calculated in Å and the stalk base components have been colored accordingly. Elements rebuilt in the current model were excluded from the calculation and are shown in grey.
(D) An enlarged view of the area in the black box of Fig. 3A is shown. mtEFG1 domain V extensively interacts with multiple elements of the GAC at 5 sites that have been color-coded. The orange, pink and blue clusters interact with 16S rRNA helices H89, H43 and H44, respectively. The red cluster stacks onto the tip of the sarcin-ricin loop (SRL) and the green cluster contacts the uL11m N-terminal domain (NTD).
(E) Close-ups of the five interaction sites of mtEFG1 domain V with the SRL (red), uL11m-NTD (green), 16S rRNA helices H43 (magenta), H44 (blue) and H89 (orange). The respective EM densities of the POST state are shown at σ=4.

Data collection & image processing p12 line 9: words missing "unsupervised 3D classification using a the 55S mitoribosome"

ANSWER: This has been corrected to "unsupervised 3D classification using the 55S mitoribosome".

2nd Editorial Decision 8th May 2020

Thank you again for submitting your revised manuscript. Please apologize the delay in communicating this decision to you, which was due to delayed referee reports on account of the current Covid-19 pandemic. We now have the reports from the original referees (please see comments below). I am pleased to say that the referees overall find that their concerns have been satisfactorily addressed and now support publication. Referee #4 raises one more point regarding a textual change, which can be incorporated into the final revised version. In addition, I would also ask you to address a number of editorial issues that are listed in detail below. Please make any changes to the manuscript text in the attached document only using the "track changes" option. Once these remaining issues are resolved, we will be happy to formally accept the manuscript for publication.

REFEREE REPORTS

Referee #2:

After carefully reading the revised version of the manuscript, this reviewer has no more remaining critical points and has no objections to publication of this work. In my opinion, authors did an extremely good job in addressing all of the critical points of this and other reviewers. I think that the manuscript could be accepted for publication in its current form.

Referee #3:

The authors have responded satisfactorily to all the queries made by this reviewer and appear to have done the same for the other reviewers' concerns.

This is an excellent manuscript, which has been improved by these modifications. I recommend publication of this revised version.

Referee #4:

The revisions have largely addressed my concerns, except for one point: the legend to Figure 1 must explain what is being shown. As mentioned in the first review, just calling the maps "structures" is misleading and inadequate. The legend should clearly state that these are surface representations generated from the atomic structures fitted to the EM maps.

2nd Revision - authors' response 8th May 2020

The Authors have made the requested editorial changes.

Accepted 11th May 2020

Thank you again for submitting the final revised version of your manuscript. I am pleased to inform you that we have now accepted it for publication in The EMBO Journal.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Nenad Ban
Journal Submitted to: EMBO Journal
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Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- 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.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be iustified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

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 χ2 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.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	NA
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	NA
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	NA
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For animal studies, include a statement about randomization even if no randomization was used.	NA
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	NA
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5. For every figure, are statistical tests justified as appropriate?	NA
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7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	NA

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F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	EM maps and structural model have been deposited in the EMDB database or the PDB database, respectively. Accession codes for the TIPOST state are EMD-10779 and 6YDW. Acession codes for the POST state are EMD-10778 and 6YDP.
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