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Cardiolipin remodeling enables protein crowding in the inner mitochondrial membrane

Yang Xu, Hediye Erdjument-Bromage, Colin K. L. Phoon, Thomas A. Neubert, Mindong Ren, and Michael Schlame
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Corresponding author: Michael Schlame (michael.schlame@med.nyu.edu)

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

1st Editorial Decision **13th May 2021**

Dear Dr. Schlame,

Thank you for submitting your manuscript entitled "Cardiolipin remodeling enables protein crowding in the inner mitochondrial membrane" [EMBOJ-2021-108428] to The EMBO Journal. Your study has now been assessed by three reviewers, whose reports are enclosed below.

As you can see, the referees concur with us on the potential interest of your findings. However, they also raise several critical points that need to be addressed before they can support publication here.

Given the overall interest of your study, I am pleased to invite submission of a manuscript revised as indicated in the reports attached herein. I would like to point it out that addressing all referees' points in a conclusive manner, as well as a strong support from the reviewers, would be essential for publication in The EMBO Journal.

I should also add that it is our policy to allow only a single round of major revision. Therefore, acceptance of your manuscript will depend on the completeness of your responses in this revised version.

We generally grant three months as standard revision time. As we are aware that many laboratories cannot function at full capacity owing to the COVID-19 pandemic, we may relax this deadline. Also, we have decided to apply our 'scooping protection policy' to the time span required for you to fully revise your manuscript and address the experimental issues highlighted herein. Nevertheless, please inform us as soon as a paper with related content is published elsewhere.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File and will therefore be made available online. For more details on our Transparent Editorial Process, please visit our website: http://emboj.embopress.org/about#Transparent_Process

Before submitting your revised manuscript, deposit any primary datasets and computer code produced in this study in an appropriate public database (see http://msb.embopress.org/authorguide#dataavailability). Please remember to provide a reviewer password, in case such datasets are not yet public. The accession numbers and database names should be listed in a formal "Data Availability" section (placed after Materials & Method). Provide a "Data availability" section even if there are no primary datasets produced in the study.

I thank you again for the opportunity to consider this work for publication and look forward to your revision.

Best regards,

Elisabetta

Elisabetta Argenzio, PhD
Editor
The EMBO Journal

Instructions for preparing your revised manuscript:

Please make sure you upload a letter of response to the referees' comments together with the revised manuscript.

Please also check that the title and abstract of the manuscript are brief, yet explicit, even to non-specialists.

When assembling figures, please refer to our figure preparation guideline in order to ensure proper formatting and readability in print as well as on screen:
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- a point-by-point response to the referees' comments, with a detailed description of the changes made (as a word file).
- a word file of the manuscript text.
- individual production quality figure files (one file per figure)
- a complete author checklist, which you can download from our author guidelines (https://www.embopress.org/page/journal/14602075/authorguide).
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Revision to The EMBO Journal should be submitted online within 90 days, unless an extension has been requested and approved by the editor; please click on the link below to submit the revision online before 11th Aug 2021:

Link Not Available

Referee #1:

The work by Yang Xu et al entitled "Cardiolipin remodeling enables protein crowding in the inner mitochondrial membrane" addresses the question of how cardiolipin remodeling and protein crowding in the membrane are connected. Through a set of experiments in various model organisms and in vitro data, the authors demonstrate that cardiolipin remodeling by the attachment of various fatty acids plays an important role in permitting a tight packing of OXPHOS complexes in the inner membrane. Moreover, cardiolipin remodeling is shown to be important for establishment of normal mitochondrial ultrastructure. Overall, the manuscript is very well written and reports a conceptually highly interesting finding. The experimental strategies and presented data are solid and convincing, and the conclusions drawn are coherent. The authors should address a couple of minor points to improve the manuscript:

• The electron microscopic pictures in Figure 2A are difficult to evaluate, because the technical quality of the pictures is much lower compared to other presented micrographs (e.g. in Fig. 3A). The mitochondrial ultrastructure is de facto not visible in the shown pictures. Can the authors provide micrographs with higher quality?
• Some line graphs (e.g. Fig. 1D) depict data points without showing variances. In case the data was obtained from replicates, please indicate standard deviations or standard errors, respectively.
• For the sake of reproducibility, please indicate the OD values or time points which were chosen to define the yeast cultures as logarithmic or stationary. It would be good if the authors would also add data for cells cultivated on glycerol in logarithmic phase.
• It is unclear if the represented immunoblot in Figure S5 is showing data for WT or TAZKO. Please specify this in the figure or the respective legend. It would be optimal to show a representative immunoblot for both, WT and TAZKO next to each other in this figure, as it would allow a visualization of the presented quantification.
• Currently it is unclear whether the raw data will be available to the community. Ideally, the authors could upload them in a common repository.

Referee #2:

This is a very elegant and interesting study coming from experts in CL biology. The concept that protein crowding is requiring specific CL modifications is exciting and can explain some of the pathologies associated with impaired CL remodeling. For example, this can explain how CL remodeling deficient cells develop the impaired respiratory function. The figures are not less than awesome. The writing is clear. However, there is one conceptual issue that is not addressed. While it is clear that crowing the membrane increases remodeling, it is not clear if the lack of remodeling prevents the crowding directly or indirectly by affecting other processes such as ATP production, protein assembly, redox potential, pH, and temperature. In the current manuscript, there is no experiment showing that reduced remodeling prevents the crowding in a system where proteins are provided rather than produced and imported. In this context, it will be interesting to compare the abundance of mitochondria encoded to nuclear-encoded proteins. Perhaps one can design a system where proteins are supplied in abundance and then import and crowding are tested either in isolated mitochondria or introduced into cells by various approaches.
Specific points:
Figure 1. Can the authors add an illustration of the experiment?
Figure 1a. This illustration and its legends are not very informative. Please explain in the illustration the consequences of these changes.
Figure 1b. It is not clear what in vitro means. The legends only explain the measurement, not the model.
Figure 1e. It is not clear how CL% where calculated. I suggest f comes before d.
Figure 1i. What evidence is there that these different structures are indeed being formed?

