nature portfolio

Peer Review File

Global analysis of translocon remodeling during protein synthesis at the ER

Corresponding Author: Professor Robert Keenan

Version 0:

Decision Letter:

15th Jul 2025

Dear Dr. Keenan,

Thank you again for submitting your manuscript "Global analysis of translocon remodeling during protein synthesis at the ER". I apologize for the delay in responding, which resulted from the difficulty in obtaining suitable referee reports. Nevertheless, we now have comments (below) from the 2 reviewers who evaluated your paper. You will see that a report from reviewer #1 is missing. This is due to the unforeseen inability of the reviewer to provide the referee report in a timely manner. As the expertise of Reviewer #2 and Reviewer #3 can cover the major aspects of the current study, we have reached our decision based on their comments to avoid further delay. If/when we receive the report form Reviewer #1 within a reasonable timeframe, we will send the comments to you. (and in that case we would also expect revisions to address Reviewer #1's comments')

In light of those reports, we remain interested in your study and would like to see your response to the comments of the referees, in the form of a revised manuscript.

You will see that while reviewers appreciate the results, they raise several concerns which will need to be addressed in a revision. Specifically, in line with reviewer's 3 comments, we ask that you control for protein expression levels, and comment on the role of TM domains, in the context of the previous work.

Please be sure to address/respond to all concerns of the referees in full in a point-by-point response and highlight all changes in the revised manuscript text file. If you have comments that are intended for editors only, please include those in a separate cover letter.

We are committed to providing a fair and constructive peer-review process. Do not hesitate to contact us if there are specific requests from the reviewers that you believe are technically impossible or unlikely to yield a meaningful outcome.

We expect to see your revised manuscript within approx. 2 months. If you cannot send it within this time, please contact us to discuss an extension; we would still consider your revision, provided that no similar work has been accepted for publication at NSMB or published elsewhere.

As you already know, we put great emphasis on ensuring that the methods and statistics reported in our papers are correct and accurate. As such, if there are any changes that should be reported, please submit an updated version of the Reporting Summary along with your revision.

Please follow the links below to download these files:

Reporting Summary:
https://www.nature.com/documents/nr-reporting-summary.pdf

Please note that the form is a dynamic 'smart pdf' and must therefore be downloaded and completed in Adobe Reader.

When submitting the revised version of your manuscript, please pay close attention to our href="https://www.nature.com/nature-portfolio/editorial-policies/image-integrity">Digital Image Integrity Guidelines. and to the following points below:

-- that unprocessed scans are clearly labelled and match the gels and western blots presented in figures. Please note that all key data shown in the main figures as cropped gels or blots should be presented in uncropped form, with molecular weight markers. While these data can be displayed in a relatively informal style, they must refer back to the relevant figures. These data should be submitted as source data with the last revision, prior to acceptance.
-- that control panels for gels and western blots are appropriately described as loading on sample processing controls
-- all images in the paper are checked for duplication of panels and for splicing of gel lanes.
-- For any revision that includes light microscopy data, we ask our authors to please include a completed light microscopy reporting table [https://www.nature.com/documents/Light_microscopy_reporting_table.xlsx] to ensure the methods are described thoroughly. The table will be available to reviewers and ultimately published should the manuscript be accepted at the journal.

EXTENDED DATA FIGURES

When re-submitting your manuscript, please ensure that any supplementary figures and tables that are crucial to the manuscript's conclusions are converted into Extended Data figures and tables to increase visibility of these data. Extended Data figures and tables are online-only (present in the online PDF and full-text HTML versions of the paper), peer-reviewed display items that provide essential background to the article but are not included in the main article due to space constraints. A maximum of ten Extended Data display items (figures and tables) is permitted.

Finally, please ensure that you retain unprocessed data and metadata files after publication, ideally archiving data in perpetuity, as these may be requested during the peer review and production process or after publication if any issues arise.

If there are additional or modified structures presented in the final revision, please submit the corresponding PDB validation reports.

SOURCE DATA: we urge authors to provide, in tabular form, the data underlying the graphical representations used in figures. This is to further increase transparency in data reporting, as detailed in this editorial (http://www.nature.com/nsmb/journal/v22/n10/full/nsmb.3110.html). Spreadsheets can be submitted in excel format. Only one (1) file per figure is permitted; thus, for multi-paneled figures, the source data for each panel should be clearly labeled in the Excel file; alternately the data can be provided as multiple, clearly labeled sheets in an Excel file. When submitting files, the title field should indicate which figure the source data pertains to. We encourage our authors to provide source data at the revision stage, so that they are part of the peer-review process.

