THE EMBO JOURNAL

Cristae formation is a mechanical buckling event controlled by the inner mitochondrial membrane lipidome

Kailash Venkatraman, Christopher Lee, Guadalupe Garcia, Arijit Mahapatra, Daniel Milshteyn, Guy Perkins, Keun-Young Kim, Hilda Pasolli, Sebastien Phan, Jennifer Lippincott-Schwartz, Mark Ellisman, Padmini Rangamani, and Itay Budin
DOI: 10.15252/embj.2023114054

Corresponding author(s): Itay Budin (ibudin@ucsd.edu) , Padmini Rangamani (padmini.rangamani@eng.ucsd.edu)

Review Timeline:

Submission Date:	19th Mar 23
Editorial Decision:	12th May 23
Revision Received:	2nd Sep 23
Editorial Decision:	9th Oct 23
Revision Received:	9th Oct 23
Editorial Decision:	16th Oct 23
Revision Received:	16th Oct 23
Accepted:	18th Oct 23

Editor: William Teale

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. 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 **12th May 2023**

Dear Dr Budin,

Thank you for the submission of your manuscript entitled " Cristae formation is a mechanical buckling event controlled by the inner membrane lipidome" (EMBOJ-2023-114054). I have now received three referees' reports, which are copied to the bottom of this message. Please accept my apologies for the exceptionally long time it has taken to get back to you with these reports.

At its heart, all referees agree that the work is based on a technically accomplished series of experiments. They also state unambiguously that the manuscript is timely and the topic is important. However, the feedback was not unambiguously positive. The data that you present will need to be grounded in a deeper physiological context with a more comprehensive range of validation experiments if they are to be published in EMBO Journal. Furthermore, it is my impression that the referees also sometimes struggled to follow sometimes complex and diverging lines of argument.

Nonetheless, I would like to invite you to address the comments of all referees in a revised version of the manuscript. In particular, more experiments and a refined round of modelling will be needed to advance our understanding of cristae formation, as outlined by the reviewers. If you judge that such experiments are not feasible within a reasonable time-frame, it may be in your best interests to submit the study elsewhere. Please bear in mind that the reviewers must be satisfied with the revised manuscript you submit. Our usual revision time of three months is used as a guideline, not a deadline; manuscripts frequently take longer to revise. I will be available and happy to talk next week if you have any questions, I recommend that we go over our next steps and discuss the referees' comments further over Zoom. I am also available through the revision process to discuss the manuscript's overall presentation.

I should add that it is The EMBO Journal policy to allow only a single major round of revision and that it is therefore important to resolve these concerns at this stage. I believe the concerns of the referees are reasonable and addressable, but please contact me if you have any questions, need further input on the referee comments or if you anticipate any problems in addressing any of their points. Please, follow the instructions below when preparing your manuscript for resubmission.

I would also like to point out that as a matter of policy, competing manuscripts published during this period will not be taken into consideration in our assessment of the novelty presented by your study ("scooping" protection). We have extended this 'scooping protection policy' beyond the usual 3 month revision timeline to cover the period required for a full revision to address the essential experimental issues. Please contact me if you see a paper with related content published elsewhere to discuss the appropriate course of action.

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 available online to the community. For more details on our Transparent Editorial Process, please visit our website: https://www.embopress.org/page/journal/14602075/authorguide#transparentprocess

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

Thank you very much again for the opportunity to consider your work for publication. I look forward to your revision.

Best regards,

William Teale

William Teale, Ph.D.
Editor
The EMBO Journal

When submitting your revised manuscript, please carefully review the instructions below and include the following items:

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).

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

4) a complete author checklist, which you can download from our author guidelines (https://wol-prod-cdn.literatumonline.com/pb-

assets/embo-site/Author Checklist%20-%20EMBO%20J-1561436015657.xlsx). 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.

6) We require a 'Data Availability' section after the Materials and Methods. Before submitting your revision, primary datasets produced in this study need to be deposited in an appropriate public database, and the accession numbers and database listed under 'Data Availability'. Please remember to provide a reviewer password if the datasets are not yet public (see https://www.embopress.org/page/journal/14602075/authorguide#datadeposition). If no data deposition in external databases is needed for this paper, please then state in this section: This study includes no data deposited in external repositories. Note that the Data Availability Section is restricted to new primary data that are part of this study.

Note - All links should resolve to a page where the data can be accessed.

7) 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:
http://bit.ly/EMBOPressFigurePreparationGuideline

Please remember: Digital image enhancement is acceptable practice, as long as it accurately represents the original data and conforms to community standards. If a figure has been subjected to significant electronic manipulation, this must be noted in the figure legend or in the 'Materials and Methods' section. The editors reserve the right to request original versions of figures and the original images that were used to assemble the figure.

8) For data quantification: please specify the name of the statistical test used to generate error bars and P values, the number (n) of independent experiments (specify technical or biological replicates) underlying each data point and the test used to calculate p-values in each figure legend. The figure legends should contain a basic description of n, P and the test applied. Graphs must include a description of the bars and the error bars (s.d., s.e.m.).

9) We would also encourage you to include the source data for figure panels that show essential data. Numerical data can be provided as individual .xls or .csv files (including a tab describing the data). For 'blots' or microscopy, uncropped images should be submitted (using a zip archive or a single pdf per main figure if multiple images need to be supplied for one panel). Additional information on source data and instruction on how to label the files are available at .

10) We replaced Supplementary Information with Expanded View (EV) Figures and Tables that are collapsible/expandable online (see examples in https://www.embopress.org/doi/10.15252/embj.201695874). 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 labelled 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.

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 .

Further 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:
https://bit.ly/EMBOPressFigurePreparationGuideline
See also guidelines for figure legends: https://www.embopress.org/page/journal/14602075/authorguide#figureformat

IMPORTANT: When you send the revision we will require
- 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).
- Expanded View files (replacing Supplementary Information)
Please see out instructions to authors
https://www.embopress.org/page/journal/14602075/authorguide#expandedview

Please remember: Digital image enhancement is acceptable practice, as long as it accurately represents the original data and conforms to community standards. If a figure has been subjected to significant electronic manipulation, this must be noted in the figure legend or in the 'Materials and Methods' section. The editors reserve the right to request original versions of figures and the original images that were used to assemble the figure.

Further information is available in our Guide For Authors: https://www.embopress.org/page/journal/14602075/authorguide

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 (11th Jul 2022). Please discuss the revision progress ahead of this time with the editor if you require more time to complete the revisions. Use the link below to submit your revision:

https://emboj.msubmit.net/cgi-bin/main.plex

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:
https://bit.ly/EMBOPressFigurePreparationGuideline
See also guidelines for figure legends: https://www.embopress.org/page/journal/14602075/authorguide#figureformat

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

IMPORTANT: When you send the revision we will require
- 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).
- Expanded View files (replacing Supplementary Information)
Please see out instructions to authors
https://www.embopress.org/page/journal/14602075/authorguide#expandedview

Please remember: Digital image enhancement is acceptable practice, as long as it accurately represents the original data and conforms to community standards. If a figure has been subjected to significant electronic manipulation, this must be noted in the

figure legend or in the 'Materials and Methods' section. The editors reserve the right to request original versions of figures and the original images that were used to assemble the figure.