Figure 2. It is not clear how the surface area was calculated from the Ems
I could not understand the rationale for choosing the different growth conditions. Please explain.
Figure 3. Since the distribution of the proteins within the membrane is not shown, how can the authors calculate the density based on WB without assuming even distribution, which is not likely to be the case?
Figure 4. It is not clear if the reduced protein crowdedness is due to CL deficiency or due to impaired bioenergetics. Not sure how this can be addressed at the organism level, but perhaps at a cellular level.
The description of the experiments presented in figure 4 is lacking. The criteria for defect detection and the quantifications are lacking clarity. A positive control will help too.
Figure 5. Please try to make the illustration self-explanatory. The "mismatch" is not shown in the illustration. Try showing the mismatch level by color-coding the match, maybe. A good illustration can be understood in 15 seconds without reading the legends (5 sec for New Yorkers).

Referee #3:

In the manuscript 'Cardiolipin remodeling enables protein crowding in the inner mitochondrial membrane' by Xu et al. the authors use a combination of in vitro and in vivo assays to analyze and interplay of the biosynthesize of the mitochondrial inner membrane lipid cardiolipin and protein crowding. The authors claim that high concentrations of inner membrane proteins, which would lead to protein crowding, changes the activity of the cardiolipin remodeling enzyme tafazzin. The crosstalk of lipids and proteins is, not only in mitochondria, an insufficient studied problem, which certainly would deserves the attention of the broader readership. Unfortunately, the claims of the manuscript are hardly backed-up by sufficient experimental proof.

Major concerns:
1. The in vitro approach is very important for the paper. Nonetheless, it is rather poorly conceptualized:
A. Why do the authors use two non-mitochondrial proteins (MBP and BR) to show an effect on tafazzin?
B. What do the authors mean by proteins available in a detergent-free state? How do they incorporate BR into vesicles if not by detergents? Why is that important as they reconstitute triton solubilized tafazzin anyway?
C. Can they exclude residual triton molecules within the vesicles?
D. Why do increasing amounts of BR, which by the authors definition is protein crowding, do not lead to increased tafazzin activity?
E. What is the basis for the cartoons of BR showing completely none hydrophobic mismatch while OXPHOS complexes do?
F. Can the authors exclude a direct effect of MBP on tafazzin?
G. Does MBP induce membrane curvature and could that lead to tafazzin activity rather than a protein crowding effect?

2. Page 5, first paragraph, Importantly...: It is an interesting observation but it would have to be shown that this is true for the entire IM protein content. For example, purify IM at different states and compare lipid and overall protein levels there. Otherwise, it is an over interpretation.

3. Page 5, second last paragraph, We hypothesize...: The experimental basis for this hypothesis is missing. Measurements would need to be included to confirm this premise. Membrane stiffness measurements of bilayers or vesicles with different amount of different CL species would need to be performed. Otherwise, it could go either way that increased CL stresses the membrane on its own or that it reduces stress

4. Table 1: Relative total CL abundance could be quantified in WT and TAZKO to show how much CL is lost

5. Page 6, No such effect was observed...: MICOS is not an outer membrane but an inner membrane complex. The missing change in MICOS levels might point into the direction that the lipid protein effect is more specific to CL and OXPHOS and not as broad as claimed in the title.

6. Page 6, In summary, TAZKO reduced...: Yes, but that's nothing new. CL is necessary for stability of OXPHOS complexes is the main conclusion that can be drawn and this was known before - TAZKO leads to degradation of CL --> less CL --> less proteins. What's more interesting is the shift from saturated to unsaturated CL, which could be discussed to support the hypothesis as to why CL is necessary for protein stability.

7. Page 8, As a result, remodeled CL...: This is certainly an interesting idea but as it is, it is not backed up by in vitro experiment.

8. Page 8, Using diverse experimental...: Protein crowding leading to CL remodeling only showed with liposomes and with yeast

Minot points:
1. Introduction, end of first paragraph. This would be a really important point on which the strategy of this manuscript is build on. Why do the authors only cite two review articles here?
2. Introduction: Our hypothesis is based on... - What is the hypothesis at that point?

3. Whereas in the text MBP is defined as myelin basic protein in the figure it is defined as myelin binding protein
4. Page 4, However, it is not straightforward... One would think that tight transcriptional and translational control would enable manipulation rather than make it difficult.

Referee #1:

The work by Yang Xu et al entitled "Cardiolipin remodeling enables protein crowding in the inner mitochondrial membrane" addresses the question of how cardiolipin remodeling and protein crowding in the membrane are connected. Through a set of experiments in various model organisms and in vitro data, the authors demonstrate that cardiolipin remodeling by the attachment of various fatty acids plays an important role in permitting a tight packing of OXPHOS complexes in the inner membrane. Moreover, cardiolipin remodeling is shown to be important for establishment of normal mitochondrial ultrastructure. Overall, the manuscript is very well written and reports a conceptually highly interesting finding. The experimental strategies and presented data are solid and convincing, and the conclusions drawn are coherent. The authors should address a couple of minor points to improve the manuscript:
RESPONSE: We would like to thank the reviewer for this evaluation and for all suggestions to improve the manuscript. We addressed the specific criticism as detailed below.