Data availability: this journal strongly supports public availability of data. All data used in accepted papers should be available via a public data repository, or alternatively, as Supplementary Information. If data can only be shared on request, please explain why in your Data Availability Statement, and also in the correspondence with your editor. Please note that for some data types, deposition in a public repository is mandatory - more information on our data deposition policies and available repositories can be found below:
https://www.nature.com/nature-research/editorial-policies/reporting-standards#availability-of-data

We require deposition of coordinates (and, in the case of crystal structures, structure factors) into the Protein Data Bank with the designation of immediate release upon publication (HPUB). Electron microscopy-derived density maps and coordinate data must be deposited in EMDB and released upon publication. Deposition and immediate release of NMR chemical shift assignments are highly encouraged. Deposition of deep sequencing and microarray data is mandatory, and the datasets must be released prior to or upon publication. To avoid delays in publication, dataset accession numbers must be supplied with the final accepted manuscript and appropriate release dates must be indicated at the galley proof stage.

While we encourage the use of color in preparing figures, please note that this will incur a charge to partially defray the cost of printing. Information about color charges can be found at http://www.nature.com/nsmb/authors/submit/index.html#costs

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Please use the link below to submit your revised manuscript and related files:

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Note: This URL links to your confidential home page and associated information about manuscripts you may have submitted, or that you are reviewing for us. If you wish to forward this email to co-authors, please delete the link to your homepage.

We look forward to seeing the revised manuscript and thank you for the opportunity to review your work.

Sincerely,

Katarzyna Ciazynska, PhD
(she/her)
Senior Editor
Nature Structural & Molecular Biology
https://orcid.org/0000-0002-9899-2428

Referee expertise:

Referee #1: proteostasis, protein biogensis

Referee #2: membrane protein biogenesis

Referee #3: ER, membrane protein biogenesis

Reviewers' Comments:

Reviewer #2 (Remarks to the Author):

General comments
Previously, the Hegde and Keenan groups presented in vitro data showing that the GEL, BOS and PAT complexes that make up the multipass translocon (MPT) are recruited to the Sec61 channel during the synthesis of several multispanning membrane proteins. The secretory Sec61 translocon that translocates exported proteins to the ER lumen is comprised of the TRAP complexes and the OST-A (for N-glycosylation). Though these results were significant, they were based on the analysis of a few multipass proteins and they presented a fixed view of the translocon composition.

In this submitted paper by Sundaram et al they present a comprehensive analysis on a global scale of the remodeling of the translocon during protein synthesis at the ER. The authors make several important conclusions based on using a transcriptome-wide selective ribosome profiling analysis to examine the co-translational interactions of the OST-A complex for N-glycosylation and the Multipass translocon (made up of the GEL, PAT and BOS complexes). They show that the OST-A (see below) is recruited to the Sec61 open channel necessary for translocation of long loops of proteins. In contrast, the authors found that the GEL, PAT and BOS complexes are synchronously recruited to a closed channel where they are stabilized by the interaction with the emerging transmembrane domains. They found that the translocon makeup can change dynamically and reversibly during the synthesis of the multipass membrane proteins. This work provides a significant contribution to the membrane field in understanding how secretory and membrane proteins are inserted across or into the membrane. They clearly show, in a paradigm shifting set of studies, that the Sec 61 channel plays a much more limited role for multipass membrane proteins. Strikingly, the MPT plays a central role in insertion of the multipass membrane proteins. The high impact work described in this paper is of significance to scientists studying membrane protein topogenesis in bacteria, archaea, endoplasmic reticulum, mitochondria, and chloroplasts. The paper is well written, and the data is of high quality with biological replicates shown for key data.