Further information is available in our Guide For Authors: https://www.embopress.org/page/journal/14602075/authorguide

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 (10th Aug 2023). Please discuss the revision progress ahead of this time with the editor if you require more time to complete the revisions. Use the link below to submit your revision:

https://emboj.msubmit.net/cgi-bin/main.plex

Referee #1:

This is a comprehensive paper which describes and analyzes the role of the lipidome in generating the characteristic tubulated morphology of the mitochondrial inner membrane (IMM). The initial experiment (Figure 1) is extraordinarily insightful - titration of the desaturase Ole1 by expressing it under the control of promoters of different strengths, reveals a sharp transition in mitochondrial morphology correlating with changes in lipid unsaturation. Cristae are lost and mitochondria display an onion skin appearance comprising several layers of IMM. These results are analyzed at multiple levels, yielding eventually to the conclusion that shaping mechanisms involve both proteins and the lipidome. This is a detailed exposition of an old idea where lipid compositions, lipid shapes and transbilayer lipid asymmetry are involved in membrane tubulation, budding, trafficking, etc, in conjunction with - or separately from - proteins.

The paper is encyclopedic; there are a lot of data. It could use clarification in several places to make things easier for the general reader. The figures are not always easy to sort out. Here are just some examples:

What are the scans in Figure 1, panels F and H? What does distance correspond to?
What are the k parameters (k1 and k2), and intuitive definitions of mean and deviatoric curvature in Figure 2A? These are not described in the legend.
Figure 3G - this is impossible to interpret without a struggle! How do the authors conclude from this figure that PE species are enriched around CL? The legend should describe what is being shown.
Figure S3C - the model of multiple invaginations to yield discrete, yet connected IMM shells does not make sense. The first invagination to yield IMM2 is plausible (but where does the membrane come from?), but the next and subsequent at the same site seems strange and deserves a proper explanation or a better cartoon.

Snap-through instability? Buckling? It would help to have accessible definitions of these (interchangeably used) terms in the text.

Referee #2:

This work combines experimental perturbations of some lipid synthesis pathways in yeast and multi-scale modeling to elucidate some conserved lipids' roles in the IMM's structural organization, specifically regulating mitochondrial cristae. The elegant yeast mutant studies, including combinations of defects in the CL synthesis pathway with PE/PL saturation manipulations, revealed that CL acts orthogonally to PL saturation and ATP synthase oligomerization to modulate IMM morphology. The authors also provide evidence for a long-suspected crosstalk between protein-mediated and lipid-dependent changes in the IMM curvature and formation of mitochondrial cristae.
The work is well done, and the data broadly support the authors' conclusions and provide some interesting new information. I think this is a nice story with carefully executed controls from investigators with experience in this area. The magnitude of the effects observed is compelling, and I have no issues with the experiments as reported. One can always nitpick about details that would send the authors back to the bench or computer for additional studies, but without necessarily affecting the overall flavor or conclusions of the paper. I believe authors deserve to be spared such harassment.
The only reason that keeps me from assigning a higher priority is that the role of CL in this process does not come as a surprise. Furthermore, although yeast are a widely-used model in the studies of mitochondrial structure and function, yeast can survive without the mitochondria. The significance of these findings and prediction for the mitochondrial function is less broad than if these studies were done in mammalian cells. For example, as the authors show, CL appears to be required for mitochondrial biogenesis during yeast fermentation but not in highly oxygenated conditions where the participation of mitochondria in ATP generation is more apparent. This may not be a weakness of the manuscript, per se, but rather the wish to see similar studies done in the heart or liver mitochondria. However, these are minor negative points, in my view. So: publish as is.

Referee #3:

The authors have analyzed the role of phospholipids and of the saturation of their acyl chains for cristae morphogenesis, combining yeast genetics and molecular dynamics simulations. They manipulated saturation levels of phospholipids in yeast expressing the desaturase Ole1p at different levels (SFA strains). The analysis of the various strains revealed a striking threshold effect, with subtle changes in PL saturation leading to drastically altered mitochondrial morphologies, flattened inner membranes, disassembly of the ATP synthase and impaired respiratory growth. Coarse-grained molecular dynamics simulations defined the effects of altered saturation levels on model membranes. The analysis revealed that membrane stiffness and spontaneous curvature are only modestly changed in membrane models of SFA strains, suggesting that the observed increased PE and CL levels in SFA strains (PE/PC ratio, CL) may buffer mechanical properties of the membranes. Consistent with the molecular dynamic simulations, the authors describe epistasis between PL saturation and CL abundance in yeast cells, which is reflected in morphological alterations in Crd1-deficient yeast cells in hypoxia but not normoxia. Together, the authors propose that the membrane curvature depends on the combined effect of proteins and the asymmetric distributions of lipids across the bilayer, which leads to a breaking point defined by the continuous loss of ATP synthase dimerization and the membrane lipid composition.

The manuscript describes a series of interesting yet sometimes disparate findings, which the authors led to a rather general summarizing conclusion. Although convincing, the general importance of the membrane lipidome and of membrane shaping proteins for cristae morphogenesis has been recognized in various studies. Although the approach to modulate the saturation level of PL by altering the expression level of Ole1, combined with molecular dynamic simulations in model membranes, is intriguing, it also has its limitations. How can the authors exclude indirect effects of the altered lipidome, for instance affecting non-mitochondrial membranes, affecting other proteins besides the ATP synthase? CL deficiency is known to impair the assembly of a series of IM proteins whose altered activity could impact cristae structure. How can the authors know whether the impaired dimerization of ATP synthase in SFA3 and SFA4 cells is a direct consequence of an altered PL saturation or a secondary consequence (for instance, by the altered levels of other PL or effect on other proteins)? Therefore, it remains at least questionable to which extent the (nevertheless interesting) results of molecular dynamic simulations on liposomes are applicable to the protein-rich mitochondrial inner membrane. Another major concern is related to the drastic changes in the saturation levels in PL at low Ole1 levels (SFA3 and SFA4), with SFA3 mainly used in the simulations. PL saturation levels are increased if the supply of oxygen is limited to levels similar as in SFA2 cells (Fig. 6). Therefore, SFA3 and SFA4 conditions appear to reflect extreme, likely non-physiological conditions, raising doubts about the relevance of the simulations under these conditions.

Additional points

1. The lipid profiles of mitochondria shown in Fig. S1E differ significantly from published data (for instance, CL levels below 10%). Moreover, CL levels are extremely low and PS level is extremely high in SFA3 mitochondria. This could be explained by impurity of the mitochondrial fraction analyzed. Contaminations of mitochondrial preparations can be problematic if dysfunctional mitochondria are purified by gradient sedimentation, because their densities differ from normal mitochondria. The authors should carefully evaluate the purify of mitochondria by immunoblot analysis of organelle markers or by quantitative proteomics to dispel this concern.
2. To evaluate the outcome from SFA3/4 conditions properly, careful comparison between SFA3/4 conditions and in atp20 conditions will be necessary to dissect the direct effect from the increase of saturation of PLs from those caused by disassembly of ATP synthase. Moreover, the authors should inspect the status of other protein entities (e.g. MICOS, respiratory chain super complexes, Mgm1, mtDNA nucleoids, mito-ER contacts) that affect inner membrane shape in these conditions.
3. In microaerobic conditions, the CL levels are increased (Fig. 6B). Can this be explained by adaptation of the mitochondrial mass? Are ATP synthase dimers formed in the microaerobic conditions both in WT or in crd1 ?
4. P9, in the first sentence of the new chapter. "Increasing Ole1p activity" should read "decreasing Ole1p activity"

Point-by-point Reviewer Response

Referee #1:

This is a comprehensive paper which describes and analyzes the role of the lipidome in generating the characteristic tubulated morphology of the mitochondrial inner membrane (IMM). The initial experiment (Figure 1) is extraordinarily insightful - titration of the desaturase Ole1 by expressing it under the control of promoters of different strengths, reveals a sharp transition in mitochondrial morphology correlating with changes in lipid unsaturation. Cristae are lost and mitochondria display an onion skin appearance comprising several layers of IMM. These results are analyzed at multiple levels, yielding eventually to the conclusion that shaping mechanisms involve both proteins and the lipidome. This is a detailed exposition of an old idea where lipid compositions, lipid shapes and transbilayer lipid asymmetry are involved in membrane tubulation, budding, trafficking, etc, in conjunction with - or separately from - proteins.

