

Mammalian MemPrep establishes the lipid composition of ER membranes in HEK293T cells

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Review
COMMONS

Review #1

1. Evidence, reproducibility and clarity:

Evidence, reproducibility and clarity (Required)

****Summary:****

The authors adapt MemPrep, a protocol they originally developed to purify organelle membranes from yeast, for use in human cell lines. To this end, they established immunoprecipitation strategies based on tagged versions of the ER sheet protein SEC61 β and the ER tubular protein REEP5 in HEK293T cells. Their purification strategy allowed them to generate highly pure ER sheet- and tubule-enriched fractions, which were then subjected to quantitative lipidomic and proteomic analyses.

Overall, this manuscript is well written and presents a careful interpretation of the data. It introduces MemPrep in mammalian cells as a method that will be useful for studying the membrane lipid and protein composition of organelles, with a particular focus on the ER. As such, the manuscript provides sufficient information and controls to assess the experiments in terms of reproducibility and clarity.

****Major comments:****

1. Based on the immunofluorescence images in Figure 1, it is not clear that the tagged and slightly overexpressed versions of SEC61 β and REEP5 localize specifically to ER sheets and tubules, respectively, or that these proteins are enriched in these distinct ER subdomains. Perhaps reducing the fixation time, for example to a maximum of 2 minutes, or using PFA fixation, could help to better preserve ER sheet and tubular domains.
2. Does expression of tagged SEC61 β or REEP5 influence the ER sheet:tubule ratio? In addition, does expression of these constructs affect the lipidome or proteome of the cells?
3. Apart from hypotonic swelling and douncing, could the authors use alternative methods for cell disruption to exclude the possibility that mechanical stress confounds the interpretation of the data?
4. What is the total amount of lipids and proteins isolated with REEP5- or SEC61 β -based MemPrep? Are there differences in the total lipid:protein ratio between these isolates, and could this reflect differences in the ER sheet:tubule ratio?
5. The combined analysis of lipid and protein composition demonstrates the capacity of the method. To test that MemPrep can capture changes in ER membrane architecture, it would be useful to compare ER protein and lipid composition across different cellular

states, such as stressed versus unstressed cells, or growing versus resting cells.

****Minor comment:****

1. In line 335, the authors state: "To address this possibility, we performed a new round of REEP5 and SEC61 β MemPreps for a direct comparison of the isolates (Fig. 5A, B)." It is unclear whether the MemPrep protocol was altered or whether this refers simply to an additional round of purification. Please clarify.

2. Significance:

Significance (Required)

General assessment:

The manuscript establishes MemPrep for mammalian cells as an important discovery tool to investigate how cells coordinate membrane lipid composition with membrane protein composition, and vice versa. This is a rapidly growing research field, which attracts a lot of interest.

MemPrep is based on an immuno-isolation strategy using tagged versions of the ER sheet protein SEC61 β and the ER tubular protein REEP5 in HEK293T cells. The purification strategy allowed to generate highly pure ER sheet- and tubule-enriched fractions, which were then subjected to quantitative lipidomic and proteomic analyses.

The results show that the protein composition differs between the SEC61 β - and REEP5-enriched fractions. Yet the lipid composition of ER sheets and tubules is largely indistinguishable. Both fractions are dominated by PC alongside other monounsaturated GPL, and hydroxylated ceramides. These physicochemical properties of the ER lipid bilayer are matched by ER-resident membrane proteins.

Thorough bioinformatic analysis of a subset of ER membrane proteins further revealed that their transmembrane domains have reduced hydrophobicity and increased polarity compared with those of plasma membrane proteins, matching the ER lipidome.

Hence the combined analysis of lipid and protein composition demonstrates the capacity of the method. Many variations of this approach will be possible in the future to understand on the molecular level how cells assemble and control their membranes.

Advance: Other immuno-isolation methods, or "organelle immunoprecipitation" approaches, have been established for lysosomes, the Golgi apparatus, and other organelles.

MemPrep is an important and complementary addition to the technical toolbox for organelle isolation, with a particular focus on the analysis of membrane lipid and protein content.

Audience:

The manuscript will be of broad interest to researchers in basic biology as well as clinical and translational research.

Reviewer's field of expertise:

Molecular membrane biology.

3. How much time do you estimate the authors will need to complete the suggested revisions:

Estimated time to Complete Revisions (Required)

(Decision Recommendation)

Between 1 and 3 months

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No

Review #2

1. Evidence, reproducibility and clarity:

Evidence, reproducibility and clarity (Required)

Jain and colleagues develop a biochemical fractionation procedure in which ER microsomes are enriched through small epitope tags. The manuscript is pitched around the concept that there are ER sheets and tubules and ER proteins differentially localise to them. The authors use REEP5 as a 'tubule' bait and SEC61beta as a 'sheet' bait. These baits are immunoisolated after a sensible membrane fractionation and ER membranes purified. There is a convincing ER proteome as a result, and this is used to compare the TMD properties of the organelles resident membrane proteins. The authors make the interesting observation that the transmembrane domains are more polar in the ER. They then compare the two sheet and tubule preparations and see a difference in the proteome, before comparing the lipidome. There is no difference observed between the lipidome of the sheet and tubule preps, however they see a difference in the whole cell lysate and use that to compare the ER lipidome against the whole cell.

Overall the manuscript has an interesting premise and the data is well presented, the experiments well performed and the interpretations appropriate. I think there are some issues with the mechanistic insight and novelty, and essentially although the premise is with regards to sheets and tubules there is limited progress in that direction in terms of results. I am reluctant to be too critical overall as there are certainly interesting observations that may be insightful for future studies in the field. I have some more specific comments below:

1. The authors cite Nixon-Abell, but they do not mention the major point of that manuscript which is that the 'sheets' in the cellular periphery are instead dense tubular networks. I think this is quite an omission for the introduction, as it points to the premise not being as clear as stated.
2. The first section when the protocol is discussed essentially relies on looking at other papers to understand. As the manuscript is centrally about this protocol, I think a brief but clear description is more appropriate.
3. In figure 1C the two markers are supposed to localise to sheets and tubules differentially. To me they look very similar. This, of course, is a major concern. Have the authors co-expressed them (at the same levels in these lines) and seen that indeed they do differentially localise?
4. I found the TMD polarity section very interesting, but it was not clear to me why they needed their proteomics for this? Could this not be done with annotated ER membrane proteins?
5. It was not clear to me based on the results section text the difference between the figure 5 proteomics and the previous runs.
6. Again in figure 5- are the authors sure that the difference was not due to the over-

expression (albeit mild) of their protein.

7. There were no differences in the ER lipidome between the two baits. This may be because there is no difference between the lipid profile of sheets and tubules, but it is very hard to conclude that.

8. I do not see it as my job as a reviewer to propose reorganisations and rewrites, so I encourage the authors to feel free to ignore this comment. To me the lipidome and TMD polar observations are the key manuscript findings, and there is very limited insight into the tubules and sheets line of inquiry. I wonder if it would be worth changing the focus of the manuscript overall to rather be about the ER, and not the tubules and sheets.

2. Significance:

Significance (Required)

Overall the manuscript has an interesting premise and the data is well presented, the experiments well performed and the interpretations appropriate. I think there are some issues with the mechanistic insight and novelty, and essentially although the premise is with regards to sheets and tubules there is limited progress in that direction in terms of results. I am reluctant to be too critical overall as there are certainly interesting observations that may be insightful for future studies in the field.