• The electron microscopic pictures in Figure 2A are difficult to evaluate, because the technical quality of the pictures is much lower compared to other presented micrographs (e.g. in Fig. 3A). The mitochondrial ultrastructure is de facto not visible in the shown pictures. Can the authors provide micrographs with higher quality?
RESPONSE & REVISION: Because of the relative impermeability to fixatives, electron micrographs of yeast samples were of lower quality than electron micrographs of mouse and fly samples. This problem has also been encountered by other researchers (Wright: Transmission electron microscopy of yeast, MICROSCOPY RESEARCH AND TECHNIQUE 51:496 –510, 2000). To better document the changes in ultrastructure under different growth conditions, we selected more electron micrographs and moved these data into Figure EV1. We limited the extent of the yellow labels in order to show the unobscured internal structure of the mitochondria. The new micrographs demonstrate that mitochondria can be recognized by their double membrane and that cristae are present in YPDstat and YPGEstat but not in YPDlog.

• Some line graphs (e.g. Fig. 1D) depict data points without showing variances. In case the data was obtained from replicates, please indicate standard deviations or standard errors, respectively.
RESPONSE & REVISION: The data of Figure 1F of the revised manuscript (corresponding to Figure 1d of the original submission) were obtained from duplicate measurements. We revised the figure to include the individual measurements, from which the standard error (range) is evident. In all other revised figures, individual measurements are displayed together with mean values and standard deviations. In Figures 1C, 1D, 1I, and 2E, regression curves are shown instead of mean values.

• For the sake of reproducibility, please indicate the OD values or time points which were chosen to define the yeast cultures as logarithmic or stationary. It would be good if the authors would also add data for cells cultivated on glycerol in logarithmic phase.
RESPONSE & REVISION: In the Methods section of the revised manuscript, under Yeast, we added the missing information: "Cells were harvested either in the logarithmic (OD=0.6-1.3) or in the stationary (OD>3) phase of growth. Repeated OD measurements were made to confirm that the cultures no longer expanded during the stationary phase." We did not include cells cultivated on glycerol in the logarithmic phase (YPGE log) because we had found in preliminary experiments (PNAS 116:11235-11240, 2019) that the CL composition of YPGE log cells was in between that of YPD stat cells and YPGE stat cells. The latter two are relatively close (Fig 2E), which seemed to make it redundant to add the YPGE log data.

• It is unclear if the represented immunoblot in Figure S5 is showing data for WT or TAZKO. Please specify this in the figure or the respective legend. It would be optimal to show a representative immunoblot for both, WT and TAZKO next to each other in this figure, as it would allow a visualization of the presented quantification.
RESPONSE & REVISION: The figure was revised to show a representative immunoblot of both WT and TAZKO (Figure EV4).

• Currently it is unclear whether the raw data will be available to the community. Ideally, the authors could upload them in a common repository.
RESPONSE & REVISION: The raw data were uploaded to the public database MassIVE (https://massive.ucsd.edu/ProteoSAFe/static/massive.jsp). Access information is provided in the Data Availability section in accordance with EMBO J requirements (Username: MSV000087602, Password: a).

Referee #2:

This is a very elegant and interesting study coming from experts in CL biology. The concept that protein crowding is requiring specific CL modifications is exciting and can explain some of the pathologies associated with impaired CL remodeling. For example, this can explain how CL remodeling deficient cells develop the impaired respiratory function. The figures are not less than awesome. The writing is clear. However, there is one conceptual issue that is not addressed. While it is clear that crowing the membrane increases remodeling, it is not clear if the lack of remodeling prevents the crowding directly or indirectly by affecting other processes such as ATP production, protein assembly, redox potential, pH, and temperature. In the current manuscript, there is no experiment showing that reduced remodeling prevents the crowding in a system

where proteins are provided rather than produced and imported. In this context, it will be interesting to compare the abundance of mitochondria encoded to nuclear-encoded proteins. Perhaps one can design a system where proteins are supplied in abundance and then import and crowding are tested either in isolated mitochondria or introduced into cells by various approaches.

RESPONSE & REVISION: We thank the reviewer for these comments and for the suggestion to compare proteins encoded by mitochondria to proteins encoded by the nucleus. We performed an additional analysis of the proteomics data, which demonstrated that nuclear OXPHOS subunits (requiring import + assembly) and mitochondrial OXPHOS subunits (requiring assembly only) are equally affected by impaired CL remodeling (see specific point below and Figure EV5 of the revised manuscript). This result supports the idea that remodeling affects the incorporation of proteins into the membrane. Of course, due to the interconnectedness of synthesis, import, and assembly and due to regulatory interactions between OXPHOS subunits, we cannot definitely exclude indirect effects. Tight control of mitochondrial protein import (Eisenberg-Bord & Schuldiner, Ground control to major TOM: mitochondria–nucleus communication, FEBS J 284:196–210, 2017) unfortunately precludes an over-supply of proteins to mitochondria. These limitations are stressed in the revised manuscript (4th paragraph of the Discussion).

Specific points:

Figure 1. Can the authors add an illustration of the experiment?

RESPONSE & REVISION: Figure 1 was revised thoroughly in response to several points raised by the 3 referees. A simple illustration of the experiment was added (Fig. 1A). The illustration no longer shows the suggested re-arrangements of the lipid packing because they were inferred from the literature but not directly demonstrated by the present experiments.