Evaluation of paper
To define the cotranslational substrates of OST-A and MPT globally the authors performed a ribosome profiling analysis using HEK293 cells expressing endogenous levels of Flag tagged subunits of OSTA (OST48 and RPN2) and the GEL (TMCO1), PAT (CCDC47), and BOS (Nicalin). Ribosome protected mRNA fragments from total membrane and affinity purified fractions (using Flag antiserum against the above Flagged tagged subunits) were prepared and sequenced in biological replicates. They determined the enrichment ratios for each flag immunoprecipitated sample in two datasets and the proteins were identified using DeepTMHMM to scan the proteome and detect the type of membrane protein or secretory protein. From this analysis the native substrates of the Sec61OSTA or MPT were determined (Fig. 1). OST48 and RPN2 (OST-A) enriched substates using the anti-flag antiserum contained long translocated segments that included secretory proteins (SP-only), type I and type II single pass proteins, and type I multipass proteins. The MPT (containing the GEL, PAT, and BOS complexes using Flag specific antiserum) enriched substrates included type I, type II, and type III multipass proteins, all of which had two or more transmembrane domains. Most single pass membrane proteins were not enriched.

By examining the interactions of OST-A along the sequence of the synthesized protein from the OST-A enrichment data (Fig. 2) it was determined that OST-A is recruited during translocation of long segments of proteins through the Sec61 open channel regardless of whether they had an N-glycosylation acceptor site. The OST-A was released from the Sec channel after completion of translocation for SP-only and type II single pass proteins, or after the TM segment emerged into the membrane of type I single pass protein.

By analyzing the interactions of the MPT complexes along the amino acid sequence of multipass membrane protein (Fig. 3) it was found that there is synchronized recruitment of the GEL, PAT and BOS complexes to the membrane proteins. Also, they found that MPT does not engage the type I or type II multipass protein if the first translocated segment is long; however, it can engage the membrane substrate following the translocation of the long loop when the downstream TM segments are

synthesized. If the first translocated segment is short, the analysis showed that the MPT binding was stabilized following the emergence of the first transmembrane domain or two transmembrane domain pair. By analyzing several multipass proteins with a large number of transmembrane domain (12, 14, or 17 TM segments) it was discovered that the MPT remain bound to the inserting substrate until complete synthesis of the protein.

There is a striking interplay between OST-A and MPT during the synthesis of multipass membrane proteins (Fig. 4). The study showed that when OST-A is engaged with the protein (to translocate long loops) MPT is not; when MPT engages the multipass membrane protein, there is no interaction of the protein with OST-A. The authors showed that MPT becomes engaged with the inserting substrate following the translocation of the loop but then dissociates when another long loop is translocated by the open Sec channel with OST-A bound. MPT also dissociates from the multipass membrane substrate if it contains a long cytoplasmic region.

In the last study (Fig 5), the authors show that the Sec61 is required for translocation of long hydrophilic loops of multipass membrane proteins containing a variety of topologies and different number of TM segments. To determine that the Sec61 channel opening is required for translocation of the long loop, they added Apratoxin A, an inhibitor that binds to the lateral gate of Sec61, locking it in a closed state. They performed a dual color ratiometric assay to monitor the substrate amounts in the cell with or without the inhibitor. Only when a long loop was present was the multipass protein sensitive to the drug. Based on this study and the other results presented, the authors come to the conclusion that the Sec61 channel remains closed during the topogenesis of membrane proteins that do not have long loops.

Minor Comments
1.On paper 4 (line 130), do you mean exposure of TMD to lipid? On page 4 (line165), do you mean emergence of the first TMD or closely-spaced TMD pair into the membrane? Please clarify.
2.For the Fig. 4B study, define for the general reader, in a few words, what a heat map is.
3.In Figure 5, how is the Log10 (translation control) determined?
4.Fig. S2. There is a typo. Change title to “Quality control …..”

Reviewer #3 (Remarks to the Author):

While the Sec61 complex constitutes the central component of the ER translocation machinery, the role of additional, Sec61-associated (accessory), proteins in the translocation, membrane-insertion and maturation of different substrates has garnered increasing attention and is providing a more complex picture of translocation/membrane protein insertion than previously imagined. This complexity appears to be in line with the wide variety of ER-directed substrates, including membrane proteins with multiple transmembrane domains (multipass membrane proteins), whose insertion into the bilayer appears particularly problematic. The group of Keenan previously characterised a novel assembly of three complexes (PAT-GEL-BOS) associated with the RNC-translocon, and, in collaboration with Ramanujan Hegde and collaborators characterized its role in the insertion into the ER membrane of multipass membrane proteins. The authors suggested that the biogenesis of these proteins, if lacking long translocated domains, occurs through the PAT-GEL-BOS complex (termed multipass translocon- MPT) independently from the Sec61 channel. This hypothesis generated a transformative conceptual framework for membrane protein biogenesis.