3. How much time do you estimate the authors will need to complete the suggested revisions:

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Review #3

1. Evidence, reproducibility and clarity:

Evidence, reproducibility and clarity (Required)

****Summary:****

Jain et al., provide a clear and thorough manuscript that extends their prior biochemical analysis of the yeast ER-lipidome (MEMPREP) to mammalian cells. They use detergent free lysis and differential speed centrifugation from 293T cells bearing reporters with affinity handles targeted to sheet-like or tubular-like subdomains of the ER and enrich membranes and membrane-embedded proteins from these sites. The lipidomics reveals a distinct ER-lipidome, heavily enriched in PC and PI, contains predominantly mono-unsaturated phospholipids and is surprisingly invariant across sheet-like and tubule-like domains. Additional hydrophobicity analysis suggests that ER-localised TMDs are more polar and shorter than PM-resident TMDs, and the authors speculate about co-evolution of the lipidome and proteome to ensure targeting.

****Major comments:****

I think the data are solid, clear and convincing. The similarity of the lipidomes from sheet and tubule regions of the ER give good indication of the robustness of the technique. Whilst the yield is low, the authors go to good lengths to demonstrate purity of ER capture and de-enrichment of other cellular membranes. There is good discussion of the limitations of the technique and good comparison to recent data from other labs, most notably, a recent preprint and I think the manuscripts support each other well. There's a fair amount of speculation in the manuscript, e.g., about lipid headgroup charge density being inferred by the charge distribution on the -1 position, but the speculation is clearly acknowledged.

1. I think that blotting for SEC61B would really help. A clear comparison to endogenous SEC61B would be helpful. I appreciate that the authors lacked an antibody here, but there are several on CiteAb that seem to detect endogenous protein.

2. It's not brilliantly easy to see the 'sharp decline' in relative frequency of hydrophobic amino acids at 21 aa for ER and Golgi; whilst the individual amino acid information is interesting (and some comment could be made about the favouring of Leucines in ER and Golgi TMDs), would this be clearer if the relative frequencies were binned into hydrophobic/aromatic, polar, positive, negative?

3. Related to this point, it's hard to correlate the degree of polar amino acid incorporation in the TMDs of Golgi, ER, PM proteins (which don't appear to vary in 4E, 4F and 4G) with the variance described in 4C. Is there a better way of displaying this data, or are the polarity

measurements calculated by some other metric in 4C?

****Minor comments:****

1. Panel 2D isn't labelled on the figure
2. There is limited co-enrichment of non-ER proteins in the ER-affinity preps, and the authors have done well to deal with misannotated GO terms. It might be worthwhile adding to the discussion that all TMD proteins that localise at steady-state to post-ER compartments must necessarily pass through the ER during biosynthesis. As such, detection of non-ER proteins in ER fractions is not inherently unexpected.
3. I didn't understand the line on L377 about the new round of extraction featuring inherently less complex proteomes.
4. For line L390-391, in the speculation about progressively more unsaturation as you move ER-Golgi-postGolgi, is there any (published) data from ER-FLIPPR that could inform about the degree of membrane fluidity/packing as you traverse the secretory pathway?

2. Significance:

Significance (Required)

The strengths of the study are the conceptual novelty and information provided - I think this is the first comprehensive reporting of the ER lipidome. This is a major organelle and I think as the lipid biology field develops, resources like this are really important. Moreover, the MEMPREP protocol is applicable for protein extraction from these domains, which will help with functional characterisation of ER subdomains and is a strong technical advance.

Weaknesses relate to the single cell type and overexpression (albeit mild) methodologies. I'm not hugely fussed about this as this manuscript describes an important 1st step.

I'm a cell biologist studying the ER

3. How much time do you estimate the authors will need to complete the suggested revisions:

Estimated time to Complete Revisions (Required)

(Decision Recommendation)

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Full Revision



Manuscript number: RC-2026-03492

Corresponding author(s): Prof. Dr. Robert Ernst

1. General Statements

We thank the reviewer for their positive assessment of our manuscript and for identifying critical issues, which we have carefully addressed during the revision.

2. Point-by-point description of the revisions

Reviewer #1

The authors adapt MemPrep, a protocol they originally developed to purify organelle membranes from yeast, for use in human cell lines. To this end, they established immuno-isolation strategies based on tagged versions of the ER sheet protein SEC61 β and the ER tubular protein REEP5 in HEK293T cells. Their purification strategy allowed them to generate highly pure ER sheet- and tubule-enriched fractions, which were then subjected to quantitative lipidomic and proteomic analyses.

Overall, this manuscript is well written and presents a careful interpretation of the data. It introduces MemPrep in mammalian cells as a method that will be useful for studying the membrane lipid and protein composition of organelles, with a particular focus on the ER. As such, the manuscript provides sufficient information and controls to assess the experiments in terms of reproducibility and clarity.

We thank the reviewer for a positive, thorough assessment and for raising important points that helped us to improve the manuscript.

Major comments:

1. Based on the immunofluorescence images in Figure 1, it is not clear that the tagged and slightly overexpressed versions of SEC61 β and REEP5 localize specifically to ER sheets and tubules, respectively, or that these proteins are enriched in these distinct ER subdomains. Perhaps reducing the fixation time, for example to a maximum of 2 minutes, or using PFA fixation, could help to better preserve ER sheet and tubular domains.

To address the localization of the bait proteins in the ER membrane network, we added new co-localization microscopy data and quantifications to the revised manuscript (**new Figure 1E,F; new Supplementary Figure S1C,D**). Despite its low level of overexpression (**new Figure 1C; new Suppl. Fig. S1A**), SEC61 β localizes to the entire ER membrane network including ER tubules and the nuclear envelope (**new Fig. 1E,F**). **Considering the new data, we have carefully rephrased all sections regarding the subcellular localization of bait-SEC61 β . In the revised manuscript, we use SEC61 β as a general ER marker.**

Intriguingly, quantitative proteomics of the SEC61 β MemPrep isolate demonstrates a selective enrichment of ER sheet-associated proteins compared to the REEP5 MemPrep, which selectively enriches proteins associated with ER tubules (**Fig. 5**). While we do not claim to 'isolate' ER subdomains, we enrich ER subdomains.

We have performed additional microscopy experiments and adjusted our fixation protocol as suggested by the reviewer (**Revision Fig. 1**). Shortening the fixation time has no apparent impact on the ER structure, while any PFA fixation seems to largely disrupt the ER.

Figure for referee with unpublished data and its description has been removed upon request by the authors.

2. Does expression of tagged SEC61 β or REEP5 influence the ER sheet:tubule ratio? In addition, does expression of these constructs affect the lipidome or proteome of the cells?

The reviewer raises an important point, which is experimentally not easy to address. Our imaging modality is not sufficient to make a firm statement about the sheet:tubule ratio in HEK293T cells. We are not aware of any study that firmly quantifies the relative content of sheets and tubules in HEK293T cells. Imaging the ER in HEK293T cells is challenging and most studies on the ER membrane networks use other cell types to study the impact of ER-shaping protein on the ER membrane network.