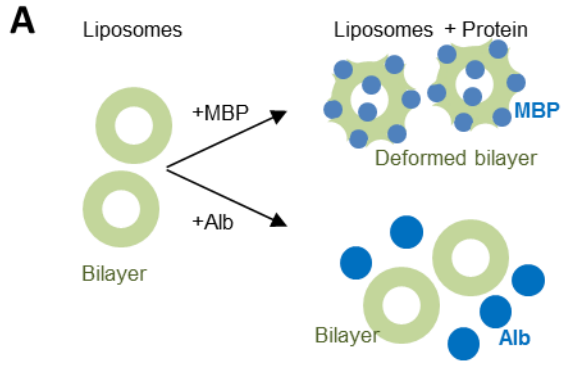


Figure 1a. This illustration and its legends are not very informative. Please explain in the illustration the consequences of these changes.

RESPONSE & REVISION: Figure 1a of the original manuscript was replaced by a new Fig 1A (see above). This illustration was designed to show that MBP (i) binds to liposomes and (ii) deforms the shape of the lipid bilayer. It also shows that albumin (Alb) does not bind to liposomes and therefore leaves the bilayer unaltered. The legend of Figure 1 was revised to include a better description of the experiment.

Figure 1b. It is not clear what in vitro means. The legends only explain the measurement, not the model.

RESPONSE & REVISION: The legend was revised to explain the experiment. It reads: "Myelin basic protein (MBP) or albumin (Alb) were added to liposomes that contained purified tafazzin (TAZ). Acyl

transfer reactions were measured by mass spectrometry."

Figure 1e. It is not clear how CL% where calculated. I suggest f comes before d.
RESPONSE & REVISION: CL% was calculated as the relative amount of the two CL species (percent of total CL). However, this figure was deleted because Fig 1F of the revised manuscript shows the chemical amounts of the two CL species, which makes the CL% data redundant. The reaction scheme was placed before the data as suggested by the reviewer.

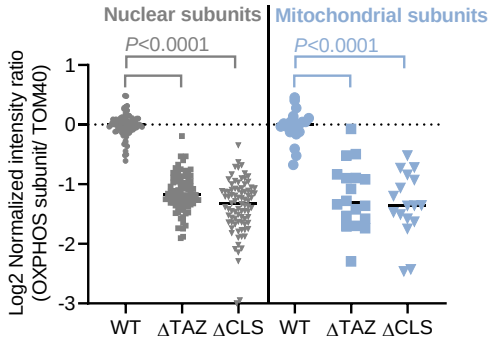
Figure 1i. What evidence is there that these different structures are indeed being formed?
RESPONSE & REVISION: The structures shown in the original manuscript were supported by the literature. Specifically, it has been shown that calcium converts CL bilayers into hexagonal structures and that transmembrane proteins convert hexagonal structures back into bilayers (e.g. Taraschi TF, de Kruijff B, Verkleij AJ: The effect of an integral membrane protein on lipid polymorphism in the cardiolipin-Ca2+ system. Eur J Biochem 129:621-625, 1983). Nevertheless, the scheme was deleted because we did not confirm these structures by our own experiments. The point we wish to make is that membrane proteins alter the packing order of lipids, not how the packing looks like.

Figure 2. It is not clear how the surface area was calculated from the Ems
I could not understand the rationale for choosing the different growth conditions. Please explain.
RESPONSE & REVISION: In the Methods section of the revised manuscript, under Transmission electron microscopy, we added the following explanation: "To determine IM/OM surface area ratios, the OM and IM were traced in randomly collected electron micrographs using the software package Image J. The ratio was calculated by dividing the sum of all IM perimeters (cristae plus inner boundary membrane) by the OM perimeter." In Results (page 5, first paragraph), we added a justification for choosing the growth conditions: "These conditions were chosen to produce a progressive shift from the fermentative to the respiratory state because respiration is higher in the stationary than in the logarithmic phase and is higher in glycerol/ethanol than in dextrose."

Figure 3. Since the distribution of the proteins within the membrane is not shown, how can the authors calculate the density based on WB without assuming even distribution, which is not likely to be the case?
RESPONSE & REVISION: We completely agree with the reviewer. An even distribution of proteins is unlikely. Therefore, our densities represent weighted averages of different membrane domains. To stress that point, we revised the last paragraph on page 4: "To determine the protein concentration of the IM, we measured the surface area of the IM by electron microscopy and the abundance of IM proteins (OXPHOS complexes and solute carriers) by mass spectrometry using label-free quantitation. These data permitted the calculation of relative values that were proportional to the average surface density of proteins in the IM."

Figure 4. It is not clear if the reduced protein crowdedness is due to CL deficiency or due to impaired bioenergetics. Not sure how this can be addressed at the organism level, but perhaps at a cellular level.
The description of the experiments presented in figure 4 is lacking. The criteria for defect detection and the quantifications are lacking clarity. A positive control will help too.
RESPONSE & REVISION: This criticism corresponds to the main point expressed by the reviewer (see our response above). If impaired bioenergetics was the cause of the reduced crowdedness, nuclear subunits should be more affected than mitochondrial subunits because the former require an additional energy-dependent step, which is protein import. To test this, we compared mitochondrial OXPHOS subunits to

nuclear OXPHOS subunits and found that they were equally affected by impaired CL remodeling (Figure EV5 of the revised manuscript). This supports the idea that the lack of remodeling prevents crowding directly by inhibiting the incorporation of proteins into the membrane rather than indirectly by impairing bioenergetics.