The paper is encyclopedic; there are a lot of data. It could use clarification in several places to make things easier for the general reader. The figures are not always easy to sort out. Here are just some examples:

In addition to the specific cases below, we generally streamlined the entire paper to improve readability, make specific sections more concise, and clarify key terms and concepts throughout. We think the revised version is thus more accessible to a wide audience.

What are the scans in Figure 1, panels F and H? What does distance correspond to?

We have updated the figure captions to clarify that this refers to the distance along the intensity profile analysis across the mitochondria.

What are the k parameters (k1 and k2), and intuitive definitions of mean and deviatoric curvature in Figure 2A? These are not described in the legend.

We have added intuitive definitions for these parameters in the main text.

Figure 3G - this is impossible to interpret without a struggle! How do the authors conclude from this figure that PE species are enriched around CL? The legend should describe what is being shown.

Thank you for pointing this out. We rewrote the legend to clarify and color key to explicitly state what is being shown (the increased likelihood of one type of lipid being within 1.5 nm of another). We also added boxes around interactions that exceed a 5% enrichment threshold, which guides the readers to the referenced interaction between PE and CL shown by the green square.

Figure S3C - the model of multiple invaginations to yield discrete, yet connected IMM shells does not make sense. The first invagination to yield IMM2 is plausible (but where does the membrane come from?), but the next and subsequent at the same site seems strange and deserves a proper explanation or a better cartoon.

We have adjusted the figure panels (now appendix S3) to posit that the subsequent membranes come from additional membrane biogenesis (or lipid synthesis). We have also re-written the figure caption to clarify this.

Snap-through instability? Buckling? It would help to have accessible definitions of these (interchangeably used) terms in the text.

We have provided accessible definitions within the results section and discussion that explicitly states that these terms are synonymous.

Referee #2:

This work combines experimental perturbations of some lipid synthesis pathways in yeast and multi-scale modeling to elucidate some conserved lipids' roles in the IMM's structural organization, specifically regulating mitochondrial cristae. The elegant yeast mutant studies, including combinations of defects in the CL synthesis pathway with PE/PL saturation manipulations, revealed that CL acts orthogonally to PL saturation and ATP synthase oligomerization to modulate IMM morphology. The authors also provide evidence for a long-suspected crosstalk between protein-mediated and lipid-dependent changes in the IMM curvature and formation of mitochondrial cristae.

The work is well done, and the data broadly support the authors' conclusions and provide some interesting new information. I think this is a nice story with carefully executed controls from investigators with experience in this area. The magnitude of the effects observed is compelling, and I have no issues with the experiments as reported. One can always nitpick about details that would send the authors back to the bench or computer for additional studies, but without necessarily affecting the overall flavor or conclusions of the paper. I believe authors deserve to be spared such harassment.

The only reason that keeps me from assigning a higher priority is that the role of CL in this process does not come as a surprise. Furthermore, although yeast are a widely-used model in the studies of mitochondrial structure and function, yeast can survive without the mitochondria. The significance of these findings and prediction for the mitochondrial function is less broad than if these studies were done in mammalian cells. For example, as the authors show, CL appears to be required for mitochondrial biogenesis during yeast fermentation but not in highly oxygenated conditions where the participation of mitochondria in ATP generation is more apparent. This may not be a weakness of the manuscript, per se, but rather the wish to see similar studies done in the heart or liver mitochondria. However, these are minor negative points, in my view. So: publish as is.

We think that our general findings here are applicable to mitochondria outside of the yeast system and agree that highlighting this point would broaden the physiological relevance of our results. In our revised manuscript, we discuss how the interplay between CL biosynthesis and lipid saturation is important for understanding the physiological roles of CL. Additionally, we highlight recent work that has similarly observed that low oxygenation, which is characteristic of tissues like the intestine as well as in cancer tumors, inhibits lipid desaturases and implicates CL as being important under these conditions. For example, a recent CRISPR screen of tissue resident intestinal T cells identified CL metabolism as a regulator of fitness during low oxygen growth (Reina-Campos *et al*, 2023).

Based on these comments and discussions with the editor, we have also incorporated experiments showing the role of CL biosynthesis in human embryonic kidney (HEK) cells when lipid saturation is increased either by fatty acid feeding or by microaerobic conditions. We find that knockdown of human cardiolipin synthase (CRLS1), using a previously validated RNAi approach, causes loss of mitochondrial morphology in HEK293 cells grown under microaerobic conditions (1% O_2) or fed with low levels of palmitic acid (50 uM), both of which increase saturation. While these experiments currently lack the detailed molecular analyses that the yeast system affords, they suggest that the dynamics observed there could apply to a wide range of systems.