In the revised manuscript we state: *'We found no evidence that the expression of the bait constructs disrupts the tubule-to-sheet ratio or other aspects of the ER architecture, but distinguishing ER sheets and ER tubules is challenging in HEK293T cells.'*

Furthermore, we have studied if the expression of the bait constructs affects the cellular proteome (**new Suppl. Fig. S1A,B**) and lipidome (**new Suppl. Fig. S4A-H** (previously Suppl. Fig. S3)). The expression of the bait constructs has no substantial impact on the cellular proteome. Most importantly, we find no evidence that proteins characteristic for ER sheets or ER tubules (other than the bait proteins) change their expression level (**new Suppl. Fig. S1A,B**). In the revised manuscript we state:

'We decided to go one step further and compared the proteomes of wildtype HEK293T cells with the two cell lines using TMT multiplexed, untargeted protein mass spectrometry (Suppl. Fig. S1A, B). This experiment revealed that bait proteins have only a minimal, neglectable impact on the cellular proteome (Suppl. Fig. S1A, B). We did not find evidence for a systematic deregulation of proteins known to localize exclusively to ER tubules or other ER subdomains. Furthermore, quantitative proteomics validated the results from immunoblotting (Fig. 1B, C): Expression of bait-SEC61 β has barely any impact on the total cellular level of SEC61 β (Suppl. Fig. S1A) while the expression of the REEP5-bait results in a 1.8-fold overabundance of REEP5 (Suppl. Fig. S1B).'

Likewise, the expression of the bait constructs has little to no effect on the cellular lipidome as shown in Suppl. Fig. S4A-J. In the revised manuscript we state:

'As a control, we also tested the impact of the bait constructs on the HEK293T whole cell lipidome (Suppl. Fig. 4A-J). Overall, the lipid composition of the virally transduced cells was indistinguishable from HEK293T cells with only minor impact on the level of CL and lysolipids (Suppl. Fig. 4A-J).'

3. Apart from hypotonic swelling and douncing, could the authors use alternative methods for cell disruption to exclude the possibility that mechanical stress confounds the interpretation of the data?

Thanks to the reviewer's comment, we became aware of a mistake. Our cell lysis buffer is hypertonic and not hypotonic (15% sucrose w/v, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)(HEPES) pH 7.4, 300 mM NaCl, 1 mM EDTA freshly supplemented with protease inhibitor cocktail from Roche). We have corrected all relevant sections in the revised manuscript.

The reviewer is right that different means of mechanical lysis, and/or the incubation of the cells in hypo/hypertonic buffer are likely to have impact on the structure of the ER and to affect the isolation procedure. Changing such critical parameters will likely affect the purity of the preparation. Performing additional MemPrep isolations using different means of cell disruptions goes beyond the scope of this manuscript.

Upon establishing the MemPrep protocol, we have explored various mechanical cell disruptions: Different cannula, Dounce homogenizers, and a ball-bearing device. We experimented with both hypo- and hypertonic buffers. Given the costs and work associated with lipidomic and proteomic analyses, we have tried to find a suitable conditions for cell disruption without performing a full analysis each time. Therefore, we performed differential centrifugations as exemplary shown in Fig. 2B of the manuscript. Critical factors for our decision whether to further pursue a certain condition was 1) the depletion of the mitochondrial TOM22 marker, 2) the enrichment of the ER markers, and 3) the total protein yield in the P100,000 fraction.

In the revised manuscript we state: *'Compared to the MemPrep procedure in yeast, we tested various means of cell disruption and optimized the differential centrifugation protocol.'* and

'Mild cell disruption by Dounce homogenization in a hypertonic buffer is crucial for cracking cells open, but these procedures can disrupt normal ER architecture and might facilitate the undesired mixing of previously well-defined ER subdomains. Despite these limitations, our data underscore the purity of our ER membrane preparations, demonstrate a differential enrichment of ER subdomains (Fig. 5), and establish the lipid composition of the ER membrane (Fig. 6).'

4. What is the total amount of lipids and proteins isolated with REEP5- or SEC61 β -based MemPrep? Are there differences in the total lipid:protein ratio between these isolates, and could this reflect differences in the ER sheet:tubule ratio?

In response to the reviewers' question, we have included a **new Supplementary table 1** to the manuscript outlining the yield of total protein and total lipid of MemPrep.

The mammalian MemPrep protocol is not yet optimized for determining the lipid:protein ratio in the membrane. At this moment, we do not want to make a statement about the protein-to-lipid ratio in the ER or its subdomains. The isolates still contain material originating from the ER lumen.

5. The combined analysis of lipid and protein composition demonstrates the capacity of the method. To test that MemPrep can capture changes in ER membrane architecture, it would be

useful to compare ER protein and lipid composition across different cellular states, such as stressed versus unstressed cells, or growing versus resting cells.

We agree with the reviewer that a comparison of the ER under different conditions would be extremely interesting. Currently, we see it beyond the scope of this study.

Unpublished results from the lab show that various conditions that cause lipid bilayer stress have a major impact on the whole cell lipidome, while other conditions causing ER stress do not manifest in a perturbation of the cellular lipidome. We plan to use mammalian MemPrep to obtain the molecular composition of the ER for the most promising conditions.

Minor comment:

1. In line 335, the authors state: "To address this possibility, we performed a new round of REEP5 and SEC61 β MemPreps for a direct comparison of the isolates (Fig. 5A, B)." It is unclear whether the MemPrep protocol was altered or whether this refers simply to an additional round of purification. Please clarify.

Thank you. This point was also raised by reviewer 2 and 3. We have clarified our statements. In the revised manuscript we state:

'Hence, we performed a new round of REEP5 and SEC61 β MemPreps in triplicates for a direct comparison of the isolates (Fig. 5A, B) rather than comparing the changes in abundance relative to the respective cell lysates as performed in Figure 3. Knowing that non-ER proteins are less efficiently enriched by the MemPrep procedure than ER proteins (Fig. 3C, D) and that the sensitivity and comprehensiveness of mass spectrometry-based proteomics experiments are reduced with increasing sample complexity (Ting et al, 2011; Beck et al, 2011) , we were hoping to gain a better insight into the distribution of low abundant and challenging to quantify proteins in the two MemPrep isolates'.

Reviewer #1 (Significance (Required)):

General assessment:

The manuscript establishes MemPrep for mammalian cells as an important discovery tool to investigate how cells coordinate membrane lipid composition with membrane protein composition, and vice versa. This is a rapidly growing research field, which attracts a lot of interest.

MemPrep is based on an immuno-isolation strategy using tagged versions of the ER sheet protein SEC61 β and the ER tubular protein REEP5 in HEK293T cells. The purification strategy allowed to generate highly pure ER sheet- and tubule-enriched fractions, which were then subjected to quantitative lipidomic and proteomic analyses.

The results show that the protein composition differs between the SEC61 β - and REEP5-enriched fractions. Yet the lipid composition of ER sheets and tubules is largely indistinguishable. Both fractions are dominated by PC alongside other monounsaturated GPL, and hydroxylated ceramides. These physicochemical properties of the ER lipid bilayer are matched by ER-resident membrane proteins.

Thorough bioinformatic analysis of a subset of ER membrane proteins further revealed that their transmembrane domains have reduced hydrophobicity and increased polarity compared with those of plasma membrane proteins, matching the ER lipidome.

Hence the combined analysis of lipid and protein composition demonstrates the capacity of the method. Many variations of this approach will be possible in the future to understand on the molecular level how cells assemble and control their membranes.

Advance: Other immuno-isolation methods, or "organelle immunoprecipitation" approaches, have been established for lysosomes, the Golgi apparatus, and other organelles.

MemPrep is an important and complementary addition to the technical toolbox for organelle isolation, with a particular focus on the analysis of membrane lipid and protein content.

Audience:

The manuscript will be of broad interest to researchers in basic biology as well as clinical and translational research.

Reviewer's field of expertise:

Molecular membrane biology.