To improve the description of the experiment in Fig 4, we revised the first paragraph of page 7: "To test the effect of tafazzin on IM proteins in a different model, we created a tafazzin mutant (ΔTAZ) in *Drosophila melanogaster*. We studied mitochondria of indirect flight muscles because, like mouse heart, it is a tissue with very high OXPHOS capacity. We compared ΔTAZ with the wild-type and with another mutant, in which CL was deleted (inactivation of CL synthase, ΔCLS) in order to confirm that CL was indeed involved in the mechanism. Again, we measured IM surface area and IM protein abundance, and we analyzed the mitochondrial morphology in the mutants and the wild-type." Also, the quantification of defects was described in the last paragraph of the revised Methods section: "In the Drosophila experiments, mitochondrial cross sections were classified as lamellar (parallel longitudinal cristae), tubular (circular cross-sectional cristae), or defective (vacuole-like areas). The areas of these sections were quantified in Image J."

Figure 5. Please try to make the illustration self-explanatory. The "mismatch" is not shown in the illustration. Try showing the mismatch level by color-coding the match, maybe. A good illustration can be understood in 15 seconds without reading the legends (5 sec for New Yorkers).
RESPONSE & REVISION: Thank you for the suggestion (and for the trust expressed in New Yorkers). We agree that conceptual figures should be self-explanatory. The figure was completely re-drawn to illustrate the mismatch and to enhance clarity.

Referee #3:

In the manuscript 'Cardiolipin remodeling enables protein crowding in the inner mitochondrial membrane' by Xu et al. the authors use a combination of in vitro and in vivo assays to analyze and interplay of the biosynthesize of the mitochondrial inner membrane lipid cardiolipin and protein crowding. The authors claim that high concentrations of inner membrane proteins, which would lead to protein crowding, changes the activity of the cardiolipin remodeling enzyme tafazzin. The crosstalk of lipids and proteins is, not only in mitochondria, an insufficient studied problem, which certainly would deserves the attention of the broader readership. Unfortunately, the claims of the manuscript are hardly backed-up by sufficient experimental proof.
RESPONSE & REVISION: We appreciate the comments of the reviewer. The manuscript was revised

extensively in order to address all points. We hope that the revised paper gives a more nuanced account of the subject and that the overall presentation is more convincing than in the previous version.

Major concerns:

1. The in vitro approach is very important for the paper. Nonetheless, it is rather poorly conceptualized:

A. Why do the authors use two non-mitochondrial proteins (MBP and BR) to show an effect on tafazzin?

RESPONSE & REVISION: The in vitro approach was meant to be a proof of principle. We wanted to determine whether it is possible in general for membrane proteins to affect the tafazzin reaction and not whether this is a specific property of mitochondrial proteins. Mitochondrial proteins were not chosen because they are not commercially available and they contain large amounts of detergents when isolated from tissue, which is problematic for our experiments (see below). MBP and BR were selected because (i) they can be purchased in high quantity at high purity, (ii) they are known to alter the packing state of lipids, and (iii) they can interact with lipids in the absence of detergents. The manuscript was revised to explain why non-mitochondrial proteins were selected: "…. the question arises as to whether membrane proteins, by affecting the physical state of lipids, are able to alter quality and quantity of tafazzin-catalyzed transacylations. This question is not specific to mitochondrial proteins but applies to all membrane proteins that may interfere with the packing order of lipids. Here we determined the effect of myelin basic protein (MBP) and bacteriorhodopsin (BR) because they have a strong influence on their lipid environment (Botelho et al 2006; Epand & Moscarello, 1982; Verchère et al 2017) and because they are among the few membrane proteins available in a detergent-free state." (first paragraph of Results).

B. What do the authors mean by proteins available in a detergent-free state? How do they incorporate BR into vesicles if not by detergents? Why is that important as they reconstitute triton solubilized tafazzin anyway?

RESPONSE & REVISION: Since detergents activate the tafazzin reaction, we kept the Triton concentration low during the purification of tafazzin. As a result, our tafazzin preparation contained only 79 molecules of Triton per enzyme molecule (Nature Chem Biol 8:862-869, 2012; JBC 292:5499-5506, 2017). Since we added 1 molecule of enzyme per 2000 molecules of lipids, our reaction mixtures contained 4 molecules of Triton X-100 per 100 lipid molecules. Triton in such low concentration does not affect the bilayer state of lipids (Dennis 1974, Arch Biochem Biophys 165:764-773). Consistent with that, the reaction could be activated by exogenous Triton (Fig 1G). Bacteriorhodopsin (BR) was added to inverted micelles in the absence of detergents, which had a strong effect on the quantity and quality (species composition) of the reaction products. In preliminary studies, we had tried to reconstitute bacteriorhodopsin (BR) with detergents (β-octylglucoside or Triton X-100) but we were unable to separate the protein effect from the detergent effect. Consequently, these experiments were not included in the paper. We revised the manuscript to specify the Triton X-100 concentration of the enzyme preparation (first paragraph of the Methods section).

C. Can they exclude residual triton molecules within the vesicles?

RESPONSE & REVISION: The residual Triton concentration in the reaction mixtures was 40 μM and the Triton-to-phospholipid ratio was 1:25 (see above), which is way below the threshold for the transition into the micellar state (Dennis 1974, Arch Biochem Biophys 165:764-773).

D. Why do increasing amounts of BR, which by the authors definition is protein crowding, do not lead to increased tafazzin activity?