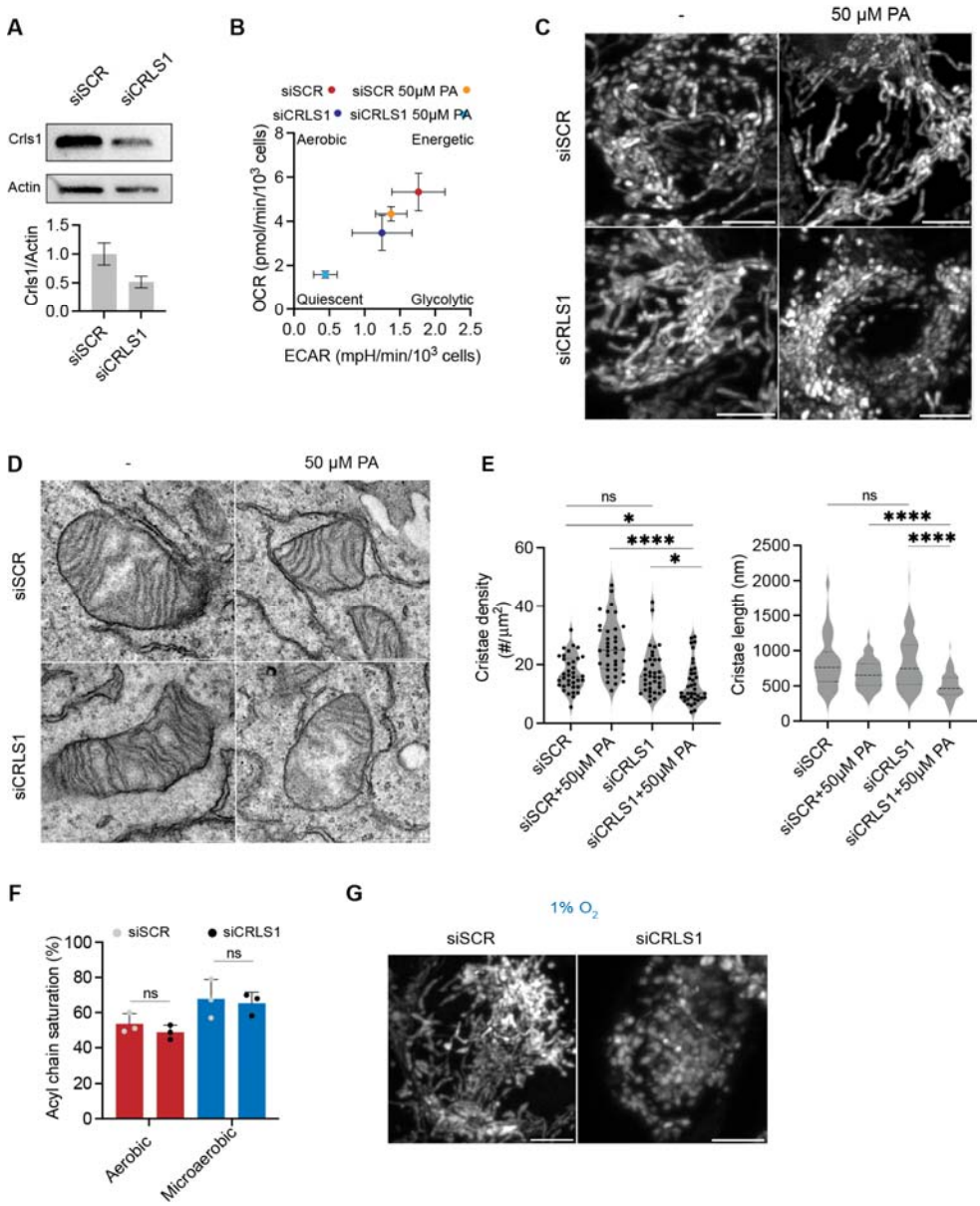

Figure R1.1: Epistasis between SFA and CL in HEK293 cells. Also in Figure EV6.

Referee #3:

The authors have analyzed the role of phospholipids and of the saturation of their acyl chains for cristae morphogenesis, combining yeast genetics and molecular dynamics simulations. They manipulated saturation levels of phospholipids in yeast expressing the desaturase Ole1p at different levels (SFA strains). The analysis of the various strains revealed a striking threshold effect, with subtle changes in PL saturation leading to drastically altered mitochondrial morphologies, flattened inner membranes, disassembly of the ATP synthase and impaired respiratory growth. Coarse-grained molecular dynamics simulations defined the effects of altered saturation levels on model membranes. The analysis revealed that membrane stiffness and spontaneous curvature are only modestly changed in membrane models of SFA strains, suggesting that the observed increased PE and CL levels in SFA strains (PE/PC ratio, CL) may buffer mechanical properties of the membranes. Consistent with the molecular dynamic simulations, the authors describe epistasis between PL saturation and CL abundance in yeast cells, which is reflected in morphological alterations in Crd1-deficient

yeast cells in hypoxia but not normoxia. Together, the authors propose that the membrane curvature depends on the combined effect of proteins and the asymmetric distributions of lipids across the bilayer, which leads to a breaking point defined by the continuous loss of ATP synthase dimerization and the membrane lipid composition.

The manuscript describes a series of interesting yet sometimes disparate findings, which the authors led to a rather general summarizing conclusion. Although convincing, the general importance of the membrane lipidome and of membrane shaping proteins for cristae morphogenesis has been recognized in various studies. Although the approach to modulate the saturation level of PL by altering the expression level of Ole1, combined with molecular dynamic simulations in model membranes, is intriguing, it also has its limitations. How can the authors exclude indirect effects of the altered lipidome, for instance affecting non-mitochondrial membranes, affecting other proteins besides the ATP synthase?

While larger perturbations to Ole1 expression can cause pleiotropic effects across organelles, we chose this set of strains specifically because they did not appear to have any deleterious effects on non-mitochondrial components, as indicated by microscopy of ER, Golgi, and Vacuole membranes (Fig. EV1A) and fitness analysis on respiratory vs. glycolytic conditions (Fig. 1B). In contrast, much weaker OLE1 promoter mutants, such as those we previously published (Degreif *et al*, 2017) do show phenotypes in the ER and other organelles.

To further assess the effect of the altered lipidome in the strains analyzed here on non-mitochondrial membranes, we have measured induction of the unfolded protein response (UPR) using a Hac1-GFP splice reporter as previously described (Leber *et al*, 2004; Rüegsegger *et al*, 2001). The UPR has long been observed to be induced by accumulation of saturated lipids in the ER (Pineau *et al*, 2009; Ariyama *et al*, 2010; Volmer *et al*, 2013) so measuring its activation is a sensitive assay to detect general saturated lipid stress. Like the mitochondria, the ER membrane is composed predominantly of glycerophospholipids (lacking sphingolipids and abundant sterols), and thus it is most susceptible to saturated lipid stress.

Using the Hac1-GFP reporter, we observe that the transition from SFA2 to SFA3 — which corresponds to the loss of IMM structure — does not correspond to an UPR activation, with both SFA2 and SFA3 cells showing a similar level of GFP expression as WT. As a positive control, WT cells treated with DTT are included in the comparison. This additional experiment indicates that loss of mitochondrial morphology is not tied to ER stress.

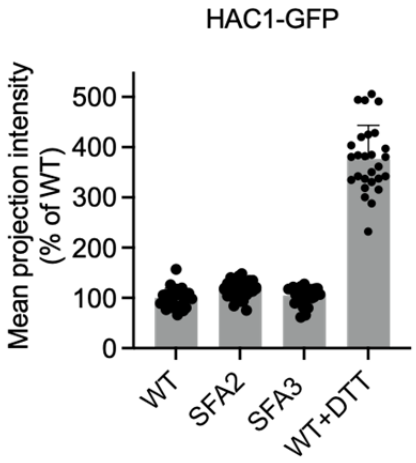

Figure R1.2: Loss of cristae does not correspond to an increase in ER stress as measured by a UPR reporter. Also, in Figure EV1B

To look at non-ATP synthase proteins, we have analyzed Mgm1, Mic60 of the MICOS complex, mtDNA nucleoids, and ETC complexes, as described further below. In all, we do not observe any changes to protein levels or associations from SFA2 to SFA3 cells that were not observed for atp20 cells. We also show that SFA3 cells retain both Mgm1 isoforms, Mic60, and levels of ETC complexes and supercomplexes. These results are consistent with a specific effect of phospholipid saturation on cristae membranes through ATP synthase oligomerization.

CL deficiency is known to impair the assembly of a series of IM proteins whose altered activity could impact cristae structure. How can the authors know whether the impaired dimerization of ATP synthase in SFA3 and SFA4 cells is a direct consequence of an altered PL saturation or a secondary consequence (for instance, by the altered levels of other PL or effect on other proteins)? Therefore, it remains at least questionable to which extent the (nevertheless interesting) results of molecular dynamic simulations on liposomes are applicable to the protein-rich mitochondrial inner membrane.

There is a long history of studies where significant changes in the lipid profile of the mitochondria do not induce impairments of ATP synthase dimerization, which are summarized in the table below. For example, PEM2 knockouts, which have a 15 fold decrease in mitochondrial PC, still retain ATP synthase dimers. Even more strikingly, PSD1 knockouts, which have <5 fold decreases in mitochondrial PE, still retain ATP synthase dimers (Baker *et al*, 2016). In our own experiments, loss of CL (in WT or SFA2 background) also does not change ATP synthase dimerization state (Fig. 4E).

In terms of other cristae shaping proteins, Mic60 KOs and yeast deficient in all 6 MICOS subunits still retain ATP synthase dimers (Friedman *et al*, 2015). Strains lacking Dnm1p (yeast homologue of Drp1), which have largely altered mitochondrial morphology, also still retain ATP synthase dimers (Harner *et al*, 2016). Similarly the loss of the ATP/ADP transporter, AAC2 (Pet9), also does not change ATP synthase dimerization (Claypool *et al*, 2008b). To our knowledge the only mutations that directly cause defects in ATP synthase dimerization are in ATP20 (subunit g) and TIM11 (subunit e), which are both physically present in the dimerization region (Arnold *et al*, 1998; Paumard *et al*, 2002; Arselin *et al*, 2004). Thus, the effect of lipid saturation modulates ATP synthase dimerization is rather unique and is unlikely to be due to secondary effects on these other components.

