EMBO reports

Molecular view of ER membrane remodeling by the Sec61/TRAP translocon

Sudeep Karki, Matti Javanainen, Shahid Rehan, Dale Tranter, Juho Kellosalo, Juha Huiskonen, Lotta Happonen, and Ville Paavilainen
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Corresponding author(s): Ville Paavilainen (ville.paavilainen@helsinki.fi)

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Referee #1 Review
Received: 7th Jun 23

Report for Author:
The authors have addressed my concerns. I have a few minor comments.

It is unclear why the authors did not show the immunoblotting of INS1 TRAPa KO complemented with TRAPa mutants in Fig. 3E but rather show samples from HEK293 cells in Fig. 3D.
Fig. 3D should be better labeled. How do we interpret the labeling of TRAPa(+/-) and FLAG-tag (-/F)? The authors may want to label non-transfected, WT TRAPa, M1, M2, etc.
The authors state that membrane deformations are costly for the cell on page 10. Since there is no energy involved here, it is not understood why this is costly.

Referee #2 Review
Received: 26th May 23

Report for Author:
The authors have addressed my concerns, and I find the Ms suitable for publication.

Referee #3 Review
Received: 19th Jun 23

Report for Author:
The revised manuscript by Karki et al. presents the structure of a mammalian ribosome in complex with the Sec61/TRAP

translocon, highlighting two key findings. First, cryo-EM imaging of detergent-solubilized microsomes was used to determine the overall architecture of the core Sec61/TRAP complex bound to a ribosome. Second, through molecular dynamics (MD) simulations, it was observed that the Sec61/TRAP complex induces local membrane curvature of the lumenal membrane leaflet. In my initial feedback on the manuscript, I focused on the lack of supporting data that would validate the authors' main conclusions regarding modeling quality and membrane deformation. In this revised version, the authors present a new validation experiment (Fig. 3C-E) and provided further clarification on their approaches to characterize membrane deformation. It is important to note that while the membrane deformation analysis is a novel aspect of this paper, there is still a lack of functional data demonstrating the mechanistic importance of this analysis. Consequently, the manuscript primarily serves as a descriptive overview of the complex and its embedded membrane.

Major comments

1. Structural validation of membrane thinning. As I had requested, the authors attempted to analyze their own structures obtained from detergent-solubilized micelles in order to validate membrane deformation. However, due to the unsuitability of the LMNG detergent as a mimic of a membrane bilayer, they were unable to draw conclusive evidence of membrane deformation. The analysis presented in Reviewer Figure 2 was a valiant attempt to visualize curvature but was inherently limited by the fact that the detergent only covers the transmembrane regions, lacking a continuous bilayer representation. Consequently, the authors' MD results lack supporting evidence from their own structures.

The authors' main source of complementary evidence for membrane deformation lies in the analysis of a previous low-resolution reconstruction of the membrane-bound ribosome-TRAP complex (Martinez-Sanchez et al., Nature Methods 2020, EMD-0084). In the original submission, I expressed dissatisfaction with this analysis due to the extremely low resolution of the map (~22 Å), which could complicate the delineation of membrane boundaries. However, I understand the authors' rationale for utilizing this map, as my suggestion to compare it with higher-resolution OST-containing maps would introduce an additional confounding factor (OST). While acknowledging the authors' good-faith efforts to maximize map interpretability given the available data, I propose two alternative suggestions to further strengthen their conclusion:

a. Conduct a "negative control" analysis of the membrane radius of curvature on a membrane-bound Sec61 structure without TRAP. This additional analysis would provide a baseline comparison to evaluate the specific impact of TRAP on membrane curvature.
b. Perform an analysis using the recently published 6.5 Å membrane-bound 80S-Sec61-TRAP complex (Gemmer et al., Nature 2023, EMD-15885). This higher-quality map would be expected to offer a more robust analysis of membrane curvature, thus reinforcing the authors' findings.