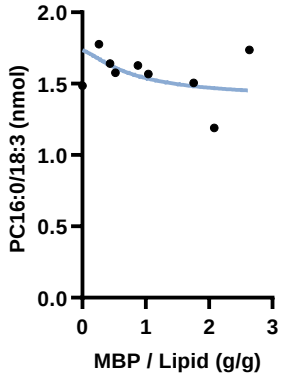
RESPONSE & REVISION: Different proteins had different effects: MBP increased transacylations in the bilayer state whereas BR decreased transacylations in the hexagonal state. The data support the notion that crowding alters the qualitative and quantitative outcome of the tafazzin reaction (BR not only suppressed the amount but also changed the composition of the reaction products) and that the effect is dependent on the lipid phase state and the type of protein. The data do not suggest that crowding always increases the extent of the tafazzin reaction. We speculate that BR suppresses the reaction by preventing the hexagonal phase state (Taraschi et al, Eur J Biochem 129:621-625, 1983), but we concede that our data do not provide sufficient evidence to make that claim. To make these points clearer, we revised the 6th paragraph of the Discussion: "While in their entirety our data support the conclusion that tafazzin is sensing membrane stress, different proteins (MBP and BR) had opposite effects and our study was not designed to elucidate the underlying mechanisms."

E. What is the basis for the cartoons of BR showing completely none hydrophobic mismatch while OXPHOS complexes do?

RESPONSE & REVISION: We agree that we do not have sufficient information about the extent of hydrophobic mismatch between BR and lipids in our experiments. Thank you for pointing out this flaw. The cartoon was removed.

F. Can the authors exclude a direct effect of MBP on tafazzin?

RESPONSE & REVISION: MBP was only effective at high concentrations, at which it was in large excess over tafazzin (>100 molecules of MBP per molecule of tafazzin). This makes a direct effect on tafazzin very unlikely. However, to address this issue more rigorously, we performed an additional experiment where we tested the effect of MBP on the tafazzin reaction in micelles (85% LPC + 15% PC). MBP had no effect on the micellar reaction, suggesting that MBP did not change the activity of tafazzin. These data were added in Fig. 1D to the revised manuscript.



The fact that MBP had an effect on liposomes but not on micelles confirms that the physical state of lipids and not the enzyme activity is critical for the MBP mechanism of action.

G. Does MBP induce membrane curvature and could that lead to tafazzin activity rather than a protein crowding effect?

RESPONSE & REVISION: Yes, we think that the induction of curvature is a possible explanation. However, the biophysical literature shows that the curvature mechanism and the crowding mechanism are not mutually exclusive (e.g. Brown: Soft matter in lipid-protein interactions. Annu Rev Biophys 46:379-410, 2017). Figure 5 was revised to better illustrate how protein crowding may induce membrane curvature.

2. Page 5, first paragraph, Importantly...: It is an interesting observation but it would have to be shown that this is true for the entire IM protein content. For example, purify IM at different states and compare lipid and overall protein levels there. Otherwise, it is an over interpretation.
RESPONSE & REVISION: We agree. The term "IM proteins" was substituted by the more specific term "solute carriers and OXPHOS proteins". In the revised manuscript the paragraph reads: "By tracing mitochondrial membranes in electron micrographs, we determined that the IM/OM area ratio also increased from YPD log to YPD stat but not any further from YPD stat to YPGE stat (Figure 2A). Finally, we found that the relative abundance of solute carriers and OXOHOS proteins increased from YPD log to YPD stat and then increased further from YPD stat to YPGE stat (Figure 2B). Importantly, the abundance of carriers and OXPHOS proteins rose by a much larger factor than the IM surface area, suggesting that their concentration was higher in YPD stat than in YPD log and even higher in YPGE stat." (p. 5, first paragraph in the revised manuscript). The suggested experiment would only be useful if we were able to isolate inner membranes consistently with very high purity from yeast at different growth stages. This would be very difficult because yeast mitochondria are contaminated with other organelles and the degree of contamination is expected to vary in different growth stages because of changes in the composition of intracellular membranes.

3. Page 5, second last paragraph, We hypothesize...: The experimental basis for this hypothesis is missing. Measurements would need to be included to confirm this premise. Membrane stiffness measurements of bilayers or vesicles with different amount of different CL species would need to be performed. Otherwise, it could go either way that increased CL stresses the membrane on its own or that it reduces stress
RESPONSE & REVISION: We agree that this is not sufficiently supported by experimental evidence. The sentence was deleted. In the revised manuscript, this section is introduced by the following, which avoids any reference to mechanistic speculations: "If protein crowding induces CL remodeling, remodeled CL may confer an advantage to protein-crowded membranes. If that is true, genetic ablation of the remodeling enzyme tafazzin (TAZKO) may prevent the membrane from reaching a high protein concentration. To test this conjecture, we ….."

4. Table 1: Relative total CL abundance could be quantified in WT and TAZKO to show how much CL is lost
RESPONSE & REVISION: We revised Table 1 to include the relative total CL abundance as suggested by the reviewer.