Reviewer #2

Jain and colleagues develop a biochemical fractionation procedure in which ER microsomes are enriched through small epitope tags. The manuscript is pitched around the concept that there are ER sheets and tubules and ER proteins differentially localise to them. The authors use REEP5 as a 'tubule' bait and SEC61 β as a 'sheet' bait. These baits are immunoisolated after a sensible membrane fractionation and ER membranes purified. There is a convincing ER proteome as a result, and this is used to compare the TMD properties of the organelles resident membrane proteins. The authors make the interesting observation that the transmembrane domains are more polar in the ER. They then compare the two sheet and tubule preparations and see a difference in the proteome, before comparing the lipidome. There is no difference observed between the lipidome of the sheet and tubule preps, however they see a difference in the whole cell lysate and use that to compare the ER lipidome against the whole cell.

Overall the manuscript has an interesting premise and the data is well presented, the experiments well performed and the interpretations appropriate. I think there are some issues with the mechanistic insight and novelty, and essentially although the premise is with regards to sheets and tubules there is limited progress in that direction in terms of results. I am reluctant to be too critical overall as there are certainly interesting observations that may be insightful for future studies in the field. I have some more specific comments below:

We thank the reviewer for a thorough, constructive assessment and for highlighting important points that helped us improve the manuscript.

1) The authors cite Nixon-Abell, but they do not mention the major point of that manuscript which is that the 'sheets' in the cellular periphery are instead dense tubular networks. I think this is quite an omission for the introduction, as it points to the premise not being as clear as stated.

In the revised manuscript we refer to the Nixon-Abell study and two additional studies from the Jokitalo lab. Notably, the Nixon-Abell study does not rule out the existence of ER sheets.

In the revised manuscript we state: '*[...] dense tubular networks in the cell periphery can appear like ER sheets in diffraction-limited microscopy (Nixon-Abell et al, 2016). Furthermore, the edges of ER sheets are populated by curvature-stabilizing proteins also found in ER tubules (Shibata et al, 2010; Shemesh et al, 2014), and ER sheets show different degrees of fenestration dependent on the cell type and the cell cycle phase (Puhka et al, 2007, 2012; Nixon-Abell et al, 2016). Consistent with our microscopic data (Fig. 1E, F) and because ER sheets may be biochemically inseparable from ER tubules, we use SEC61 β as a general ER marker.*'

We performed additional co-localization studies of the bait proteins with RTN4 and CLIMP63 (**new Fig. 1E,F**) suggesting that SEC61B can localize across many ER subdomains including ER tubules and the nuclear envelope.

We have carefully revised our manuscript accordingly and shifting the focus of our discussion away from a molecular description of discrete ER subdomains.

2) The first section when the protocol is discussed essentially relies on looking at other papers to understand. As the manuscript is centrally about this protocol, I think a brief but clear description is more appropriate.

We agree with the reviewer. We added a short section to the results section providing an overview over the MemPrep procedure. We now state:

*'To this end, we adapted the MemPrep procedure originally developed for the isolation of organelle membranes from *Saccharomyces cerevisiae* (*S. cerevisiae*) (Reinhard et al, 2023, 2024). Mammalian MemPrep relies on a gentle, detergent-free, mechanical lysis of the cells in a*

hypertonic buffer followed by differential centrifugation to separate ER-derived microsomes from mitochondria-derived membranes. Next, larger organelle fragments are disrupted by brief pulses of sonication, and the resulting vesicles are subjected to affinity purification using magnetic dynabead-coupled antibodies directed against the cleavable tag of the bait protein. Specifically bound, ER-derived membrane vesicles are washed with harsh, urea-containing buffers and selectively released by proteolytically cleaving the bait tag.'

3) In figure 1C the two markers are supposed to localise to sheets and tubules differentially. To me they look very similar. This, of course, is a major concern. Have the authors co-expressed them (at the same levels in these lines) and seen that indeed they do differentially localise?

The reviewer raises an important point regarding the localization of the bait proteins. While we have not co-expressed the bait proteins in cells, we have performed additional co-localization experiments with RTN4 and CLIMP63 as markers for ER tubules and ER sheets, respectively (**new Figure 1E,F; new Suppl. Fig. S1C,D**). The implications of these data are discussed in the manuscript.

In light of these new data, we do not refer to SEC61 β as an ER sheet marker any longer, instead we refer to SEC61 β as a general ER marker. We carefully revised our discussion of the data throughout the manuscript along the line suggested by the reviewer in point 8.

4) I found the TMD polarity section very interesting, but it was not clear to me why they needed their proteomics for this? Could this not be done with annotated ER membrane proteins?

The reviewer is correct. The same type of analysis could have been performed with an even bigger dataset of all ER annotated proteins. One of the co-authors, Joseph Lorent, has performed such analysis at this larger scale (PMID: 40326394). The study by Lorent *et al.* addressed TMH length and side chain bulkiness (PMID: 40326394) in the ER, Golgi apparatus, and the PM. This work is referenced in the manuscript.

We focused our analysis on the smaller dataset of 83 single-pass proteins found in our proteomics experiments, because we initially planned to perform a comparative analysis of ER proteins in either of the two isolates.

In line of the reviewers' suggestion, we validate our new finding on the TMH hydrophobicity in the ER using a larger dataset covering all single pass TMHs of ER proteins (215 instead of 83), Golgi apparatus proteins (260), and plasma membrane proteins (1322) (**Suppl. Fig. S3D**).

5) It was not clear to me based on the results section text the difference between the figure 5 proteomics and the previous runs.

This point was also raised by reviewer 1 and 3. We clarified our statement in the revised manuscript:

'Hence, we performed a new round of REEP5 and SEC61 β MemPreps in triplicates for a direct comparison of the isolates (Fig. 5A, B) rather than comparing the changes in abundance relative to the respective cell lysates as performed in Figure 3. Knowing that non-ER proteins are less efficiently enriched by the MemPrep procedure than ER proteins (Fig. 3C, D) and that the sensitivity and comprehensiveness of mass spectrometry-based proteomics experiments are reduced with increasing sample complexity (Ting et al, 2011; Beck et al, 2011) , we were hoping to gain a better insight into the distribution of low abundant and challenging to quantify proteins in the two MemPrep isolates.'

6) Again in figure 5- are the authors sure that the difference was not due to the over-expression (albeit mild) of their protein.

After performing an important control experiment, we are sure that the mild over-expression of the bait proteins has no impact.

We have compared HEK293T WT cells with the bait protein expressing cell lines by quantitative proteomics (**new Suppl. Fig. S1A,B**). The bait proteins have no impact of the cellular proteome and do not affect the abundance of proteins known to be enriched in ER sheets or ER tubules. Hence, the enrichment of these proteins in our MemPrep isolates as shown in Fig. 5 suggests that some of the identity of ER sheets and ER tubules is maintained in our preparations even though they are not resolved by our microscopy experiments (Fig. 1). In the revised manuscript, we carefully discuss the implications of these findings.

7) There were no differences in the ER lipidome between the two baits. This may be because there is no difference between the lipid profile of sheets and tubules, but it is very hard to conclude that.

The reviewer has a point. Even though our findings suggest that we can differentially enrich for ER subdomains (the proteomics data in Fig. 5 on MemPrep isolates can be regarded as a golded standard for this statement), we do not have any knowledge about their biochemical purity. Hence, we have carefully toned down our statements on the basis of new imaging data (**Fig. 1E,F; Suppl. Fig. S1C,D**) and new proteomics data (**Suppl. Fig. S1A,B**).