2. Cell-based validation of TRAPalpha interacting residues. The authors present new results of insulin secretion from cells expressing various TRAPalpha mutation constructs (new Fig. 3C-E) and demonstrate that several mutations impair insulin secretion. However, the presented results of this experiment are flawed due to the following reasons:

a. Lack of loading controls in panels C and D: The absence of loading controls in panels C and D hinders the accurate assessment of protein expression levels or potential variations in sample loading.
b. Apparently inconsistent TRAPalpha expression levels (panel D): The observed inconsistencies in TRAPalpha expression levels in panel D raise concerns regarding the reliability and reproducibility of the experiment.
c. Uncertainty regarding whether insulin secretion in panel E is confounded by variable TRAPalpha expression levels in panel D. Given the apparent variability in TRAPalpha expression levels, it becomes uncertain whether the observed differences in insulin secretion can be attributed solely to the introduced TRAPalpha mutations or if they are influenced by variable TRAPalpha expression levels across the samples.
d. Only a single biological replicate was included (N=1). The lack of replication makes it impossible to determine if the observed effects are reproducible.
e. Poor presentation of the M1-M5 mutations: It was challenging to go back-and-forth between the figure and the main text to understand the relevance of each mutant. Additionally, there is a lack of discussion regarding whether M1-M5 are expected to be compared among each other.

3. The refined model indicates an extremely high Clashscore (26.02, Table S3), but details of the clashes are not available in the PDB validation report. The high Clashscore raises concerns about the quality of modeling.

4. The main text indicates that the Sec61/TRAP model was validated by XL-MS, but it seems odd that there is only one interprotein crosslink reported (Fig. 2B).

Minor comments

5. The main text indicates the reconstruction was resolved to an overall resolution of 2.6 Å, but the supplemental data indicates a resolution of 2.7 Å.

6. The use of "high" vs. "low" contour levels appear to be swapped in the manuscript. "High" contour refers to a display at higher threshold levels (i.e., displaying less density).

Dear Dr. Paavilainen

Thank you for the transfer of your research manuscript to our journal. As my colleague at The EMBO Journal, senior editor Hartmut Vodermaier, informed you on July 25th, we are interested in considering your study further for potential publication in EMBO Reports, as long as a final revision could be achieved no later than September 1st (given that related papers have already been published).

Hartmut already informed you about the required revisions, so that you could start the experiments immediately, while I checked your manuscript from the editorial side. I apologize for the delay in this regard, but I was on leave and just came back to the office. As outlined before, the final revision should experimentally address all remaining concerns from referee 1, as well as point 2 from referee 3 - for which it would be sufficient to add the requested "two more biological repeats and a simple statistical analysis" to strengthen the functional validation shown in Figure 3. All other concerns would need to be addressed in the discussion, by toning down the conclusions and discussing the limitations of the current dataset, in particular with regard to the data on membrane thinning.

Please address all referee concerns also in a complete point-by-point response. Your manuscript will be evaluated again by one of the referees and acceptance of the manuscript will depend on a positive outcome of this additional round of re-review.

Please feel free to contact me any time to discuss the revision progress. I am also happy to discuss the revision further via e-mail or a video call, if you wish.

A) You find our general formatting guidelines below, but let me add these important points:

1) Please provide links that resolve to the deposited datasets in the Data Availability section.

2) Please do not calculate statistics on technical replicates.

3) If n = 2, show the individual datapoints instead of the mean/medium and error bars.

4) Always define whether "n" refers to biological/independent or technical replicates in the figure legends.

5) Remove the author contributions from the manuscript text. Please make sure that the author contributions in our online submission system are correct and up-to-date. The information you specified in the system will be automatically retrieved and typeset into the article.