We have incorporated these references and comment on the uniqueness of this effect in the revised text. To our knowledge, this is the first evidence that a lipidic (or any other molecular component that is not a ATP synthase subunit) alteration has been observed to cause disassembly of ATP synthase dimers in yeast, and this is better explained and highlighted in the revised text.

Table R1.1

Strain	Effect	ATP synthase organization (dimer/monomer)	Reference
pem2	15 fold decrease in mitochondrial PC	Dimer	Baker et. al. 2016.
psd1	5 fold decrease in mitochondrial PE	Dimer	Baker et. al. 2016.
crd1	Loss of CL, accumulation of PG	Dimer	Baker et. al. 2016, this study.
taz1	MLCL accumulation. Decreased CL levels.	Dimer	Claypool et. al. 2008.

aac2	Defective ADP/ATP transport.	Dimer	Claypool et. al. 2008.
dnm1	Hyperfused, aberrant mitochondrial morphology	Dimer	Harner et. al. 2016.
mic60	Altered mitochondrial morphology, no crista junctions.	Dimer	Friedman et. al. 2015
MICOS KO	Loss of all MICOS subunits, altered cristae structure	Dimer	Friedman et. al. 2015.

Another major concern is related to the drastic changes in the saturation levels in PL at low Ole1 levels (SFA3 and SFA4), with SFA3 mainly used in the simulations. PL saturation levels are increased if the supply of oxygen is limited to levels similar as in SFA2 cells (Fig. 6). Therefore, SFA3 and SFA4 conditions appear to reflect extreme, likely non-physiological conditions, raising doubts about the relevance of the simulations under these conditions.

Thank you for this comment. We agree that SFA4 is indeed an extreme, non-physiological condition. As a result, we have not reported a lipidome from SFA4 mitochondria and instead report the SFA3 mitochondrial lipidome, as this is the initial strain where we observe a breakage of mitochondrial ultrastructure and function. We note that the level of saturation in SFA3 however, is only slightly higher than in microaerobic conditions (<5% acyl chain saturation difference), which illustrates our overarching point that very small changes in PL saturation lead to abrupt breakages in IMM structure.

We note that our simulations were carried out on SFA2 as well as SFA3 lipidomes. Although the SFA3 lipidomes are not physiological, they provided insight that mechanical properties, specifically bending rigidity, could affect ATP synthase dimerization. Importantly, they also provided a potential mechanism by which CL acts in SFA2 lipidomes, which closely mimic those of cells grown in microaerobic conditions.

Additional points

1. The lipid profiles of mitochondria shown in Fig. S1E differ significantly from published data (for instance, CL levels below 10%). Moreover, CL levels are extremely low and PS level is extremely high in SFA3 mitochondria. This could be explained by impurity of the mitochondrial fraction analyzed. Contaminations of mitochondrial preparations can be problematic if dysfunctional mitochondria are purified by gradient sedimentation, because their densities differ from normal mitochondria. The authors should carefully evaluate the purify of mitochondria by immunoblot analysis of organelle markers or by quantitative proteomics to dispel this concern.

Thank you for this important point. We have added immunoblot analysis of purified mitochondria from the strains we report lipidomics on in our manuscript (Appendix Figure S5). We find only trace contamination from other organelles, at a level consistent with previous studies (Liao *et al*, 2018; Meisinger *et al*), and note these do not increase in SFA3 mitochondria that show elevated PS. In WT, the mitochondrial lipid profile we report is almost identical in phospholipid composition (PC/PE/PI/PS) to seminal yeast mitochondria lipidomes such as those presented in (Zinser *et al*, 1991). Thus, we are confident that the changes in SFA3 lipidomes are biological.

Below we display western blot analysis of isolated mitochondria from WT and SFA2/3 which depict the absence of ER and vacuole contamination from each strain. As a control, we also verified the presence of the identical vacuole, mitochondria and ER proteins in the whole cell yeast fraction.

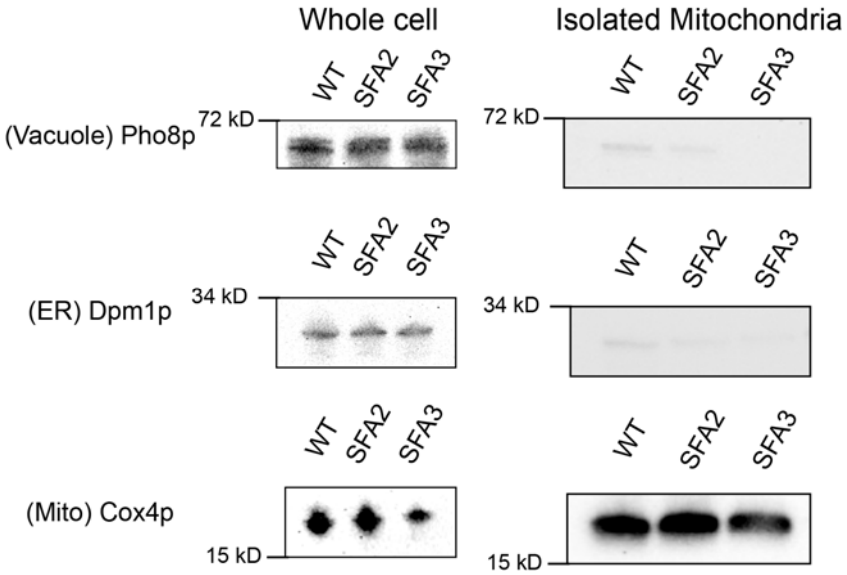

Figure R1.3: Purity of mitochondrial isolation from SFA strains used for lipidomics analysis. Also in Appendix Figure S5.

To our knowledge, several studies report a CL level from isolated yeast mitochondria in the same range as what we report in our manuscript – 7 to 8% (Claypool *et al*, 2008b; Baile *et al*, 2014; Ball *et al*, 2018; Claypool *et al*, 2011, 2008a). These levels are summarized in the table below. In regards to the low levels of CL in SFA3 mitochondria, we also observe a 2-fold reduction in CL levels in whole cell yeast (Appendix Figure S1B, bottom right panel), so we do not think this is a purification artifact. Our hypothesis, that we are currently exploring, is that saturated CL accumulates in this strain, which is then cleared by a CL-specific phospholipase.

The increase in PS in SFA3 mitochondrial is indeed quite interesting. In yeast, PS decarboxylation to PE by Psd1p is the primary mechanism for PE generation in the mitochondria. Our model is that increased PE is required in SFA3 mitochondria to maintain curvature of the IMM and therefore causes increased PS import to drive PE biosynthesis in the IMM.

Table R1.2

Reported WT CL levels from isolated yeast mitochondria	Reference
5-7%	Claypool *et. al.* 2008.
9-10%	Claypool *et. al.* 2008
8%	Claypool *et. al.* 2011.
7-8%	Baile *et. al.* 2014
8-10%	Basu Ball *et. al.* 2018

7%	This study

2. To evaluate the outcome from SFA3/4 conditions properly, careful comparison between SFA3/4 conditions and in atp20 conditions will be necessary to dissect the direct effect from the increase of saturation of PLs from those caused by disassembly of ATP synthase. Moreover, the authors should inspect the status of other protein entities (e.g. MICOS, respiratory chain super complexes, Mgm1, mtDNA nucleoids, mito-ER contacts) that affect inner membrane shape in these conditions.