Along the reasoning of the reviewer, we also rephrased our statements on the difference/similarity of ER subdomains.

8) I do not see it as my job as a reviewer to propose reorganisations and rewrites, so I encourage the authors to feel free to ignore this comment. To me the lipidome and TMD polar observations are the key manuscript findings, and there is very limited insight into the tubules and sheets line of inquiry. I wonder if it would be worth changing the focus of the manuscript overall to rather be about the ER, and not the tubules and sheets.

Again, the reviewer raises an important point that we did not want 'to ignore'. We have carefully revised the manuscript and toned down our interpretations. In the revised manuscript we put more emphasis on the ER lipidome and less so on the composition of specific ER subdomains.

Reviewer #2 (Significance (Required)):

Overall the manuscript has an interesting premise and the data is well presented, the experiments well performed and the interpretations appropriate. I think there are some issues with the mechanistic insight and novelty, and essentially although the premise is with regards to sheets and tubules there is limited progress in that direction in terms of results. I am reluctant to be too critical overall as there are certainly interesting observations that may be insightful for future studies in the field.

Reviewer #3

Summary:

Jain et al., provide a clear and thorough manuscript that extends their prior biochemical analysis of the yeast ER-lipidome (MEMPREP) to mammalian cells. They use detergent free lysis and differential speed centrifugation from 293T cells bearing reporters with affinity handles targeted to sheet-like or tubular-like subdomains of the ER and enrich membranes and membrane-embedded proteins from these sites. The lipidomics reveals a distinct ER-lipidome, heavily enriched in PC and PI, contains predominantly mono-unsaturated phospholipids and is surprisingly invariant across sheet-like and tubule-like domains. Additional hydrophobicity analysis suggests that ER-localised TMDs are more polar and shorter than PM-resident TMDs, and the authors speculate about co-evolution of the lipidome and proteome to ensure targeting.

Major comments:

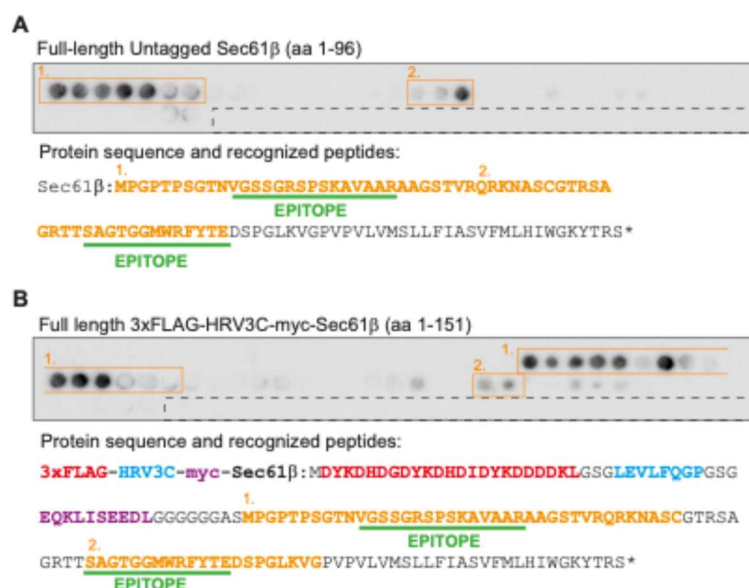
I think the data are solid, clear and convincing. The similarity of the lipidomes from sheet and tubule regions of the ER give good indication of the robustness of the technique. Whilst the yield is low, the authors go to good lengths to demonstrate purity of ER capture and de-enrichment of other cellular membranes. There is good discussion of the limitations of the technique and good comparison to recent data from other labs, most notably, a recent preprint and I think the manuscripts support each other well. There's a fair amount of speculation in the manuscript, e.g., about lipid headgroup charge density being inferred by the charge distribution on the -1 position, but the speculation is clearly acknowledged.

1. I think that blotting for SEC61B would really help. A clear comparison to endogenous SEC61B

would be helpful. I appreciate that the authors lacked an antibody here, but there are several on CiteAb that seem to detect endogenous protein.

Following the reviewers' advice, we added new data using a commercial antibody directed against SEC61 β (**new Fig. 1C**). We also added proteomics data comparing HEK293T WT cells with the bait expressing cell lines (**new Suppl. Fig. S1A,B**).

We also characterized the commercial Proteintech (15087-1-AP) antibody to make sure it recognizes the same epitopes in the tagged and untagged variant of SEC61 β .



Revision Figure 1: Characterization of the SEC61 β antibody Proteintech (15087-1-AP) using immobilized peptide fragments. (A) Peptide fragments covering the entire sequence of untagged SEC61 β were spotted on a solid matrix. The reactivity of the SEC61 β antibody was scored by immunoblotting to identify the epitopes recognized by the antibody. (B) Peptide fragments covering the entire sequence of bait-SEC61 β were spotted on a solid matrix. The reactivity of the SEC61 β antibody was scored by immunoblotting to identify the epitopes recognized by the antibody. The antibody recognizes the same number of epitopes in both tagged and untagged SEC61 β .

2. It's not brilliantly easy to see the 'sharp decline' in relative frequency of hydrophobic amino acids at 21 aa for ER and Golgi; whilst the individual amino acid information is interesting (and some comment could be made about the favouring of Leucines in ER and Golgi TMDs), would this be clearer if the relative frequencies were binned into hydrophobic/aromatic, polar, positive, negative?

The reviewer is right. We have removed our statement regarding a 'sharp decline'. In fact, the decline is rather gradual for ER and Golgi TMHs, but more clear for PM TMHs. This is also reflected in the data shown in **Suppl. Fig. S3D** and discussed in the revised manuscript.

We state: Confirming our expectations based on the predicted TMH length (Suppl. Fig. S3A), we observed a gradual decline in the relative frequency of hydrophobic and aromatic residues at about 21 amino acids for ER (Fig. 4E) and Golgi-associated TMHs (Fig. 4F). Such decline was more clearly defined for plasma membrane TMHs but only after 24 aa or more (Fig. 4G).'

We also state: *'We therefore challenged our finding and performed an additional analysis using this larger dataset of all annotated human single-pass TMHs (Fig. S3D) and compared the hydrophobicity profiles of TMHs from the ER (215), the Golgi apparatus (260), and the PM (1322) (Lorent et al, 2025). This analysis further substantiated our finding that the ER and the Golgi apparatus host less hydrophobic TMHs compared to the plasma membrane. Furthermore, we observed that the ER and Golgi profiles display a conical shape with hydrophobic maxima at the center of the membrane's hydrophobic core, while the PM TMH's possess higher hydrophobicity in the cytoplasmic part of the membrane, compared to the exoplasmic part (Fig. S3D).'*

We decided to keep the Fig. 4 with its single amino acid 'resolution' as it was in the original manuscript, because we feel that this representation still has its value. It helps connecting physicochemical parameters of an average TMH in an organelle (Fig. 4A-D; Suppl. Fig. S3A-D) with the preferred amino acid composition and distribution (Fig. 4E-G). Nevertheless, some 'noise' is inherent to the data and we hope that the adaptations to the text avoids any possible confusion of the reader.

The frequency of leucine residues in TMHs from the PM (24.5%) is comparable to the frequency of TMHs from the ER (24.1%) and from the Golgi apparatus (26.3%). Our attempts to identify an organelle-selective usage of certain amino acids did not yield robust and significant results.