6) Please add a 'Disclosure and competing interests statement'. For more information see
https://www.embopress.org/page/journal/14693178/authorguide#conflictsofinterest

7) This is our reference style and use et al if there are more than 10 authors:
Miller I, Bentham A, Lorem I (year) Title. Journal name (bold, italics) issue (bold): pages

8) Figure legends: if something applies to all panels (such as in Fig. 2 "in all figures") use the prefix "Data information: xxx"

9) All materials and methods must be part of the main manuscript. The MD method might remain part of the Appendix, though, but the rest should be incorporated into the main Methods section. The Supplementary information should be transformed into an Appendix. The Appendix pdf contains all figures and their legends, a title page with a table of content and page numbers. Nomenclature is "Appendix Fig S#" or "Appendix Table S#".

10) We need all figures as individual files and the manuscript as a Word file.

11) Please supply a synopsis image (550 pixels width, height between 200 and 600 px), a short summary (1-2 sentences) and up to 4 bullet points, describing key results.

12) You will receive an e-mail from our source data coordinator Hannah Sonntag. You state in your manuscript that you will upload all raw data to Zenodo. This is sufficient in this case (with a reference to the data in the Data Availability section).

13) Reduce the keywords to only 5.

B) General formatting guidelines:

When submitting your revised manuscript, we will require:

1) a .docx formatted version of the manuscript text (including legends for main figures, EV figures and tables). Please make sure that the changes are highlighted to be clearly visible.

2) individual production quality figure files as .eps, .tif, .jpg (one file per figure).
Please download our Figure Preparation Guidelines (figure preparation pdf) from our Author Guidelines pages https://www.embopress.org/page/journal/14693178/authorguide for more info on how to prepare your figures.

3) a .docx formatted letter INCLUDING the reviewers' reports and your detailed point-by-point responses to their comments. As part of the EMBO Press transparent editorial process, the point-by-point response is part of the Review Process File (RPF), which will be published alongside your paper.

4) a complete author checklist, which you can download from our author guidelines (<https://www.embopress.org/page/journal/14693178/authorguide>). Please insert information in the checklist that is also reflected in the manuscript. The completed author checklist will also be part of the RPF.

5) Please note that all corresponding authors are required to supply an ORCID ID for their name upon submission of a revised manuscript (<https://orcid.org/>). Please find instructions on how to link your ORCID ID to your account in our manuscript tracking system in our Author guidelines (<https://www.embopress.org/page/journal/14693178/authorguide#authorshipguidelines>)

6) We replaced Supplementary Information with Expanded View (EV) Figures and Tables that are collapsible/expandable online. A maximum of 5 EV Figures can be typeset. EV Figures should be cited as 'Figure EV1, Figure EV2" etc... in the text and their respective legends should be included in the main text after the legends of regular figures.

- For the figures that you do NOT wish to display as Expanded View figures, they should be bundled together with their legends in a single PDF file called *Appendix*, which should start with a short Table of Content. Appendix figures should be referred to in the main text as: "Appendix Figure S1, Appendix Figure S2" etc. See detailed instructions regarding expanded view here: <https://www.embopress.org/page/journal/14693178/authorguide#expandedview>

- Additional Tables/Datasets should be labeled and referred to as Table EV1, Dataset EV1, etc. Legends have to be provided in a separate tab in case of .xls files. Alternatively, the legend can be supplied as a separate text file (README) and zipped together with the Table/Dataset file.

7) (See above) Please note that a Data Availability section at the end of Materials and Methods is now mandatory.
See also < https://www.embopress.org/page/journal/14693178/authorguide#dataavailability>). Please note that the Data Availability Section is restricted to new primary data that are part of this study.

8) (but see above) At EMBO Press we ask authors to provide source data for the main figures. Our source data coordinator will contact you to discuss which figure panels we would need source data for and will also provide you with helpful tips on how to upload and organize the files.

Additional information on source data and instruction on how to label the files are available <https://www.embopress.org/page/journal/14693178/authorguide#sourcedata>.