5. Page 6, No such effect was observed...: MICOS is not an outer membrane but an inner membrane complex. The missing change in MICOS levels might point into the direction that the lipid protein effect is more specific to CL and OXPHOS and not as broad as claimed in the title.
RESPONSE & REVISION: We apologize for the ambiguous wording. We did not mean to imply that MICOS is an outer membrane protein. The sentence "No such effect was observed on proteins residing in other mitochondrial compartments, specifically not on the OM-associated VDAC, the mitochondrial contact site and cristae organizing system (MICOS), or matrix proteins (Figure 3e)" was revised to "No such effect was observed on proteins residing in the OM, cristae junctions, or the matrix (Figure 3E)." We also revised Figures 3 & 4, in which MICOS was mislabeled as OM protein. We agree with the reviewer that we specifically observed effects on OXPHOS proteins and carriers rather than on IM proteins in general. A review of the literature shows that OXPHOS proteins make up the majority of IM proteins (Table I in BBA

Bioenergetics 1862:148305, 2021) and therefore are most responsible for protein crowding. However, for the sake of accuracy, we limited our claim to OXPHOS proteins in the revised manuscript. The revised abstract reads: "In vitro, the incorporation of large amounts of proteins into liposomes altered the outcome of the remodeling reaction. In yeast, the concentration of proteins of oxidative phosphorylation (OXPHOS) correlated with the CL composition. Genetic ablation of either CL remodeling or CL biosynthesis caused a substantial drop in the surface density of OXPHOS proteins in the inner membrane of mouse heart and Drosophila flight muscle mitochondria. Our data suggest that OXPHOS protein crowding induces CL remodeling and vice versa remodeled CL supports the high concentration of these proteins in the inner mitochondrial membrane." In the results section, we revised the concluding sentences: "In summary, TAZKO reduced the mitochondrial concentration of OXPHOS proteins and carriers but not the IM surface area." (p. 6) and "Taken together, our data demonstrate a drop in the surface density of IM proteins in mutant mitochondria, with little difference between ΔTAZ and ΔCLS. This conclusion follows from the reduced abundance of OXPHOS proteins and carriers in the face of an unchanged IM surface area." (p7).

6. Page 6, In summary, TAZKO reduced...: Yes, but that's nothing new. CL is necessary for stability of OXPHOS complexes is the main conclusion that can be drawn and this was known before - TAZKO leads to degradation of CL --> less CL --> less proteins. What's more interesting is the shift from saturated to unsaturated CL, which could be discussed to support the hypothesis as to why CL is necessary for protein stability.
RESPONSE & REVISION: It was known that CL promotes the assembly of OXPHOS complexes to supercomplexes but not that CL is necessary to achieve a high concentration of OXPHOS proteins in the membrane. Also our data support a novel mechanism by which CL affects OXPHOS protein abundance, which is mitigation of crowding stress. We therefore believe that the information in our manuscript is novel. However, we followed the suggestion of the reviewer and expanded the discussion on the role of unsaturated versus saturated CL: " … CL … is therefore ideally suited to mitigate the stress of crowding in mitochondrial IMs. Remodeled (unsaturated) CL is better equipped for this function than non-remodeled (saturated) CL because it has higher intrinsic curvature (Sankaram et al, 1989). The high intrinsic curvature of remodeled CL is likely to reduce the energy required for lipid bending (Figure 5D)." (4th paragraph of the Discussion in the revised manuscript).

7. Page 8, As a result, remodeled CL...: This is certainly an interesting idea but as it is, it is not backed up by in vitro experiment.
RESPONSE & REVISION: We agree that this is speculative. The sentence ("As a result, remodeled CL creates the necessary flexibility to allow local adaptations to the shape of proteins.") was revised: "Furthermore, the acyl chains of remodeled CL may be more flexible than the acyl chains of non-remodeled CL and therefore might theoretically adapt better to the shape of proteins." (4th paragraph of the Discussion in the revised manuscript).

8. Page 8, Using diverse experimental...: Protein crowding leading to CL remodeling only showed with liposomes and with yeast
RESPONSE & REVISION: We agree. The sentence ("Using diverse experimental models, including liposomes, yeast, flies, and mice, we observed robust effects in either direction") was removed.

Minot points:
1. Introduction, end of first paragraph. This would be a really important point on which the strategy of this manuscript is build on. Why do the authors only cite two review articles here?

RESPONSE & REVISION: We agree that this is an important foundation of our work, one that is well established in the literature. More citations were added (Lewis & McElhany, 2009; Mårtensson et al, 2017; Mileykovskaya & Dowhan, 2009; Pennington et al, 2019). We apologize in advance if the list of review articles is incomplete.

2. Introduction: Our hypothesis is based on... - What is the hypothesis at that point?
RESPONSE & REVISION: The hypothesis was made more explicit in the revised sentence: "The idea that the protein content of the IM requires CL remodeling is based on four premises:" (4th paragraph of the Introduction)

3. Whereas in the text MBP is defined as myelin basic protein in the figure it is defined as myelin binding protein
RESPONSE & REVISION: Thank you for catching this error. It is of course myelin basic protein. The error was corrected.

4. Page 4, However, it is not straightforward... One would think that tight transcriptional and translational control would enable manipulation rather than make it difficult.
RESPONSE & REVISION: We agree that the sentence ("However, it is not straightforward to manipulate the protein concentration of mitochondria because their protein expression is under tight transcriptional and translational control.") is misleading. Mitochondria tightly regulate protein import, which precludes arbitrary increases in the expression levels of IM proteins. The sentence was changed: "However, it is not straightforward to increase the protein concentration of mitochondria because tight import regulation prevents the over-accumulation of proteins (Friedman & Nunnari, 2014; Harbauer et al, 2014)."

Dear Dr Schlame,

Thank you for submitting your revised manuscript (EMBOJ-2020-108428R) to The EMBO Journal. Please accept again our sincere apologies for getting back to you with this unusual delay due to protracted reviewer input. Your amended study was sent back to all referees for re-evaluation and we have received re-reports from two of them, whose comments I enclose below. Please note that while reviewer #2 was at this time not able to reassess the work, we have carefully considered your response to this expert editorially, and found the critique raised to be satisfactorily addressed. As you will see, the other reviewers stated that their issues have been reasonably considered and they are now in favour of publication.