The previous work by the authors investigated the interaction of nascent multipass-proteins with the MPT by using truncated constructs stalled at various stages of translation/membrane integration. These studies demonstrated that interaction occurred after emergence of the signal peptide or first TMD from the Sec61 lateral gate, and provided evidence for the dynamic remodelling of the translocon during translation/translocation, depending on the substrate and, importantly, on what part of the polypeptide is being synthesised (lumenal translocated domain, cytosolic domain, or transmembrane domain). In the submitted manuscript, the authors have now extended their investigation to a genome-wide analysis and by RNase footprinting analysed recruitment of the MPT as well as of the oligosaccharyl transferase (OST) to thousands of ER-associated nascent polypeptides. The impressive results of this technically brilliant work, while confirming the previous conclusions and excluding that artefacts due to truncated constructs could explain them, generalise the significance of the previous work and provide a solid basis for a novel paradigm on membrane protein biogenesis at the ER. The results also clarify the mechanism of recruitment of the OST to the translocon, a subject that was previously incompletely understood.

The methodology, statistics and presentation are all in place. Previous work appears to be cited correctly. Before publication, however, a few points need clarification.Before publication, however, a few points need clarification.

Major concerns:
1. Characterization/description of cell lines used in the study.
On line 72, the authors state that “Stably integrated HEK293 cell lines expressing near-endogenous levels of Flag-tagged subunits of OST-A (OST48 and RPN2) and the GEL (TMCO1), PAT (CCDC47) and BOS (Nicalin) complexes were constructed”; data or reference to previous work should be provided to support the statement that the levels of the expressed proteins are near endogenous. In addition, there is a difference between the cell lines expressing tagged MPT components and those expressing tagged OST components: the former were produced in KO cells, thus there are no endogenous competing proteins; the OSTA tagged constructs are expressed in the presence of the untagged proteins. One would like to know levels of the tagged proteins compared to the endogenous ones. Because of the presence of untagged competing OST48 and RPN2, the sensitivity of the OST cell lines in FLAG pulldown assays may be lower than in the case of the MPT cells.

2. Fig. 5: from the results of this figure, the authors conclude that features of the transmembrane domains do not determine whether insertion is via the Sec61 lateral gate or not. Yet, their previous work has shown that a hydrophilic transmembrane helix favours interaction with Asterix. In the current genome-wide study, TM hydrophobicity was not identified as a factor affecting the interaction with MPT, if I understand correctly. The authors should comment on this discrepancy.
Fig. 5B, shows that the presence of a large lumenal loop between TM9 and 10 effects transition of Sec61 from the closed to the open state. Since opening must be triggered by the interaction of TM9 with Sec61, this implies (as the authors state) that the same TM engages or does not engage Sec61, depending on the downstream sequence (long translocated or short loop). In other words, as I understand it, TM9 senses what is arriving behind it. This might be explained a little more clearly.

Minor:
1. Figure S1B and C: please specify the relative amounts of input, flowthrough and eluates loaded on the gel.
2. Fig. 4B: could the authors please explain how the heatmap was constructed? Also, is this really a heatmap? In my understanding, colours in heatmaps are related to the intensity of the signal/variable. In the heatmap of Fig. 4B, the colours represent the different complexes and are not related to intensity.
3. Dual colour ratiometric assay (Figs. 5 and S7). Although the authors have used this assay in their previous publications, they could be a little more reader-friendly by spending just a few words to explain the assay. In describing the utilized constructs in the Methods section (p.18, line 538), the authors refer to a previous publication, which is not cited. In Fig. S7B, the abscissa and ordinate presumably represent RFP and GFP fluorescence, respectively; please, specify this.
4. Fig. 3B legend: please refer to the Methods section for the definition/calculation of peaks.
5. Text, p. 6, lines 241 – 250. The authors provide a list of proteins, whose stability was either affected or unaffected by Apra1 treatment. For two of them (signal peptide-containing secretory protein and tail-anchored proteins), the data are not shown. Please show the data in the supplementary figure or provide appropriate reference.
6. There are lots of typos: here are a few that I caught:
(i) Legend to Fig. S5, lines 791-792: “mean enrichment (line) and range (shaded) are shown. Median (dark magenta line) and range (light magenta shading) for n=6 replicates are indicated”. Maybe the “mean enrichment (line)” refers to panel A (SLC7A11)?
(II) Legend to Fig. S6: the word “for” is duplicated
(III) Legend to Fig. S2: “qualify” should read “quality”
(iv) Legends to Fig. S1 and Fig. 1: MNase ??
(v): p. 20 line 20: by “nutator” I suppose that “rotator” is meant.
(vi) p.21, lines 669 and 673: “SMAT” should read “SMART”