9) The journal requires a statement specifying whether or not authors have competing interests (defined as all potential or actual interests that could be perceived to influence the presentation or interpretation of an article). In case of competing interests, this must be specified in your disclosure statement. Further information: https://www.embopress.org/competing-interests

10) Figure legends and data quantification:
The following points must be specified in each figure legend:

- the name of the statistical test used to generate error bars and P values,
- the number (n) of independent experiments (please specify technical or biological replicates) underlying each data point,
- the nature of the bars and error bars (s.d., s.e.m.)

- If the data are obtained from n {less than or equal to} 5, show the individual data points in addition to the SD or SEM.
- If the data are obtained from n {less than or equal to} 2, use scatter blots showing the individual data points.

Discussion of statistical methodology can be reported in the materials and methods section, but figure legends should contain a basic description of n, P and the test applied.

See also the guidelines for figure legend preparation:

https://www.embopress.org/page/journal/14693178/authorguide#figureformat

- Please also include scale bars in all microscopy images.

11) Our journal encourages inclusion of *data citations in the reference list* to directly cite datasets that were re-used and obtained from public databases. Data citations in the article text are distinct from normal bibliographical citations and should directly link to the database records from which the data can be accessed. In the main text, data citations are formatted as follows: "Data ref: Smith et al, 2001" or "Data ref: NCBI Sequence Read Archive PRJNA342805, 2017". In the Reference list, data citations must be labeled with "[DATASET]". A data reference must provide the database name, accession number/identifiers and a resolvable link to the landing page from which the data can be accessed at the end of the reference. Further instructions are available at <https://www.embopress.org/page/journal/14693178/authorguide#referencesformat>.

12) As part of the EMBO publication's Transparent Editorial Process, EMBO Reports publishes online a Review Process File to accompany accepted manuscripts. This File will be published in conjunction with your paper and will include the referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript.

You are able to opt out of this by letting the editorial office know (emboreports@embo.org). If you do opt out, the Review Process File link will point to the following statement: "No Review Process File is available with this article, as the authors have chosen not to make the review process public in this case."

We would also welcome the submission of cover suggestions, or motifs to be used by our Graphics Illustrator in designing a cover.

I look forward to seeing a revised form of your manuscript when it is ready and please let me know if you have questions or comments regarding the revision.

Yours sincerely,

Martina Rembold, PhD
Senior Editor
EMBO reports

Response to the reviewers

Reviewer(s)' Comments to Author:

Referee #1:

The authors have addressed my concerns. I have a few minor comments.

It is unclear why the authors did not show the immunoblotting of INS1 TRAPa KO complemented with TRAPa mutants in Fig. 3E but rather show samples from HEK293 cells in Fig. 3D.
Fig. 3D should be better labeled. How do we interpret the labeling of TRAPa(+/-) and FLAG-tag (-/F)? The authors may want to label non-transfected, WT TRAPa, M1, M2, etc.

The INS-1 832/13 cell line which we obtained was contaminated with mycoplasma, and proved challenging to grow in culture and transfect.For this reason we carried out the immunoblot analysis of the different TRAPα mutants using HEK293 cells to obtain results in a timely manner for the previous submission. We have since removed the contaminating mycoplasma and have now carried out the same immunoblotting in INS-1 832/13 cells, which shows that all of the mutant TRAPα constructs express to a similar level as transfected wild-type TRAPα. The Western blotting data is now included as new Figures 3D and 3E. As suggested, we have now improved labeling of the Western blot samples and indicated where the mutated residues localize in the Sec61/TRAP structure in Figures 3C.

The authors state that membrane deformations are costly for the cell on page 10. Since there is no energy involved here, it is not understood why this is costly.

We thank the reviewer for the opportunity to elaborate upon this point. The bending (just as other deformations) of lipid membranes costs energy as defined by their elastic properties. However, due to the complexity of the processes involved from protein synthesis to lipid–protein interactions, we decided to not speculate on the energetics of membrane bending in the framework of the cell. Thus, we have removed the sentence in question in the revised version of the manuscript.