To address this question, we have carried out all the specified analyses and found the following (Figure R1.3, Figure EV2):

1) BN-PAGE analysis showed no apparent changes in the abundances of individual ETC complexes (V, CII, CIII, CIV) between SFA2/3 strains. We analyzed the formation of SCs specifically by BN-PAGE followed by immunoblotting with anti-Cox1p (CIV). We observed that WT and SFA2/3 still retained the formation of the major SCs while atp20 mitochondria showed a partial loss of SCs.

2) To determine the effect of saturation on mtDNA nucleoids, we stained cells with SYBR Green I (SGI), which preferentially localizes to mtDNA rather than nuclear DNA in live cells and has been previously utilized in yeast (Dua *et al*, 2022). We found that while WT and SFA2 retained the same number of mtDNA nucleoids per cell, both SFA3 and atp20 cells had increased numbers of mtDNA nucleoids.

3) We also inspected the relative levels of the MICOS component Mic60 and Mgm1 via immunoblot. We found that SFA3 retained both short and long forms of Mgm1 while atp20 mitochondria showed a decrease in the long form of Mgm1. SFA3 shows a partial loss of Mic60, which is further lost in atp20. Since SFA3 shows a partial loss of ATP synthase dimerization, this is consistent with Mic60 being lost as a result of ATP synthase dimerization.

4) We assayed ER-mitochondria contacts by thin-section EM, where we measured the shortest distance between mitochondria and ER in a given cell in SFA2, SFA3 and atp20 conditions. While SFA2 showed an average distance of <200 nm by thin-section EM, both SFA3 and atp20 showed an increased ER-mito distance. Despite this increase, there are still examples within SFA3 and atp20 cells where ER-mito distance is <200 nm, so we think that contact between the organelles is maintained (and is required for the IMM lipidome).

In sum, these analyses show that mitochondrial molecular phenotypes are shared between dimer-deficient SFA3 and dimer-absent atp20 — though are generally more severe in the latter — which is consistent with the model that lipid saturation acts through a reduction in ATP synthase dimerization. We did not observe any phenotypes that are found in SFA3 mitochondria but not atp20 mitochondria, which argues against alternative mechanisms by which lipid saturation acts on these features.

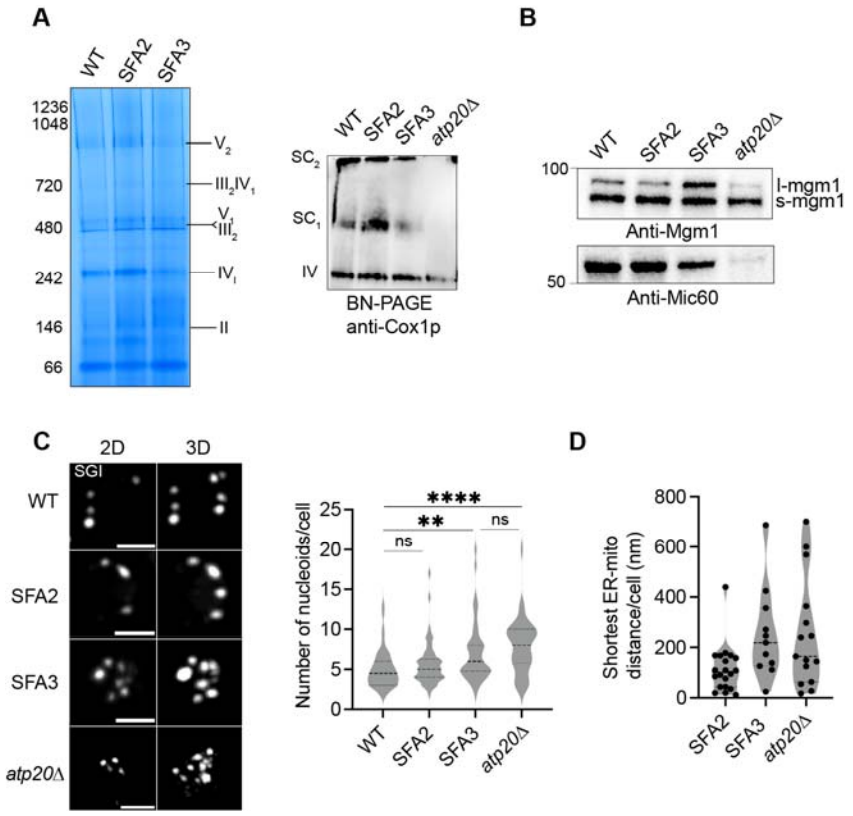

Figure R1.4: Additional molecular characterization of SFA mitochondria. Panels are also in Figure EV2C-F.

3. In microaerobic conditions, the CL levels are increased (Fig. 6B). Can this be explained by adaptation of the mitochondrial mass? Are ATP synthase dimers formed in the microaerobic conditions both in WT or in crd1?

This is a very valid point, given the challenges in isolation of pure mitochondria from microaerobic cells.

To address this concern (Figure R1.5, Figure EV5), we first tested whether mitochondrial mass changes during microaerobic growth through image analysis with Mitograph software (Viana *et al*, 2015). We found no changes in mitochondrial volume or the total mitochondrial length when comparing cells grown under microaerobic and aerobic conditions, suggesting that changes in CL content in the lipidomics data are not due to changes in mitochondrial volume.

To test the amounts of CL more directly in the mitochondria under these conditions, we stained cells with the CL-binding dye, nonyl acridine-orange (NAO). We quantified the intensities of NAO across and found a significant increase in intensity in microaerobic cells compared to aerobic conditions. These data support the hypothesis that CL levels increase under microaerobic conditions (Figure 6B) independently of mitochondrial abundance.

To analyze ATP synthase dimerization under microaerobic conditions, we carried out BN-PAGE on crude mitochondrial extracts. We found that in microaerobic conditions, both WT and crd1 retained ATP synthase dimers at similar levels to SFA2 cells. We included atp20 mitochondria as a monomeric control. This supports the model that, in microaerobic conditions, CL shapes cristae morphology independently of ATP synthase dimerization, as it does in SFA2 cells under aerobic conditions.

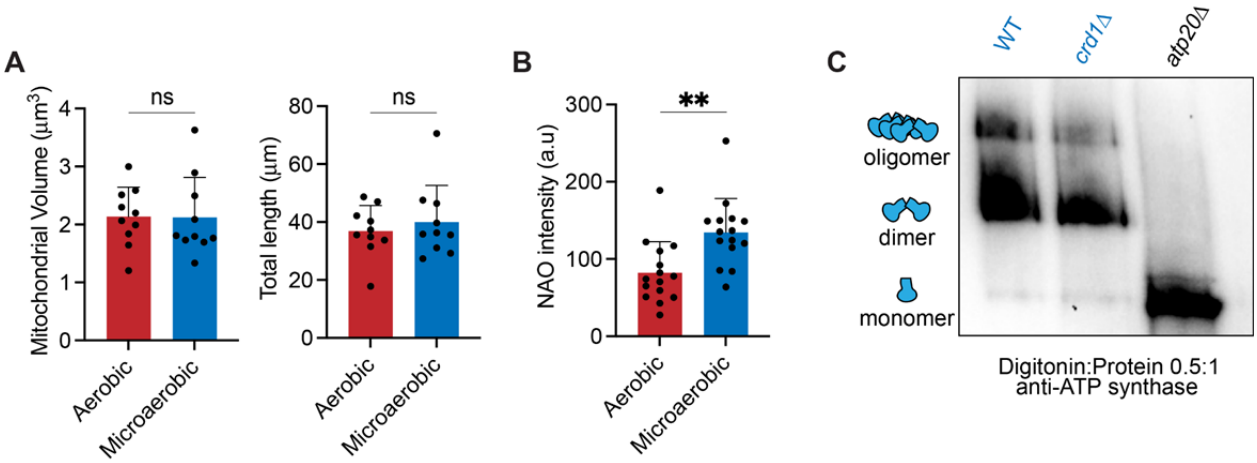

Figure R1.5: Further characterization of microaerobic mitochondria. Also shown in Figure EV5C-E.