Version 1:

Decision Letter:

Our ref: NSMB-A51226A

4th Aug 2025

Dear Dr. Keenan,

Thank you for submitting your revised manuscript "Global analysis of translocon remodeling during protein synthesis at the ER" (NSMB-A51226A). It has now been seen by the original referees and their comments are below. The reviewers find that the paper has improved in revision, and therefore we'll be happy in principle to publish it in Nature Structural & Molecular Biology, pending minor revisions to satisfy the referees' final requests and to comply with our editorial and formatting guidelines.

We are now performing detailed checks on your paper and will send you a checklist detailing our editorial and formatting requirements in about 2-3 weeks. Please do not upload the final materials and make any revisions until you receive this additional information from us.

Thank you again for your interest in Nature Structural & Molecular Biology Please do not hesitate to contact me if you have any questions.

Sincerely,
Kat

Katarzyna Ciazynska, PhD
(she/her)
Senior Editor
Nature Structural & Molecular Biology
https://orcid.org/0000-0002-9899-2428

Reviewer #2 (Remarks to the Author):

The authors have addressed all my previous concerns and comments.

Reviewer #3 (Remarks to the Author):

The authors have addressed my major and minor concerns, however, I recommend a clarification in the part of the text discussing the role of the downstream translocated segment in determining whether the preceding TMD opens or does not open the Sec61 channel (lines 289 – 293). Here it is stated that “the length of the downstream translocated segment influences whether the preceding TMD will open the Sec61 channel: LONG TRANSLOCATED SEGMENTS OPEN THE CHANNEL, AND SHORT TRANSLOCATED SEGMENTS DO NOT.” (capitals mine). In fact, it is not the translocated segments that open the channel, rather the upstream TMD; furthermore, the downstream segments are not translocated yet, rather, they are destined for translocation. Thus, to avoid confusion, one might refer to these segments as segments “to be translocated”, but for sure it is incorrect to state that they themselves open the channel; I realize that a more precise description may be grammatically more complicated, but I think that more precision would help the reader to fully appreciate this work.

Version 2:

Decision Letter:

17th Sep 2025

Dear Dr. Keenan,

We are now happy to accept your revised paper "Global analysis of translocon remodeling during protein synthesis at the ER" for publication as an Article in Nature Structural & Molecular Biology.

Acceptance is conditional on the manuscript's not being published elsewhere and on there being no announcement of this work to the newspapers, magazines, radio or television until the publication date in Nature Structural & Molecular Biology.

Over the next few weeks, your paper will be copyedited to ensure that it conforms to Nature Structural & Molecular Biology style. Once your paper is typeset, you will receive an email with a link to choose the appropriate publishing options for your paper and our Author Services team will be in touch regarding any additional information that may be required.

After the grant of rights is completed, you will receive a link to your electronic proof via email with a request to make any corrections within 48 hours. If, when you receive your proof, you cannot meet this deadline, please inform us at rjsproduction@springernature.com immediately.

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If you have any questions about our publishing options, costs, Open Access requirements, or our legal forms, please contact ASJournals@springernature.com

Sincerely,
Kat

Katarzyna Ciazynska, PhD
(she/her)
Senior Editor
Nature Structural & Molecular Biology
https://orcid.org/0000-0002-9899-2428

Response to referees

Reviewer #2 (Remarks to the Author):

General comments
Previously, the Hegde and Keenan groups presented in vitro data showing that the GEL, BOS and PAT complexes that make up the multipass translocon (MPT) are recruited to the Sec61 channel during the synthesis of several multispanning membrane proteins. The secretory Sec61 translocon that translocates exported proteins to the ER lumen is comprised of the TRAP complexes and the OST-A (for N-glycosylation). Though these results were significant, they were based on the analysis of a few multipass proteins and they presented a fixed view of the translocon composition.