Referee #2:

The authors have addressed my concerns, and I find the Ms suitable for publication.

We thank the reviewer for their recommendations.

ADDITIONAL REFEREE 2 INPUT

Both Referee #1 and #3 raise some issues with the new Fig 3C-E. Unfortunately, the authors do not (or, rather, cannot) draw a clear conclusion from this new data. They state that "Among the TRAPα mutants mutation R150A, located at the lumenal TRAPα/β interface (Table S5), failed to rescue insulin production, whereas every other TRAPα point mutation restored insulin production to a substantial degree yet not to WT levels", but they leave it unsaid whether they consider this sufficient proof that the mutants indeed provide support for the interactions they propose based on the MD modelling. The culprit is the wording "yet not to WT levels" (which somehow implies a significant effect) but they haven't carried out any statistical analysis to support that there is indeed a difference between the mutants and the WT (which would require a couple more biological repeats and, possibly, better quantification of the relative expression levels of the different mutants). As far as I understand it, the data points in Fig 3E represent four repeat measurements of insulin levels from one cell culture (N=1), meaning that the SDs pertain to the ELISA kit itself, not the variation stemming from the biological system.

We appreciate the concern raised regarding the strength of the data in previous Figures 3C–E. We have now addressed these points by repeating the mutant TRAPα experiment in INS-1 832/13 cells which have been cleared of contaminating mycoplasma, generating three independent replicates, and applying appropriate statistical testing to determine which populations differ significantly in TRAP-mediated insulin production. The new transient transfection data now clearly demonstrates which of the TRAPα mutants are produced to a similar level as wild-type TRAPα. The new data is now included in new Figures 3D–3F.

Since, in the Ms, they put so much weight on the MD and the suggested interactions, asking for two more biological repeats and a simple statistical analysis would not seem inappropriate, I think. Or, the authors would have to say something like "these effects are in the direction expected if the mutations indeed affect TRAPa interactions with the other subunits, but they must be further validated before firm conclusions can be drawn".

See above, we have now carried out the requested repeat experiments and the suggested statistical analyses.

Referee #3:

The revised manuscript by Karki et al. presents the structure of a mammalian ribosome in complex with the Sec61/TRAP translocon, highlighting two key findings. First, cryo-EM imaging of detergent-solubilized microsomes was used to determine the overall architecture of the core Sec61/TRAP complex bound to a ribosome. Second, through molecular dynamics (MD) simulations, it was observed that the Sec61/TRAP complex induces local membrane curvature of the lumenal membrane leaflet. In my initial feedback on the manuscript, I focused on the lack of supporting data that would validate the authors' main conclusions regarding modeling quality and membrane deformation. In this revised version, the authors present a new validation experiment (Fig. 3C-E) and provided further clarification on their approaches to characterize membrane deformation. It is important to note that while the membrane deformation analysis is

a novel aspect of this paper, there is still a lack of functional data demonstrating the mechanistic importance of this analysis. Consequently, the manuscript primarily serves as a descriptive overview of the complex and its embedded membrane.

We thank the reviewer for their assessment.

Major comments

1. Structural validation of membrane thinning. As I had requested, the authors attempted to analyze their own structures obtained from detergent-solubilized micelles in order to validate membrane deformation. However, due to the unsuitability of the LMNG detergent as a mimic of a membrane bilayer, they were unable to draw conclusive evidence of membrane deformation. The analysis presented in Reviewer Figure 2 was a valiant attempt to visualize curvature but was inherently limited by the fact that the detergent only covers the transmembrane regions, lacking a continuous bilayer representation. Consequently, the authors' MD results lack supporting evidence from their own structures.