Thus, we are pleased to inform you that your manuscript has been accepted in principle for publication in The EMBO Journal.

We need you to take care of a number of minor points related to formatting and data representation as detailed below, which should be addressed at re-submission.

Please contact me at any time if you have additional questions related to below points.

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Again, please contact me at any time if you need any help or have further questions.

Kind regards,

Daniel Klimmeck

Daniel Klimmeck PhD
Senior Editor
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****

Formatting changes required for the revised version of the manuscript:

>> Please limit keywords to maximally five and add a 'Conflict of Interest' section to your manuscript.

>> Add a 'Statistical analysis' section to the Material & Methods, detailing the algorithms applied.

>> Clarify number and type of replicates integrated into Figures 1F and 1I by amending the figure legend.

>> Dataset EV legends: Table 1 needs renaming to 'Table EV1'. Please adjust callouts in text and legends accordingly.

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Referee #1:

The authors did a great job to clarify the few points we had on the initial version. A really interesting study!

Referee #3:

The authors made an effort to answer all points raised by the reviewers. I acknowledge that I wasn't as positive as the other two reviewers from the beginning and in part some of my criticism stands. This is also due to the fact that many issues were discussed rather than experimentally addressed (of course many of the experiments would have been quite difficult and time consuming). Nonetheless, with the changes made I do think that the paper includes an interesting concept that certainly should be out there for discussion. I'm therefor happy to recommend publication.

The author performed the requested editorial changes.

Dear Dr Schlame,

Thank you for submitting the revised version of your manuscript. We have now evaluated your amended manuscript and concluded that the remaining minor concerns have been sufficiently addressed.

Thus, I am pleased to inform you that your manuscript has been accepted for publication in the EMBO Journal.

Please note that it is EMBO Journal policy for the transcript of the editorial process (containing referee reports and your response letter) to be published as an online supplement to each paper.

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On a different note, I would like to alert you that EMBO Press is currently developing a new format for a video-synopsis of work published with us, which essentially is a short, author-generated film explaining the core findings in hand drawings, and, as we believe, can be very useful to increase visibility of the work. This has proven to offer a nice opportunity for exposure i.p. for the first author(s) of the study. Please see the following link for representative examples and their integration into the article web page:
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Please let me know, should you be interested to engage in commissioning a similar video synopsis for your work. According operation instructions are available and intuitive.

If you have any questions, please do not hesitate to call or email the Editorial Office.

Thank you again for this contribution to The EMBO Journal and congratulations on a successful publication! Please consider us again in the future for your most exciting work.

Kind regards,

Daniel Klimmeck

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The EMBO Journal
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PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

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

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

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

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

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1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Sample size was chosen on the basis of the difference of the mean and the standard deviation
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	We chose the minimal sample size to ensure statistical significance
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No animals had to be excluded from the analysis
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	No
For animal studies, include a statement about randomization even if no randomization was used.	No formal randomization was used.
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	No
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6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	NDUFB6 antibody: Anti-NDUFB6 antibody [21C11BC11] (ab110244) (Abcam); UQCRC2 antibody: Anti-UQCRC2 antibody [13G12AF12BB11] (ab14745) (Abcam); ATP5A1 antibody: Anti-ATP5A antibody [15H4C4] - Mitochondrial Marker (ab14748) (Abcam); TOM70 antibody: Cat #PA5-82508 (Invitrogen)
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	The S. cerevisiae cell line was obtained from the laboratory of Dr. ML Greenberg (Wayne State Univ, Detroit). The P. pastoris cell line was obtained from Invitrogen. Mycoplasma infections are not a common problem in yeast cultures.

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D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	Mice: Mice were housed under temperature-controlled conditions under a 12-hour light/dark cycle with free access to drinking water and food. The TAZKO mouse model was developed by D. Strathdee of the Cancer Research UK Beatson Institute (Glasgow, UK). In this model, two loxP sites flank exons 5–10 of the TAZ gene. We obtained floxed sperm in order to generate the KO model by Cre recombination. The model was maintained on a C57BL/6 background. TAZKO (Taz-/y) male mice were generated from crosses between wild type (Taz+/y) males and heterozygote (Taz+/-) females. Flies: Strain w1118; PBac{PB} CG4774c01874/TM6B, Tb1, with a transposon insertion in the coding region of the last exon of the cardiolipin synthase gene (ΔCLS), was obtained from the Bloomington Drosophila Stock Center (No. 10741). The tafazzin mutant (ΔTAZ) and the precise-excision control (WT) were created in our laboratory (Xu et al, 2006b). To avoid confounding effects, we re-derived all strains in identical genetic backgrounds. To this end, the ΔCLS and ΔTAZ alleles were backcrossed to the WT control background for 6 generations. The ΔCLS allele was identified by the mini-w eye color. The ΔTAZ allele was identified by PCR genotyping with the primers: 5′-GTC CAA ACA TCA GTT GGA TG-3′ and 5′-GAA GTA GTT AGG CAA CAG TC-3′, which yields a 230 bp DNA fragment. The isogenic strains were re-balanced by crossing with w;Sco/CyO;Sb/TM6B. Fly culture and crosses were performed on a standard fly food containing yeast, cornmeal, and molasses in 3-inch culture vials at 24 °C.
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E- Human Subjects

11. Identify the committee(s) approving the study protocol.	Not applicable
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F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	Proteomics data are available in MAssIVE: https://massive.ucsd.edu/ProteoSAFe/static/massive.jsp, accession number MSV000087602
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