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Evaluation of paper
To define the cotranslational substrates of OST-A and MPT globally the authors performed a ribosome profiling analysis using HEK293 cells expressing endogenous levels of Flag tagged subunits of OSTA (OST48 and RPN2) and the GEL (TMCO1), PAT (CCDC47), and BOS (Nicalin). Ribosome protected mRNA fragments from total membrane and affinity purified fractions (using Flag antiserum against the above Flagged tagged subunits) were prepared and sequenced in biological replicates. They determined the enrichment ratios for each flag immunoprecipitated sample in two datasets and the proteins were identified using DeepTMHMM to scan the proteome and detect the type of membrane protein or secretory protein. From this analysis the native substrates of the Sec61OSTA or MPT were determined (Fig. 1). OST48 and RPN2 (OST-A) enriched substates using the anti-flag antiserum contained long translocated segments that included secretory proteins (SP-only), type I and type II single pass proteins, and type I multipass proteins. The MPT (containing the GEL, PAT, and BOS complexes using Flag specific antiserum) enriched substrates included type I, type II, and type III multipass proteins, all of which had two or more transmembrane domains. Most single pass membrane proteins were not enriched.

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the TM segment emerged into the membrane of type I single pass protein.

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Minor Comments:

1. On paper 4 (line 130), do you mean exposure of TMD to lipid? On page 4 (line165), do you mean emergence of the first TMD or closely-spaced TMD pair into the membrane? Please clarify.

This is now clarified to state "...emergence of the TMD from the ribosome" and "...emergence of the first TMD or closely-spaced TMD pair from the ribosome".

2. For the Fig. 4B study, define for the general reader, in a few words, what a heat map is.

On advice of the other referee, we now refer to these plots as "peak maps" to show factor recruitment. The legend for the Fig. 4b panel has been adjusted to indicate what this means:

“OST-A and MPT peak maps for the indicated multipass protein types, sorted by the first MPT peak (length >1% of coding sequence). Each row represents a single transcript, colored to indicate positions where either OST-A, MPT or both are enriched. The number (N) of each type are indicated. Type II multipass proteins were further classified by the length of the first translocated segment.”

3. In Figure 5, how is the Log10 (translation control) determined?

The translation control is the level of fluorescent protein that is not fused to the protein of interest, but is encoded on the same mRNA separated by the ribosome-skipping P2A sequence. The assay is now more fully explained in the revised Fig. 5a and Ext. Data Fig S7 legends.

4. Fig. S2. There is a typo. Change title to “Quality control …..”

Thank you for spotting this error. This has been changed.

Reviewer #3 (Remarks to the Author):

While the Sec61 complex constitutes the central component of the ER translocation machinery, the role of additional, Sec61-associated (accessory), proteins in the translocation, membrane-insertion and maturation of different substrates has garnered increasing attention and is providing a more complex picture of translocation/membrane protein insertion than previously imagined. This complexity appears to be in line with the wide variety of ER-directed substrates, including membrane proteins with multiple transmembrane domains (multipass membrane proteins), whose insertion into the bilayer appears particularly problematic. The group of Keenan previously characterised a novel assembly of three complexes (PAT-GEL-BOS) associated with the RNC-translocon, and, in collaboration with Ramanujan Hegde and collaborators characterized its role in the insertion into the ER membrane of multipass membrane proteins. The authors suggested that the biogenesis of these proteins, if lacking long translocated domains, occurs through the PAT-GEL-BOS complex (termed multipass translocon- MPT) independently from the Sec61 channel. This hypothesis generated a transformative conceptual framework for membrane protein biogenesis.

The previous work by the authors investigated the interaction of nascent multipass-proteins with the MPT by using truncated constructs stalled at various stages of translation/membrane integration. These studies demonstrated that interaction occurred after emergence of the signal peptide or first TMD from the Sec61 lateral gate, and provided evidence for the dynamic remodelling of the translocon during translation/translocation, depending on the substrate and, importantly, on what part of the polypeptide is being synthesised (lumenal translocated domain, cytosolic domain, or transmembrane domain). In the submitted manuscript, the authors have now extended their investigation to a genome-wide analysis and by RNase footprinting analysed recruitment of the MPT as well as of the oligosaccharyl transferase (OST) to thousands of ER-associated nascent polypeptides. The impressive results of this technically brilliant work, while confirming the previous conclusions and excluding that artefacts due to truncated constructs could explain them, generalise the significance of the previous work and provide a solid basis for a novel paradigm on membrane protein biogenesis at the ER. The results also clarify the mechanism of recruitment of the OST to the translocon, a subject that was previously incompletely understood.