The authors' main source of complementary evidence for membrane deformation lies in the analysis of a previous low-resolution reconstruction of the membrane-bound ribosome-TRAP complex (Martinez-Sanchez et al., Nature Methods 2020, EMD-0084). In the original submission, I expressed dissatisfaction with this analysis due to the extremely low resolution of the map (~22 Å), which could complicate the delineation of membrane boundaries. However, I understand the authors' rationale for utilizing this map, as my suggestion to compare it with higher-resolution OST-containing maps would introduce an additional confounding factor (OST). While acknowledging the authors' good-faith efforts to maximize map interpretability given the available data, I propose two alternative suggestions to further strengthen their conclusion:
a. Conduct a "negative control" analysis of the membrane radius of curvature on a membrane-bound Sec61 structure without TRAP. This additional analysis would provide a baseline comparison to evaluate the specific impact of TRAP on membrane curvature.
b. Perform an analysis using the recently published 6.5 Å membrane-bound 80S-Sec61-TRAP complex (Gemmer et al., Nature 2023, EMD-15885). This higher-quality map would be expected to offer a more robust analysis of membrane curvature, thus reinforcing the authors' findings.

Although it goes beyond what the editor has requested, we appreciate the reviewer's curiosity regarding observed experimental effects of TRAP-induced ER membrane curvature in an intact membrane setting. We agree that the resolution for the isolated Sec61/TRAP from existing available cryo-ET analyses does not permit one to draw detailed conclusions on TRAP-induced membrane curving and we have modified the text to de-emphasize the strength of our cryo-ET membrane analysis. We expect that future advancements in cryo-ET analysis of rough ER, including in intact cells, will allow a comprehensive analysis of local membrane structure Sec61 and TRAP.

2. Cell-based validation of TRAPalpha interacting residues. The authors present new results of insulin secretion from cells expressing various TRAPalpha mutation constructs (new Fig. 3C-E)

and demonstrate that several mutations impair insulin secretion. However, the presented results of this experiment are flawed due to the following reasons:
a. Lack of loading controls in panels C and D: The absence of loading controls in panels C and D hinders the accurate assessment of protein expression levels or potential variations in sample loading.

We have now repeated this experiment in the insulin-secreting INS-1 832/13 cells and carried out more extensive Western blot analysis of TRAPα expression, including the needed loading controls. The new data shows that all of the tested mutations express to comparable levels as WT TRAPα and is now included as new Figures 3D and 3E.

b. Apparently inconsistent TRAPalpha expression levels (panel D): The observed inconsistencies in TRAPalpha expression levels in panel D raise concerns regarding the reliability and reproducibility of the experiment.

See above. The new experiment with optimized transient transfection shows that all of the tested TRAPα mutants express to virtually identical levels compared to wild-type FLAG-tagged TRAPα.

c. Uncertainty regarding whether insulin secretion in panel E is confounded by variable TRAPalpha expression levels in panel D. Given the apparent variability in TRAPalpha expression levels, it becomes uncertain whether the observed differences in insulin secretion can be attributed solely to the introduced TRAPalpha mutations or if they are influenced by variable TRAPalpha expression levels across the samples.

See above response.

d. Only a single biological replicate was included (N=1). The lack of replication makes it impossible to determine if the observed effects are reproducible.

We have now carried out the insulin secretion assay with three independent biological replicates (N=3). Each independent experiment produced a similar result for the tested mutations and we are showing the new data and associated statistical analysis in new Figure 3F. The raw data points for the individual experiments are also provided per journal guidelines.

e. Poor presentation of the M1-M5 mutations: It was challenging to go back-and-forth between the figure and the main text to understand the relevance of each mutant. Additionally, there is a lack of discussion regarding whether M1-M5 are expected to be compared among each other.

We have now included a map indicating which interfaces the mutants are located in as Figure 3C. In each case, we were only interested in comparing effects of a single mutant to wild-type TRAPα.

3. The refined model indicates an extremely high Clashscore (26.02, Table S3), but details of the clashes are not available in the PDB validation report. The high Clashscore raises concerns about the quality of modeling.