4. P9, in the first sentence of the new chapter. "Increasing Ole1p activity" should read "decreasing Ole1p activity"

Thank you for pointing this out; it is now fixed.

References

Ariyama H, Kono N, Matsuda S, Inoue T & Arai H (2010) Decrease in membrane phospholipid unsaturation induces unfolded protein response. *J Biol Chem* 285: 22027–22035

Arnold I, Pfeiffer K, Neupert W, Stuart RA & Schägger H (1998) Yeast mitochondrial F1F0-ATP synthase exists as a dimer: identification of three dimer-specific subunits. *EMBO J* 17: 7170–7178

Arselin G, Vaillier J, Salin B, Schaeffer J, Giraud M-F, Dautant A, Brèthes D & Velours J (2004) The modulation in subunits e and g amounts of yeast ATP synthase modifies mitochondrial cristae morphology. *J Biol Chem* 279: 40392–40399

Baile MG, Sathappa M, Lu Y-W, Pryce E, Whited K, Michael McCaffery J, Han X, Alder NN & Claypool SM (2014) Unremodeled and Remodeled Cardiolipin Are Functionally Indistinguishable in Yeast. *Journal of Biological Chemistry* 289: 1768–1778 doi:10.1074/jbc.m113.525733 [PREPRINT]

Baker CD, Basu Ball W, Pryce EN & Gohil VM (2016) Specific requirements of nonbilayer phospholipids in mitochondrial respiratory chain function and formation. *Mol Biol Cell* 27: 2161–2171

Ball WB, Baker CD, Neff JK, Apfel GL, Lagerborg KA, Žun G, Petrovič U, Jain M & Gohil VM (2018) Ethanolamine ameliorates mitochondrial dysfunction in cardiolipin-deficient yeast cells. *J Biol Chem* 293: 10870–10883

Claypool SM, Boontheung P, McCaffery JM, Loo JA & Koehler CM (2008a) The cardiolipin transacylase, tafazzin, associates with two distinct respiratory components providing insight into Barth syndrome. *Mol Biol Cell* 19: 5143–5155

Claypool SM, Oktay Y, Boontheung P, Loo JA & Koehler CM (2008b) Cardiolipin defines the interactome of the major ADP/ATP carrier protein of the mitochondrial inner membrane. *J Cell Biol* 182: 937–950

Claypool SM, Whited K, Srijumnong S, Han X & Koehler CM (2011) Barth syndrome mutations that cause tafazzin complex lability. *J Cell Biol* 192: 447–462

Degreif D, de Rond T, Bertl A, Keasling JD & Budin I (2017) Lipid engineering reveals regulatory roles for membrane fluidity in yeast flocculation and oxygen-limited growth. *Metab Eng* 41: 46–56

Dua N, Seshadri A & Badrinarayanan A (2022) DarT-mediated mtDNA damage induces dynamic reorganization and selective segregation of mitochondria. *J Cell Biol* 221

Friedman JR, Mourier A, Yamada J, McCaffery JM & Nunnari J (2015) MICOS coordinates with respiratory complexes and lipids to establish mitochondrial inner membrane architecture. *Elife* 4

Harner ME, Unger A-K, Geerts WJ, Mari M, Izawa T, Stenger M, Geimer S, Reggiori F, Westermann B & Neupert W (2016) An evidence based hypothesis on the existence of two pathways of mitochondrial crista formation. *Elife* 5

Leber JH, Bernales S & Walter P (2004) IRE1-independent gain control of the unfolded protein response. *PLoS Biol* 2: E235

Liao P-C, Boldogh IR, Siegmund SE, Freyberg Z & Pon LA (2018) Isolation of mitochondria from Saccharomyces cerevisiae using magnetic bead affinity purification. *PLoS One* 13: e0196632

Meisinger C, Pfanner N & Truscott KN Isolation of Yeast Mitochondria. *Yeast Protocols*: 033–040 doi:10.1385/1-59259-958-3:033 [PREPRINT]

Paumard P, Vaillier J, Coulary B, Schaeffer J, Soubannier V, Mueller DM, Brèthes D, di Rago J-P & Velours J (2002) The ATP synthase is involved in generating mitochondrial cristae morphology. *EMBO J* 21: 221–230

Pineau L, Colas J, Dupont S, Beney L, Fleurat-Lessard P, Berjeaud J-M, Bergès T & Ferreira T (2009) Lipid-induced ER stress: synergistic effects of sterols and saturated fatty acids. *Traffic* 10: 673–690

Reina-Campos M, Heeg M, Kennewick K, Mathews IT, Galletti G, Luna V, Nguyen Q, Huang H, Milner JJ, Hu KH, *et al* (2023) Metabolic programs of T cell tissue residency empower tumour immunity. *Nature*: 1–9

Rüegsegger U, Leber JH & Walter P (2001) Block of HAC1 mRNA translation by long-range base pairing is released by cytoplasmic splicing upon induction of the unfolded protein response. *Cell* 107: 103–114

Viana MP, Lim S & Rafelski SM (2015) Quantifying mitochondrial content in living cells. *Methods Cell Biol* 125: 77–93

Volmer R, van der Ploeg K & Ron D (2013) Membrane lipid saturation activates endoplasmic reticulum unfolded protein

response transducers through their transmembrane domains. *Proc Natl Acad Sci U S A* 110: 4628–4633

Zinser E, Sperka-Gottlieb CD, Fasch EV, Kohlwein SD, Paltauf F & Daum G (1991) Phospholipid synthesis and lipid composition of subcellular membranes in the unicellular eukaryote Saccharomyces cerevisiae. *Journal of Bacteriology* 173: 2026–2034 doi:10.1128/jb.173.6.2026-2034.1991 [PREPRINT]

1st Revision - Editorial Decision **9th Oct 2023**

Dear Itay,

Thank you for submitting a revised version of your manuscript. It was sent to all three of the original referees; I have now heard back from the referee with the most substantive concerns. This report is attached to the bottom of this email. As you will see, you have addressed all of the key original concerns adequately; therefore, barring any unexpected and important interventions from either Referee 1 or Referee 2, I am happy to proceed towards publication with your manuscript. There are some remaining editorial issues which need to be addressed. In this regard, would you please:

- change the red coloured text to black,
- include missing funding information in our online submission system for National Institutes of Health Award number S10OD023527, The National Institutes of Health grants R24GM137200 and U24NS120055, the Department of Energy grant DE-SC0022954, and Howard Hughes Medical Institute,
- remove the author credit section,
- include callouts in the text for Figure 2E, Appendix Table S4 and Movies EV1-EV7
- upload "Lipidomics Dataset" individually as Dataset EV1-EV2 with the legends uploaded as a separate sheet in each Excel file,
- include page numbers in the table of contents in Appendix 1 ToC, captions for movies should be removed from the Appendix file, renaming with the nomenclature Appendix Table S1-S4 in the table legends,
- include a p-value for "**" in the figure legend for figure EV2,
- attend to the following: the legend of figure EV4b refers to a line profile analysis; however, no such analysis is provided in the figure panel,
- indicate the statistical test used for data analysis in the legends of figures 6c, EV4a, EV5d and EV6f,
- define error bars in the legend of figures 1h; 3c-d, f; EV1b; EV3d-g; EV6a,
- define sample size in the legend of figures 3c-d, f; EV3d-g; EV6a, and
- describe the nature of entity for 'n' in the legend of figure EV6f.