The methodology, statistics and presentation are all in place. Previous work appears to be cited correctly. Before publication, however, a few points need clarification.Before publication, however, a few points need clarification.

Major concerns:

1. Characterization/description of cell lines used in the study.
On line 72, the authors state that “Stably integrated HEK293 cell lines expressing near-endogenous levels of Flag-tagged subunits of OST-A (OST48 and RPN2) and the GEL (TMCO1), PAT (CCDC47) and BOS (Nicalin) complexes were constructed”; data or

reference to previous work should be provided to support the statement that the levels of the expressed proteins are near endogenous. In addition, there is a difference between the cell lines expressing tagged MPT components and those expressing tagged OST components: the former were produced in KO cells, thus there are no endogenous competing proteins; the OSTA tagged constructs are expressed in the presence of the untagged proteins. One would like to know levels of the tagged proteins compared to the endogenous ones. Because of the presence of untagged competing OST48 and RPN2, the sensitivity of the OST cell lines in FLAG pulldown assays may be lower than in the case of the MPT cells.

We apologize for not properly explaining the expression level data, which are embedded in the experiment shown in Extended Data Fig. 1b,c. Here, immunoblotting is performed on solubilized microsomes (the "input" lanes) for each tagged cell line and compared directly to cell lines tagged for other subunits. Inspection of the data shows that in each case, the tagged subunit is expressed at roughly the endogenous levels seen in cell lines where that subunit is not tagged, and also that there is no detectable untagged (endogenous) population of the subunit. Taking RPN2 as an example (Ext. Data Fig. 1c), one can see that the amount of Flag-RPN2 in the “input” is comparable to endogenous RPN2 levels in the wild-type (WT) control and FLAG-OST48 samples, and that we observe little untagged (endogenous) RPN2 in the Flag-RPN2 cell line. The same is seen for all other tagged subunits by making similar comparisons. This is now explained in the revised legend.

2. Fig. 5: from the results of this figure, the authors conclude that features of the transmembrane domains do not determine whether insertion is via the Sec61 lateral gate or not. Yet, their previous work has shown that a hydrophilic transmembrane helix favours interaction with Asterix. In the current genome-wide study, TM hydrophobicity was not identified as a factor affecting the interaction with MPT, if I understand correctly. The authors should comment on this discrepancy.

We apologise for any confusion, but there is no discrepancy with earlier work. The key point is that the TMD insertion route (which depends on the length of the flanking translocated segment) does not influence TMD interaction with Asterix (which depends only on partial hydrophilicity) following insertion. As shown previously, the first TMD of Rhodopsin, which is normally inserted by a Sec61-independent route (PMID 30415835), interacts with Asterix in a hydrophilicity-dependent manner (PMID 36261528). Routing insertion of this same TMD via Sec61 did not affect Asterix interaction (PMID 36261528). The genome-wide profiling analysis shows that MPT recruitment is strongly correlated with multipass proteins, which invariably contain one or more TMDs with partial hydrophilic character, consistent with earlier work showing hydrophilicity as a parameter influencing Asterix interaction.

3. Fig. 5B, shows that the presence of a large lumenal loop between TM9 and 10 effects transition of Sec61 from the closed to the open state. Since opening must be triggered by the interaction of TM9 with Sec61, this implies (as the authors state) that the same TM engages or does not engage Sec61, depending on the downstream sequence (long translocated or short loop). In other words, as I understand it, TM9 senses what is arriving behind it. This might be explained a little more clearly.

Thank you for this suggestion. We now explain that a TMD's use of Sec61 is influenced by the length of the downstream translocated domain.

Minor:

1. Figure S1B and C: please specify the relative amounts of input, flowthrough and eluates loaded on the gel.

This is now indicated in each panel.

2. Fig. 4B: could the authors please explain how the heatmap was constructed? Also, is this really a heatmap? In my understanding, colours in heatmaps are related to the intensity of the signal/variable. In the heatmap of Fig. 4B, the colours represent the different complexes and are not related to intensity.