We thank the reviewer for raising this matter. We have now reanalyzed our model and corrected the parts of the models that had most close contacts (mostly located in the poorly ordered TRAP lumenal domains). The new model with a lower clash score (6.48) has now been deposited to the PDB, and we are including a new PDB validation report with this submission. Refinement statistics have been now updated in the table S2.8. The reviewer can access the PDB and EMDB deposition using the following login information:

[Reviewer access]

4. The main text indicates that the Sec61/TRAP model was validated by XL-MS, but it seems odd that there is only one interprotein crosslink reported (Fig. 2B).

We did carry out XL-MS to attempt to further validate our structural model that was derived from cryo-EM modeling and MD. However, we only observed a single statistically significant crosslink which we are showing in Fig. 2B. Access to the proteomic dataset is available at

[Reviewer access]

Minor comments

5. The main text indicates the reconstruction was resolved to an overall resolution of 2.6 Å, but the supplemental data indicates a resolution of 2.7 Å.

The overall resolution of the reconstruction of ribosome/Sec61/TRAP complex is at 2.69 Å (FSC 0.143 cutoff). We have revised the sentence in the main text as :

“The extracted particles were further refined using heterogeneous and homogeneous 3D refinement to yield a reconstruction of the entire ribosome/Sec61/TRAP complex with an overall resolution of 2.7 Å“.

6. The use of "high" vs. "low" contour levels appear to be swapped in the manuscript. "High" contour refers to a display at higher threshold levels (i.e., displaying less density).

We thank the reviewer for their observation, and have corrected the manuscript accordingly.

Dear Dr. Paavilainen

Thank you for the submission of your revised manuscript to EMBO reports. We have now received the report from former referee #3 who assessed the new data and conclusions in Fig. 3D-E.

As you will see, the referee supports publication but also asks you to discuss the new, divergent data and results, i.e., that structure-informed mutations lead to insignificant phenotypic outputs. Please address this comment in the manuscript text and in a point-by-point response.

From the editorial side, there are also a few things that we need before we can proceed with the official acceptance of your study.

- Your manuscript will be published in our Scientific Reports section. Therefore, please combine the Results and Discussion section and keep our character limit of 27,000 characters (including spaces but excluding materials & methods and references) in mind.

- Manuscript text: Please,
- provide the text in a one-column layout
- add the header "Abstract"
- remove the figures and place the figure legends as a section called "Figure Legends" after the References
- label the keywords as "Keywords: Protein translocation...."
- The "Disclosure and competing interests statement" should be placed after the Acknowledgmets
- change Bibliography to References

- In general, this is the correct order of the manuscript sections:
Title page - Abstract - Introduction - Results - Discussion - Materials and Methods - Acknowledgements - Disclosure and competing interests statement - References - Figure legends - Tables and their legends (not EV tables) - Expanded View Figure legends

- Our editorial policies request that conclusions need to be supported by data that are part of the manuscript. "Data not shown" is therefore not possible. Either include the respective data or remove the conclusions based on "data not shown" (page 8, page 10).

- BioRxiv citations need to be labeled. In the text it is (preprint: Evans et al., 2021) and in the reference list you need to add [PREPRINT] at the end of this reference. (Given that it is still a preprint).

- Appendix Figure labels need to be corrected: we need Appendix Figure S1, Appendix Figure S2, etc. instead of Appendix Figure S1.1, Appendix Figure S1.2. etc. References need to be in the correct EMBO Reports format and the header needs to be changed to "References".

- All materials and methods must be part of the main methods section in the manuscript. Please move the Supplementary methods to the main methods section, unless you feel that some of these are very specialized. If you leave parts of the methods in the Appendix, please refer to the Appendix methods in the main methods section. The title should be "Appendix Methods" and then you could add the header "Appendix Results" in the table of contents, referring to Appendix Figure S# and Appendix Table S#.

- Please add callouts to Appendix Table S3 and a callout to the movie in the text where appropriate. Callouts for Appendix Figure S4 should be corrected to "Appendix Figure S4E" instead of "Fig. S4E".