3. Related to this point, it's hard to correlate the degree of polar amino acid incorporation in the TMDs of Golgi, ER, PM proteins (which don't appear to vary in 4E, 4F and 4G) with the variance described in 4C. Is there a better way of displaying this data, or are the polarity measurements calculated by some other metric in 4C?

The reviewer is right. Figure 4A-D and Figure 4E-G are based on different metrics. Figure 4A-D considers different physicochemical parameters of the amino acid sidechains (Fig. 4C: Kyte-Doolittle scale). Figure 4E-G only represents the relative frequencies. We believe that both representations can be useful.

Notably, the relative incorporation of polar and apolar amino acids is significantly different between TMHs from the ER and the Golgi versus the TMHs from the PM (**Suppl. Fig. S3B,C**).

In the revised manuscript we state: *'Our new finding that the TMHs of ER proteins are more polar than the TMHs in the plasma membrane (Fig. 4C) is also reflected by the significantly different number of apolar and polar residues in the TMHs from ER-, Golgi apparatus-, and PM-derived proteins (Suppl. S3B, C).'*

Indeed, the polarity in Fig. 4A and Fig. 4C is calculated via the Kyte-Doolittle scale, while only the normalized frequency of the amino acid is color-coded in Fig. 4E-G.

Minor comments:

1. Panel 2D isn't labelled on the figure

We represented both MemPreps in a single Panel 2C because we aimed to label in the immunoblots only a single time to avoid redundancies. We are open to change our strategy of panel labeling if our current representation is confusing.

2. There is limited co-enrichment of non-ER proteins in the ER-affinity preps, and the authors have done well to deal with misannotated GO terms. It might be worthwhile adding to the discussion that all TMD proteins that localise at steady-state to post-ER compartments must necessarily pass through the ER during biosynthesis. As such, detection of non-ER proteins in ER fractions is not inherently unexpected.

This is of course correct. In the revised manuscript we state: *'Finding non-ER proteins in an ER proteome is not surprising, because a very large number of proteins are first delivered to the ER, before they are sent to other cellular destinations.'*

3. I didn't understand the line on L377 about the new round of extraction featuring inherently less complex proteomes.

This point was also raised by reviewer 1 and 2. We clarified our statement in the revised manuscript:

'Hence, we performed a new round of REEP5 and SEC61 β MemPreps in triplicates for a direct comparison of the isolates (Fig. 5A, B) rather than comparing the changes in abundance relative to the respective cell lysates as performed in Figure 3. Knowing that non-ER proteins are less efficiently enriched by the MemPrep procedure than ER proteins (Fig. 3C, D) and that the sensitivity and comprehensiveness of mass spectrometry-based proteomics experiments are reduced with increasing sample complexity (Ting et al, 2011; Beck et al, 2011), we were hoping to gain a better insight into the distribution of low abundant and challenging to quantify proteins in the two MemPrep isolates.'

4. For line L390-391, in the speculation about progressively more unsaturation as you move ER-Golgi-postGolgi, is there any (published) data from ER-FLIPPR that could inform about the degree of membrane fluidity/packing as you traverse the secretory pathway?

We agree that mentioning evidence on the biophysical changes along the secretory pathway is helpful in this section. In the revised manuscript we state:

'These changes of the lipid acyl chains are associated with biophysical changes of the membrane properties along the secretory pathway as observed by molecular probes reporting on lipid packing and membrane tension (Goujon et al, 2019; López-Andarias et al, 2021, 2022; Wong & Budin, 2024).'

Reviewer #3 (Significance (Required)):

The strengths of the study are the conceptual novelty and information provided - I think this is the first comprehensive reporting of the ER lipidome. This is a major organelle and I think as the lipid biology field develops, resources like this are really important. Moreover, the MEMPREP protocol is applicable for protein extraction from these domains, which will help with functional characterisation of ER subdomains and is a strong technical advance.

Weaknesses relate to the single cell type and overexpression (albeit mild) methodologies. I'm not hugely fussed about this as this manuscript describes an important 1st step.

I'm a cell biologist studying the ER

Dear Prof. Ernst

Thank you for the transfer of your research manuscript to our journal. We have now received the full set of referee reports that is copied below.

As you will see, the referees acknowledge that the findings are interesting and that the conclusions are overall supported by the data presented, with suggestions only geared towards increased clarity.

Given these constructive comments, we would like to invite you to revise your manuscript with the understanding that the referee concerns (as detailed above and in their reports) must be fully addressed and their suggestions taken on board. Please address all referee concerns in a complete point-by-point response. The manuscript will not be sent out for additional review.

We realize that it is difficult to revise to a specific deadline. In the interest of protecting the conceptual advance provided by the work, we recommend a revision within 3 months (October 17th). Please discuss the revision progress ahead of this time with the editor if you require more time to complete the revisions.

I am also happy to discuss the revision further via e-mail or a video call, if you wish.

You can either publish the study as a short report or as a full article. For short reports, the revised manuscript should not exceed 27,000 characters (including spaces but excluding materials & methods and references) and 5 main plus 5 expanded view figures. The results and discussion sections must further be combined, which will help to shorten the manuscript text by eliminating some redundancy that is inevitable when discussing the same experiments twice. For a normal article there are no length limitations, but it should have more than 5 main figures and the results and discussion sections must be separate. In both cases, the entire materials and methods must be included in the main manuscript file.

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IMPORTANT NOTE:

We perform an initial quality control of all revised manuscripts before re-review. Your manuscript will FAIL this control and the handling will be delayed IN CASE the following APPLIES:

- 1) A data availability section providing access to data deposited in public databases is missing. If you have not deposited any data, please add a sentence to the data availability section that explains that.
- 2) Your manuscript contains statistics and error bars based on $n=2$. Please use scatter blots in these cases. No statistics should be calculated if $n=2$.

When submitting your revised manuscript, please carefully review the instructions that follow below. Failure to include requested items will delay the evaluation of your revision.

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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 (). 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 (). Please find instructions on how to link your ORCID ID to your account in our manuscript tracking system in our Author guidelines

()

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:

- 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) Please include a dedicated "Data Availability" section at the end of the Methods (suggested wording: "The [structural coordinates | microarray | mass spectrometry] data from this publication have been deposited to the [name of the database] database [URL] and assigned the identifier [accession | permalink | hashtag]."). Should this not apply, this should still be stated as "This study includes no data deposited in external repositories."

8) At EMBO Press we ask authors to provide source data for the main manuscript figures. You will receive a separate email with instructions for providing source data with your revised manuscript, including how to upload and organize the files.

Additional information on source data and instruction on how to label the files are available

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 EXACT 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 and define their size in the legend, not the image.

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 .

12) All Materials and Methods need to be described in the main text. We would ask you to use 'Structured Methods', our new Materials and Methods format, which is mandatory for Methods and Articles with a strong methodological focus. According to this format, the Materials and Methods section should include a Reagents and Tools Table (listing key reagents, experimental models, software and relevant equipment and including their sources and relevant identifiers) followed by a Methods and Protocols section in which we encourage the authors to describe their methods using a step-by-step protocol format with bullet points, to facilitate the adoption of the methodologies across labs. More information on how to adhere to this format as well as downloadable templates (.doc or .xls) for the Reagents and Tools Table can be found in our author guidelines: < <https://www.embopress.org/page/journal/14693178/authorguide#manuscriptpreparation>

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Please download and fill our Reagents and Tools Table template (.docx), which you can find in our author guidelines:

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When submitting your revised manuscript, please do not include the Reagents and Tools Table in the Methods section of the manuscript but upload it as a separate file choosing the file type "Reagent Table".