Thank you for this suggestion. We now refer to these plots as "peak maps" of factor recruitment. The legend for this panel has been adjusted to clarify what it represents:

"OST-A and MPT peak maps for the indicated multipass protein types, sorted by the first MPT peak (length >1% of coding sequence). Each row represents a single transcript, colored to indicate positions where either OST-A, MPT or both are enriched. The number (N) of each type are indicated. Type II multipass proteins were further classified by the length of the first translocated segment. "

3. Dual colour ratiometric assay (Figs. 5 and S7). Although the authors have used this assay in their previous publications, they could be a little more reader-friendly by spending just a few words to explain the assay. In describing the utilized constructs in the Methods section (p.18, line 538), the authors refer to a previous publication, which is not cited. In Fig. S7B, the abscissa and ordinate presumably represent RFP and GFP fluorescence, respectively; please, specify this.

Thank you for this suggestion. We have now more fully described the assay in the legends for Fig. 5a and Ext. Data Fig. 7b. The abscissa and ordinate do not represent specific colours, but rather the fusion protein versus the translation control. Because of the different topologies being analysed in both figures, the fusion protein is sometimes GFP and other times RFP.

We have also clarified the referencing for the dual-color constructs described in earlier work.

4. Fig. 3B legend: please refer to the Methods section for the definition/calculation of peaks.

The legend has been adjusted accordingly.

5. Text, p. 6, lines 241 – 250. The authors provide a list of proteins, whose stability was either affected or unaffected by Apra1 treatment. For two of them (signal peptide-containing secretory protein and tail-anchored proteins), the data are not shown. Please show the data in the supplementary figure or provide appropriate reference.

The flow cytometry data for a signal peptide-containing translocated protein (EGFP-KDEL) and a tail-anchored protein (VAMP2) are shown in Extended Data Fig. 7b. We have modified the text to make this clearer.

6. There are lots of typos: here are a few that I caught:
(i) Legend to Fig. S5, lines 791-792: “mean enrichment (line) and range (shaded) are shown. Median (dark magenta line) and range (light magenta shading) for n=6 replicates are indicated”. Maybe the “mean enrichment (line)” refers to panel A (SLC7A11)?
(II) Legend to Fig. S6: the word “for” is duplicated
(III) Legend to Fig. S2: “qualify” should read “quality”
(iv) Legends to Fig. S1 and Fig. 1: MNase ??
(v): p. 20 line 20: by “nutator” I suppose that “rotator” is meant.
(vi) p.21, lines 669 and 673: “SMAT” should read “SMART”

We apologise for these errors. The manuscript has now been carefully proofread and these and other errors have been corrected.

Response to referees

Reviewer #2 (Remarks to the Author):

The authors have addressed all my previous concerns and comments.

Reviewer #3 (Remarks to the Author):

The authors have addressed my major and minor concerns, however, I recommend a clarification in the part of the text discussing the role of the downstream translocated segment in determining whether the preceding TMD opens or does not open the Sec61 channel (lines 289 – 293). Here it is stated that "the length of the downstream translocated segment influences whether the preceding TMD will open the Sec61 channel: LONG TRANSLOCATED SEGMENTS OPEN THE CHANNEL, AND SHORT TRANSLOCATED SEGMENTS DO NOT." (capitals mine). In fact, it is not the translocated segments that open the channel, rather the upstream TMD; furthermore, the downstream segments are not translocated yet, rather, they are destined for translocation. Thus, to avoid confusion, one might refer to these segments as segments "to be translocated", but for sure it is incorrect to state that they themselves open the channel; I realize that a more precise description may be grammatically more complicated, but I think that more precision would help the reader to fully appreciate this work.

We agree that the original paragraph was confusing and have revised it to focus on the main point, which is that many multipass proteins can be synthesized without opening the Sec61 channel. The revised text now reads as follows (lines 288-292):

"Third, synthesis of many multipass proteins does not require opening of the Sec61 channel. Whereas insertion of TMDs that are followed by long translocated segments requires Sec61, TMDs followed by short translocated segments can be inserted via MPT. Thus, biogenesis of the hundreds of multipass proteins with only short translocated segments can occur without opening the Sec61 channel or engaging OST-A."