Additionally, could you please supply the synopsis image in jpeg, TIFF or png format, and remove the bullet points for the summary form the main file

I look forward to receiving these changes. EMBO Press is an editorially independent publishing platform for the development of EMBO scientific publications.

Best wishes,

William

William Teale, PhD
Editor
The EMBO Journal
w.teale@embojournal.org

Instructions for preparing your revised manuscript:

Please 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:
https://bit.ly/EMBOPressFigurePreparationGuideline
See also figure legend guidelines: https://www.embopress.org/page/journal/14602075/authorguide#figureformat

IMPORTANT: When you send the revision we will require
- 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).
- Expanded View files (replacing Supplementary Information)
Please see out instructions to authors
https://www.embopress.org/page/journal/14602075/authorguide#expandedview

Please remember: Digital image enhancement is acceptable practice, as long as it accurately represents the original data and conforms to community standards. If a figure has been subjected to significant electronic manipulation, this must be noted in the figure legend or in the 'Materials and Methods' section. The editors reserve the right to request original versions of figures and the original images that were used to assemble the figure.

Further information is available in our Guide For Authors: https://www.embopress.org/page/journal/14602075/authorguide

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 (7th Jan 2024). Please discuss the revision progress ahead of this time with the editor if you require more time to complete the revisions. Use the link below to submit your revision:

https://emboj.msubmit.net/cgi-bin/main.plex

Referee #3:

The authors have carefully considered all points of criticisms raised on the original manuscript. Among other points, they provide now convincing evidence using a reporter that the functionality of the ER is not compromised in SFA3 cells. I am still wondering though why the reduced PE/PC ratio in these cells (Fig. 3D) does not elicit a stress response as previously observed. Moreover, they show now immunoblots of a series of marker proteins to monitor the purity of the mitochondrial fraction analyzed by lipidomics. These data are difficult to assess as the sample were apparently not analyzed on the same gels and are therefore difficult to compare (exposure times etc). However, this is only a minor point. Overall, the authors have addressed the concerns adequately. Although a general role of the mitochondrial lipidome (and of cristae shaping proteins) and the crosstalk between CL and other PLs have been recognized, the manuscript provides some novel insight (such as pivotal role of CL for cristae morphogenesis independent of the ATP synthase in low oxygen) that will be of interest to the field.

The authors addressed the minor editorial issues.

Dear Itay,

Thank you for making all of the editorial changes I requested in my last email. As we approach my accepting the manuscript for publication, there are a couple of outstanding points.

If one measurement was made for each of the two transfections of the experiment shown in figure EV6f, could you please mark each individual data point on the graph. You may link these points with a line if you wish, but please indicate in the legend that this line represents the range of measurements.

Please choose five EV figures. It would be fine to have seven main figures. Rename figure callouts in the text as appropriate.

Please reduce the size of the synopsis image, and provide the synopsis text as an individual file, re.

Please remove the Expanded View title page (currently after the References).

Please place the main figure legends between the references and EV figure legends.

It should be possible to still export this manuscript for a 2023 publication date if I receive these small changes within the next two or three days.

Best wishes,

William

William Teale, PhD
Editor
The EMBO Journal
w.teale@embojournal.org

Instructions for preparing your revised manuscript:

Please 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:
https://bit.ly/EMBOPressFigurePreparationGuideline
See also figure legend guidelines: https://www.embopress.org/page/journal/14602075/authorguide#figureformat

IMPORTANT: When you send the revision we will require
- 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).
- Expanded View files (replacing Supplementary Information)
Please see out instructions to authors
https://www.embopress.org/page/journal/14602075/authorguide#expandedview

Please remember: Digital image enhancement is acceptable practice, as long as it accurately represents the original data and conforms to community standards. If a figure has been subjected to significant electronic manipulation, this must be noted in the figure legend or in the 'Materials and Methods' section. The editors reserve the right to request original versions of figures and the original images that were used to assemble the figure.

Further information is available in our Guide For Authors: https://www.embopress.org/page/journal/14602075/authorguide

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 (14th Jan 2024). Please discuss the revision progress ahead of this time with

the editor if you require more time to complete the revisions. Use the link below to submit your revision:

https://emboj.msubmit.net/cgi-bin/main.plex

The authors addressed the remaining editorial issues.

Dear Itay,

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

Congratulations! This manuscript is a really impressive achievement.

NB. In the final version, could you please include callouts to datasets EV1 and EV2, and zip movies with the corresponding legends? Many thanks.

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. If you do NOT want this, you will need to inform the Editorial Office via email immediately. More information is available here:
https://www.embopress.org/page/journal/14602075/authorguide#transparentprocess

Your manuscript will be processed for publication in the journal by EMBO Press. Manuscripts in the PDF and electronic editions of The EMBO Journal will be copy edited, and you will be provided with page proofs prior to publication. Please note that supplementary information is not included in the proofs.

You will be contacted by Wiley Author Services to complete licensing and payment information. The required 'Page Charges Authorization Form' is available here: https://www.embopress.org/pb-assets/embo-site/tej_apc.pdf - please download and complete the form and return to embopressproduction@wiley.com

EMBO Press participates in many Publish and Read agreements that allow authors to publish Open Access with reduced/no publication charges. Check your eligibility: https://authorservices.wiley.com/author-resources/Journal-Authors/open-access/affiliation-policies-payments/index.html

Should you be planning a Press Release on your article, please get in contact with embojournal@wiley.com as early as possible, in order to coordinate publication and release dates.

If you have any questions, please do not hesitate to call or email the Editorial Office. Thank you for your contribution to The EMBO Journal.

Best wishes,

William

William Teale, PhD
Editor
The EMBO Journal
w.teale@embojournal.org

** Click here to be directed to your login page: https://emboj.msubmit.net

EMBO Press Author Checklist

Corresponding Author Name: Itay Budin
Journal Submitted to: EMBO Journal
Manuscript Number: EMBOJ-2023-114054

USEFUL LINKS FOR COMPLETING THIS FORM
The EMBO Journal - Author Guidelines
EMBO Reports - Author Guidelines
Molecular Systems Biology - Author Guidelines
EMBO Molecular Medicine - Author Guidelines

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	Materials and Methods

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	Materials and methods, Data Availability section

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.	Not Applicable	

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
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.	Not Applicable	

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.	Yes	Reagents and tools table (Appendix S3)

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	

Core facilities	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If your work benefited from core facilities, was their service mentioned in the acknowledgments section?	Yes	Acknowledgments

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)
If study protocol has been **pre-registered, provide DOI in the manuscript**. For clinical trials, provide the trial registration number **OR** cite DOI.	Not Applicable	
Report the **clinical trial registration number** (at ClinicalTrials.gov or equivalent), where applicable.	Not Applicable	

Laboratory protocol	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Provide DOI OR other citation details if **external detailed step-by-step protocols** are available.	Not Applicable	

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? If sample or data points were omitted from analysis, report if this was due to attrition or intentional exclusion and provide justification.	Not Applicable	
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	

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	
In the figure legends: define whether data describe **technical or biological replicates**.	Yes	

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 approva**l (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

The MDAR framework recommends adoption of discipline-specific guidelines, established and endorsed through community initiatives. Journals have their own policy about requiring specific guidelines and recommendations to complement MDAR.

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?	Not Applicable	
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?	Yes	
If publicly available data were reused, provide the respective **data citations in the reference list**.	Not Applicable	