An example of a Method paper with Structured Methods can be found here:

<https://www.embopress.org/doi/10.15252/msb.20178071>.

13) 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,

Kurt Weir
Editor
EMBO Reports

Referee #1:

During the revision, the authors have addressed my initial concerns. The manuscript is much improved and strengthened. Overall, it presents MemPrep for mammalian cells, with a careful evaluation of this method to purify ER for the analysis of protein and lipid content.

The revised manuscript is convincingly written and presents a careful interpretation of the data. I appreciate that the authors have adapted their conclusions based on the new results. The manuscript provides sufficient information and controls to assess the experiments in terms of reproducibility and clarity.

One minor point should be addressed regarding ether lipids: it is not clear from the Methods whether plasmalogens were analyzed separately, or whether they were grouped together with plasmalogen lipids without clear distinction. Please clarify this point. Also, in the legend for Figure 6 (line 1431) there is a typo at the very end of the end - I it should read: PE O- and not PC O-

General assessment: The manuscript establishes MemPrep for mammalian cells as an important discovery tool for investigating how cells coordinate membrane lipid composition with membrane protein composition, and vice versa, a rapidly growing field that attracts considerable interest.

The results show that while the protein composition differs between SEC61 β - and REEP5-enriched fractions, their lipid composition is largely indistinguishable. Both fractions are dominated by PC alongside other unsaturated glycerophospholipids (but not PUFAs) and hydroxylated ceramides and ether lipids. These physicochemical properties of the ER lipid bilayer are matched by ER-resident membrane proteins.

Thorough bioinformatic analysis of a subset of ER membrane proteins further reveals that their transmembrane domains have reduced hydrophobicity and increased polarity compared with those of plasma membrane proteins, matching the ER lipidome.

The combined analysis of lipid and protein composition demonstrates the method's capacity.

Advance: Other immuno-isolation ("organelle immunoprecipitation") methods have been established for lysosomes, the Golgi apparatus, and other organelles. MemPrep is an important and complementary addition to the toolbox for organelle isolation,

with a particular focus on membrane lipid and protein content.

Audience: The manuscript will be of broad interest to researchers in all field of basic and more applied life sciences.

Referee #2:

This all looks good to me, the edited changes are good and I think help with readability. The only outstanding item is Figure 2D; this is called out on L253, but the figure and legend only run to 2C. You could either refer to fig 2C in each case, or update the figure and legend to include reference to 2D.

Referee #3:

The authors have fairly addressed all my concerns, and the manuscript is suitable for publication. Congratulations to the authors.

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Rev_Com_number: RC-2026-03492

New_manu_number: EMBOR-2026-64553V1-T

Corr_author: Ernst

Title: Mammalian MemPrep establishes the lipid composition of ER membranes in HEK293T cells

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The revised manuscript is convincingly written and presents a careful interpretation of the data. I appreciate that the authors have adapted their conclusions based on the new results. The manuscript provides sufficient information and controls to assess the experiments in terms of reproducibility and clarity.

We thank the reviewer highlighting critical points and for the positive assessment our revised manuscript.

One minor point should be addressed regarding ether lipids: it is not clear from the Methods whether plasmalogens were analyzed separately, or whether they were grouped together with plasmalogen lipids without clear distinction.

We agree with the reviewer and have added the following clarification to the methods section (Mass spectrometry (MS) data acquisition for lipidomics):

'With respect to ether lipids, the analysis does not distinguish between plasmalogen and plasmalogen variants, because only the number but not the position of the double bonds relative to the ether bond can be determined via this shotgun approach.'

We also added the sentence: 'The shotgun analysis does not distinguish plasmalogen and plasmalogen lipids.' to the relevant Figure 6 and Supplementary Figure S4.

Please clarify this point. Also, in the legend for Figure 6 (line 1431) there is a typo at the very end of the end - I it should read: PE O- and not PC O-

We thank the reviewer for spotting this typo. We corrected it.

General assessment: The manuscript establishes MemPrep for mammalian cells as an important discovery tool for investigating how cells coordinate membrane lipid composition with membrane protein composition, and vice versa, a rapidly growing field that attracts considerable interest.

The results show that while the protein composition differs between SEC61 β - and REEP5-enriched fractions, their lipid composition is largely indistinguishable. Both fractions are dominated by PC alongside other unsaturated glycerophospholipids (but not PUFAs) and hydroxylated ceramides and ether lipids. These physicochemical properties of the ER lipid bilayer are matched by ER-resident membrane proteins.

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Audience: The manuscript will be of broad interest to researchers in all field of basic and more applied life sciences.

Again, we thank the reviewer for the efforts that helped us improving the manuscript.

Referee #2:

This all looks good to me, the edited changes are good and I think help with readability. The only outstanding item is Figure 2D; this is called out on L253, but the figure and legend only run to 2C. You could either refer to fig 2C in each case, or update the figure and legend to include reference to 2D.

Thank you for spotting that. We have removed the reference to the (non-existing) Figure 2D and consistently refer to Figure 2C.

Thank you for your time and efforts, which ultimately improved our manuscript.

Referee #3:

The authors have fairly addressed all my concerns, and the manuscript is suitable for publication. Congratulations to the authors.

Thank you for your time and efforts! We are very happy with the review process, which raised critical points and which helped us improving the manuscript.

Dear Prof. Ernst

Thank you for the submission of your revised manuscript to EMBO reports.

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

- As a standard procedure, we edit the title abstract of manuscripts to make them more accessible to a general readership. Please find the suggested version below my signature, which has been edited to meet our word limit requirement.
- Please update the order and the headings of the manuscript sections to: Abstract / Introduction / Results / Discussion / Methods / Data Availability / Acknowledgements / Disclosure and Competing Interests Statement / References / Figure Legends / Expanded View Figure Legends
- Please incorporate a statement on blinding in statistical analysis in the Methods section, even if no blinding was performed.
- CRediT has replaced the traditional author contributions section because it offers a systematic machine readable author contributions format that allows for more effective research assessment. Please remove the Author Contributions section from the manuscript and use the free text boxes beneath each contributing author's name in our online systems to add specific details on the author's contribution. More information is available in our guide to authors.
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The citation in the text should be: "(preprint: NAME1 et al, YEAR)"
The citation in the reference list: "Author NAME1, Author NAME2, ... (YEAR) article title. PLATFORM NAME, doi: XXX [PREPRINT]"
Please, refer to our detailed online guidelines for further information:
<https://www.embopress.org/page/journal/14602075/authorguide#referencesformat>
- Please adjust the in-text callouts for individual figures and figure panels: e.g. Fig. S3D, Suppl. Fig. 4A-J, Suppl. Table 1, Appendix Table 2, Appendix Table 3, Appendix Table 4 and Appendix Table appear to be missing.
- Please note that the specific URLs for datasets (PXD075836) are not provided in the data availability statement. This needs to be corrected.
- Our production/data editors have asked you to clarify several points in the figure legends (see below). Please incorporate these changes in the manuscript and return the revised file with tracked changes with your final manuscript submission.
 1. Please note that the legend for figure panel 5B is not provided in the manuscript. This needs to be rectified.
 2. Please note that the exact p values are not provided in the legends of figure 6A-C,E,F,G,H; EV-3A,B,C; EV-4B,C,F,I,K,L; EV-5B,C,D.
 3. Please indicate the statistical test used for data analysis in the legends of figures 3A,B; 5A; EV-1A,B.
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 - B) 2-5 short bullet points highlighting the key results, and
 - C) a synopsis image in .jpg or .png format that is exactly 550 pixels wide and 300 pixels high. Please note that the text needs to be legible at the final size. Please upload this information along with your revised manuscript (the text for A and B should be provided in one separate Word file uploaded as Cover Art/Synopsis Image).