- The movie should be named and called out in the manuscript as Movie EV1. It should be zipped up together with its legend which can be provided as a README.txt file

- I attach to this email a related manuscript file with comments by our data editors. Please address all comments and upload a revised file with tracked changes with your final manuscript submission.

- On a different note, I would like to alert you that EMBO Press offers a new format for a video-synopsis of work published with us, which essentially is a short, author-generated film explaining the core findings in hand drawings, and, as we believe, can be very useful to increase visibility of the work. This has proven to offer a nice opportunity for exposure i.p. for the first author(s) of the study. Please see the following link for representative examples and their integration into the article web page:
https://www.embopress.org/video_synopses
https://www.embopress.org/doi/full/10.15252/embj.2019103932

Please let me know, should you be interested to engage in commissioning a similar video synopsis for your work. According operation instructions are available and intuitive.

We look forward to seeing a final version of your manuscript as soon as possible.

With kind regards,

Martina Rembold, PhD
Senior Editor
EMBO reports

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Referee #1:

The revised manuscript by Karki*, Javanainen*, Rehan*, and Tranter* et al. provides an updated Figure 3 that re-assesses their analysis of insulin secretion in the context of various TRAPalpha mutants. The inclusion of additional replicates and confirmation of protein expression levels enables more reliable quantification of insulin secretion, which is notably different from their original version. Most significantly, the effects of M1, M2, M4 mutations are not significantly different from WT TRAPalpha, and so the authors should therefore elaborate on whether these residues play less important roles in protein function. What does it mean if structure-informed mutations lead to insignificant phenotypic outputs? While I do not think that these new results alter the overall conclusion of the manuscript, it would be worthwhile for the authors to comment on M1, M2, and M4. For instance, why do M4 and M5 show such different results if they are both predicted to disrupt the ribosome binding site-1? Do the authors think that the TRAPalpha-TRAPbeta interface disrupted by M1 is not important for protein function?

The effects of M3 and M5 are more convincing and their descriptions are fine as-is. Other aspects of my previous comments have been adequately addressed.

Response to the reviewer

Reviewer Comment to Author:

Referee #1:

The revised manuscript by Karki*, Javanainen*, Rehan*, and Tranter* et al. provides an updated Figure 3 that re-assesses their analysis of insulin secretion in the context of various TRAPalpha mutants. The inclusion of additional replicates and confirmation of protein expression levels enables more reliable quantification of insulin secretion, which is notably different from their original version. Most significantly, the effects of M1, M2, M4 mutations are not significantly different from WT TRAPalpha, and so the authors should therefore elaborate on whether these residues play less important roles in protein function. What does it mean if structure-informed mutations lead to insignificant phenotypic outputs? While I do not think that these new results alter the overall conclusion of the manuscript, it would be worthwhile for the authors to comment on M1, M2, and M4. For instance, why do M4 and M5 show such different results if they are both predicted to disrupt the ribosome binding site-1? Do the authors think that the TRAPalpha-TRAPbeta interface disrupted by M1 is not important for protein function?

The effects of M3 and M5 are more convincing and their descriptions are fine as-is. Other aspects of my previous comments have been adequately addressed.

We have now added a clarification to the main text regarding lack of observable effect for insulin biogenesis for TRAP-Alpha mutations M1, M2 and M4. It is possible that some of these structure-informed mutations are not sufficiently strong to effectively disrupt TRAP interactions with either the ribosome or Sec61. All of these mutants contain charged-to-alanine replacements whereas for example mutation M5 that shows the largest effect for insulin contains several charge reversal mutations. An alternative explanation that should be tested in future studies is that some TRAP interactions may be dispensable for biogenesis of specific Sec61 client proteins.

Dr. Ville Paavilainen
Univ of Helsinki
Inst of Biotechnology
Viikinkaari 1
Helsinki, Helsinki 00790
Finland

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