Kurt Weir
Editor
EMBO Reports

The endoplasmic reticulum (ER) forms a dynamic network of sheets and tubules, whose lipid composition remains incompletely defined. Using an optimized MemPrep workflow, we establish a high-confidence lipidome of the mammalian ER and selectively enrich membrane vesicles originating from ER tubules as a major ER subdomain. Quantitative lipidomics show ER membranes are dominated by phosphatidylcholine and mono-unsaturated glycerophospholipids, consistent with a highly compressible bilayer. Although proteomics suggests a functional specialization of ER tubules and an enrichment of tubule-associated proteins, the lipidome of an ER tubule-enriched isolate is indistinguishable from the general ER, indicating that principal ER architectures share a common lipid composition. Integration of lipidomic data with bioinformatic analyses of transmembrane helices demonstrates that the physicochemical features of ER lipids mirror those of ER-resident membrane proteins, including reduced hydrophobicity and increased polarity compared to plasma membrane proteins. These findings support a coordinated evolution of ER proteins and lipids based on shared biophysical constraints. Together, this work provides a definitive characterization of the mammalian ER lipidome and suggests that membrane properties are maintained across the ER.

The authors have addressed all minor editorial requests.

Prof. Robert Ernst
Saarland University
Medical Biochemistry and Molecular Biology and Center of Biophysics
Kirrberger Str. 100
Building 48.5 R02.01.02
Homburg, Saarland 66421
Germany

Dear Robert,

My colleague Kurt Weir has meanwhile left EMBO reports and I took over the handling of your manuscript. I have now checked everything and all looks fine.

I am thus very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

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Best regards,

Martina

Martina Rembold, PhD
Senior Editor
EMBO reports

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Corresponding Author Name: Prof. Dr. Robert Ernst
Journal Submitted to: EMBO Reports
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Reporting Checklist for Life Science Articles (updated January

This checklist is adapted from Materials Design Analysis Reporting (MDAR) Checklist for Authors. MDAR establishes a minimum set of requirements in transparent reporting in the life sciences (see Statement of Task: [10.31222/osf.io/9sm4x](#)). Please follow the journal's guidelines in preparing your

Please note that a copy of this checklist will be published alongside your article.

Abridged guidelines for 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.
- ideally, figure panels should include only measurements that are directly comparable to each other and obtained with the same assay.
- plots include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical
- if n<5, the individual data points from each experiment should be plotted. Any statistical test employed should be justified.
- Source Data should be included to report the data underlying figures according to the guidelines set out in the authorship guidelines on Data

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 χ^2 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.

Please complete ALL of the questions below.

Select "Not Applicable" only when the requested information is not relevant for your study.

Materials

Newly Created Materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
New materials and reagents need to be available; do any restrictions apply?	Yes	Data availability section.
Antibodies	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
For antibodies provide the following information: - Commercial antibodies: RRID (if possible) or supplier name, catalogue number and or/clone number - Non-commercial: RRID or citation	Yes	Appendix Table S5
DNA and RNA sequences	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Short novel DNA or RNA including primers, probes: provide the sequences.	Yes	Appendix Table S2
Cell materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Cell lines: Provide species information, strain. Provide accession number in repository OR supplier name, catalog number, clone number, and/OR RRID.	Yes	Materials and Methods. Structured Methods - Reagents and Tools Table.
Primary cultures: Provide species, strain, sex of origin, genetic modification status.	Not Applicable	
Report if the cell lines were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Yes	Materials and Methods - Cell cultivation.
Experimental animals	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Laboratory animals or Model organisms: Provide species, strain, sex, age, genetic modification status. Provide accession number in repository OR supplier name, catalog number, clone number, OR RRID.	Not Applicable	
Animal observed in or captured from the field: Provide species, sex, and age where possible.	Not Applicable	
Please detail housing and husbandry conditions .	Not Applicable	
Plants and microbes	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Plants: provide species and strain, ecotype and cultivar where relevant, unique accession number if available, and source (including location for collected wild specimens).	Not Applicable	
Microbes: provide species and strain, unique accession number if available, and source.	Not Applicable	
Human research participants	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If collected and within the bounds of privacy constraints report on age, sex and gender or ethnicity for all study participants.	Not Applicable	
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If your work benefited from core facilities, was their service mentioned in the acknowledgments section?	Not Applicable	

Design

Study protocol	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
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Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	Not Applicable	

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Experimental study design and statistics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Include a statement about sample size estimate even if no statistical methods were used.	Not Applicable	
Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, have they been described?	Not Applicable	
Include a statement about blinding even if no blinding was done.	Not Applicable	
Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	Not Applicable	
If sample or data points were omitted from analysis, report if this was due to attrition or intentional exclusion and provide justification.		
For every figure, are statistical tests justified as appropriate? Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. Is there an estimate of variation within each group of data? Is the variance similar between the groups that are being statistically compared?	Yes	Figure 3-6. Figure EV1, Figure EV3-5.

Sample definition and in-laboratory replication	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
In the figure legends: state number of times the experiment was replicated in laboratory.	Yes	all relevant figure legends
In the figure legends: define whether data describe technical or biological replicates .	Yes	all relevant figure legends

Ethics

Ethics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Studies involving human participants : State details of authority granting ethics approval (IRB or equivalent committee(s), provide reference number for approval.	Not Applicable	
Studies involving human participants : Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	Not Applicable	
Studies involving human participants : For publication of patient photos , include a statement confirming that consent to publish was obtained.	Not Applicable	
Studies involving experimental animals : State details of authority granting ethics approval (IRB or equivalent committee(s), provide reference number for approval. Include a statement of compliance with ethical regulations.	Not Applicable	
Studies involving specimen and field samples : State if relevant permits obtained, provide details of authority approving study; if none were required, explain why.	Not Applicable	

Dual Use Research of Concern (DURC)	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Could your study fall under dual use research restrictions? Please check biosecurity documents and list of select agents and toxins (CDC): https://www.selectagents.gov/sat/list.htm .	Not Applicable	
If you used a select agent, is the security level of the lab appropriate and reported in the manuscript?	Not Applicable	
If a study is subject to dual use research of concern regulations, is the name of the authority granting approval and reference number for the regulatory approval provided in the manuscript?	Not Applicable	

Reporting

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Adherence to community standards	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
State if relevant guidelines or checklists (e.g., ICMJE, MIBBI, ARRIVE, PRISMA) have been followed or provided.	Not Applicable	
For tumor marker prognostic studies , we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	Not Applicable	
For phase II and III randomized controlled trials , please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	Not Applicable	

Data Availability

Data availability	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Have primary datasets been deposited according to the journal's guidelines (see 'Data Deposition' section) and the respective accession numbers provided in the Data Availability Section?	Yes	Data Availability Section
Were human clinical and genomic datasets deposited in a public access-controlled repository in accordance to ethical obligations to the patients and to the applicable consent agreement?	Not Applicable	
Are computational models that are central and integral to a study available without restrictions in a machine-readable form? Were the relevant accession numbers or links provided?	Not Applicable	
If publicly available data were reused, provide the respective data citations in the reference list .	Yes	Materials and Methods, Figures, Results